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Potential Microbial Processes In An Ancient Martian Environment, An Investigation Into Bio-Signature Production And Community Ecology

Thesis

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Potential microbial processes in an ancient martian environment, an investigation into bio-signature production and community ecology

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Declaration

The research described herein was funded by STFC (Science and Technologies Funding Council) and conducted at The Open University. All of the research carried out in this thesis is my own original research, with the following exceptions:

- MiSeq sequencing (Chapters 2 and 3). Conducted by a bioinformatics company (Research and Testing Laboratory, Lubbock, Texas). All subsequent bioinformatic analyses was conducted myself.

- XRF analysis (Chapter 3). Samples were prepared myself but operation of the instrument was carried out by John Watson (Open University, UK).

- ICP-AES (Chapter 3). Operation of the instrument was carried out by Laboratory Technician Patrick Rafferty (Open University, UK). All subsequent data processing was carried out myself.

- XRD (Chapter 3). Sample preparation was carried out by myself but operation of the instrument was carried out by Dr Paul Schofield (Natural History Museum, UK). Subsequent data analysis was carried out by myself.

Abstract

This work investigated whether the River Dee estuary can be considered as an martian environmental analogue and examined the whether an understanding of microbial processes could inform future life detection missions on Mars.

The subsurface environment of the River Dee estuary, UK, and its microbial community, was characterised and compared to the palaeolake at Gale Crater, Mars. Similarities were identified in pH, temperature and Total Organic Carbon measurements, as well as potential bioessential element availability (based on comparative mineralogy of the two sites). The microbial community at the River Dee site was also characterised, indicating that a diverse bacterial community thrived there, alongside a single dominant archaeal group. This provided key insight into potential microbial communities on Mars, and associated processes that may inform future Mars research.

Since the diversity and attributes of microorganisms is directly linked to their environment, the microbial community of the River Dee estuary was used to investigate potential martian geomicrobiological processes and community dynamics within a simulated martian experimental environment. Concentrations of the bioessential elements Fe, Mg and K were seen to increase in the biological experiments when compared with abiotic controls, leading to, for example, a 143 μmol L^{-1} difference in the concentration of Fe during the stationary phase. One bacterial group, the Acidobacteria Gp9, dominated the microbial community for 400 hr during the stationary phase, accounting for ~58 % of the microbial community at its peak.

For a subsequent experiment, five bacterial species were isolated from the simulated martian environment, and characterised in order to demonstrate their growth optima and tolerance to relevant environmental extremes. *Clostridium amygdalinum* was

found to be a model organism for survival within environments like the palaeolake at Gale Crater, and is proposed as a useful biological analogue for future investigations of the potential of life in such environments on Mars.

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To Rebecca Wolsey, I owe the most. Without you I fear I would not be sitting here writing this gratuitous monologue. When you strolled into the lab in October 2013 you turned my life upside down. Working together in the lab you, without question, handed me solutions to problems you'd spent months solving. You are the source of my happiness and sanity, without which I certainly would not have made it this far.

Finally, to my family: your love and support has been invaluable. Thank you.

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Abbreviations and acronyms

ANOSIM	Analysis of Similarities
ANOVA	Analysis of Variance
BCM	Biologically controlled mineralisation
BET	Brunauer-Emmett-Teller
BIM	Biologically induced mineralisation
bp	Base Pairs
BSA	Bovine Serum Albumin
BSE	Back Scattered Electron
CB	Cumberland
CTAB	Cetrimonium bromide
ddH_2O	Double Distilled Water
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
DOC	Dissolved Organic Carbon
EDS	Electron Dispersive Spectra
EDTA	Ethylenediaminetetraacetic Acid
EMPA	Electron Microprobe Analysis
EPS	Extracellular polymeric substances
FEG-SEM	Field Electron Gun - SEM
FIB-SEM	Focussed Ion Beam - SEM
FPS	Fossilised polymeric substances
GCR	Glactic Cosmic Rays
GGA	L-glutamic acid
GLA	D- galacturonic acid
HBA	4-hydroxy benzoic acid
HBA*	γ- hydroxybutyric acid
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
IDPs	Interplanetary Dust Particle
IO	Iron Oxidising
IRB	Iron Reducing Bacteria
JK	John Klein
LB	Lysogeny Broth
LEO	Low Earth Orbit
LHB	Late Heavy Bombardment
MAHLI	Mars Hand Lens Imager

MER	Mars Exploration Rover
MGS	Mars Global Surveyor
MPS	Mineral Phosphate Solubilisation
MRO	Mars Reconnaissance Orbitor
MSL	Mars Science Laboratory
NASA	National Aeronautics and Space Association
NASA JPL	NASA Jet Propulsion Laboratory
NR	Nitrate Reducing
NRC	National Research Council
OD	Optical Density
PAHs	Polycyclic Aromatic Hydrocarbons
PAME	Pyruvic Acid Methyl Ester
PBS	Phosphate Buffered Saline
PCA	Principal component Analysis
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PS	Portage soil
R	Niche space
RPD	Redox Potential Discontinuity
rRNA	Ribosomal Ribonucleic Acid
RSL	Recurring Slope Lineae
S	Species richness
SAM	Sample Analysis at Mars
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscope
SIMPER	Analysis of Dissimilarity
SLiMEs	Subsurface Lithotrophic Microbial Ecosystems
Sn	New species
SRB	Sulfate Reducing Bacteria
TCP	Tri-Calcium Phosphate
TOC	Total Organic Carbon
TRFLP	Terminal Restriction Fragment Length Polymorphism
UV	Ultraviolet
VFA	Volatile Fatty Acids
XRD	X-Ray Diffraction
XRF	X-Ray Fluorescence

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Chapter 1- Introduction

The prospect of extra-terrestrial life has fascinated many and inspired debate since astronomers recognised the existence of planets other than Earth. Though the conception of what might constitute extra-terrestrial life may have changed, the excitement it generates has diminished little.

Contemporary efforts at life detection focus on the discovery of potentially habitable environments within our Solar System and the likelihood of microorganisms successfully living there. Presently, water is considered essential for life, and has consequently shaped NASA's space exploration efforts, leading to the "Follow the water" theme (Hubbard *et al.*, 2002). Mars and the icy moons of Jupiter and Saturn are considered promising astrobiological targets (Des Marais *et al.*, 2008; Tsou *et al.*, 2011). For example, Gale Crater, Mars has been the focus of astrobiological interest during the MSL Curiosity Rover mission (Cockell *et al.*, 2012; Grotzinger *et al.*, 2014; Vaniman *et al.*, 2014). In addition, potentially habitable environments are suggested on Europa, Enceladus and Titan (Preston and Dartnell, 2014), and future life detection missions to these bodies are currently being considered (Tsou *et al.*, 2011; Hand, 2015; Carr *et al.*, 2016).

With the discovery of extremophiles, microorganisms that can survive and grow in extreme physical and chemical conditions (Rothschild and Mancinelli, 2001; Pikuta *et al.*, 2007; Mesbah *et al.*, 2009; Olsson-Francis *et al.*, 2010; Harrison *et al.*, 2013; Soto-Padilla *et al.*, 2014; Mohammadipanah and Wink, 2016), the limits of life have been extended and the number of potentially habitable environments has increased significantly. With this in mind, a search for terrestrial analogues of extra-terrestrial environments has begun, enabling the modelling of potential biological extra-terrestrial processes (see Preston and Dartnell, (2014) for a review). This thesis aims

to propose and investigate one such environment, and identify and characterise potential microbial processes that could have occurred on Mars.

1.1. The martian environment

1.1.1. Past

1.1.1.1. Pre-Noachian (4.5-3.95 Gya)

Past conditions on Mars have varied greatly since its formation. Similarly to Earth, Mars was the subject of periodic bolide impacts in its early life (Wordsworth *et al.*, 2013), known as the Late Heavy Bombardment (LHB) (Wetherill, 1975; Kring and Cohen, 2002; Cohen *et al.*, 2005; Strom *et al.*, 2005). Levels of erosion and crater forming were initially high and evidence of water on the surface (and subsurface) is abundant (see below).

There has also been ongoing debate as to whether there was once an ocean in the northern plains existing into the Noachian (Clifford, 2001; DiBiase *et al.*, 2013; Villanueva *et al.*, 2015), although early work by Parker et al. (1993) on the apparent coastal geomorphology there was discounted by later detailed analyses (Malin and Edgett, 1999; Ghatan and Zimbelman, 2006). Recent identification of significant deuterium-enriched water in the martian atmosphere suggests a global equivalent layer of water more than 137 m deep 4.5 Gya (Villanueva *et al.*, 2015).

1.1.1.2. Noachian (3.95-3.7 Gya)

There is abundant evidence of water during the Noachian period on early Mars (Carr, 1996; McSween Jr., 2006; Murchie *et al.*, 2009; Carr and Head, 2010; Williams *et al.*,

2013; Westall *et al.*, 2015a), with well-preserved fluvial and lacustrine sediments discovered at many locations (Ori *et al.*, 2000; M. C. Malin and Edgett, 2000; Malin and Edgett, 2003; Moore *et al.*, 2003; Fassett and Head, 2005; Irwin *et al.*, 2005; Weitz *et al.*, 2006; Grant *et al.*, 2008; Burr *et al.*, 2009; Achille and Hynek, 2010; Bishop *et al.*, 2013; Ruff *et al.*, 2014; Vaniman *et al.*, 2014; Grotzinger *et al.*, 2014; Pajola *et al.*, 2015). Notably this includes an ancient lake in Gale Crater (Grotzinger *et al.*, 2014, 2015; Vaniman *et al.*, 2014; Mangold *et al.*, 2016), which is currently the focus of the MSL Curiosity Rover. In addition there is evidence of an ephemeral lake at Gusev Crater (Ruff *et al.*, 2014), long term aqueous activity at Mawrth Vallis (Bishop *et al.*, 2013) and of several ancient martian aquifers (Grotzinger *et al.*, 2005; Andrews-Hanna and Lewis, 2011; Michalski *et al.*, 2013).

Along with the aqueous features described above, large valley networks, alluvial fans, detection of mineral alteration (Mustard *et al.*, 2008; Noe Dobrea *et al.*, 2010; Gaudin *et al.*, 2011; Le Deit *et al.*, 2012) and evidence suggesting soil formation support the indication that the early Noachian must have been warm enough to sustain liquid water on the surface (Grotzinger *et al.*, 2014; Vaniman *et al.*, 2014).

A greenhouse effect, based upon a CO_2 and H_2O atmosphere was suggested as a means of maintaining such mild surface temperatures (Westall *et al.*, 2013). However, global climate simulations performed by Forget et al. (2013) and Wordsworth et al. (2013) suggested that the presence of surface water is likely to be attributable to other non-climate processes, for example meteorite impacts, volcanism and basal melting (Bishop *et al.*, 2013).

1.1.1.3. Hesperian (3.7-3.0 Gya)

Crater densities suggest that the Hesperian period extends from the end of the Late Heavy Bombardment 3.7 Gyr ago to around 3 Gyr ago (Hartmann and Neukum, 2001), which is approximately coincident with the early Archean eon on Earth.

Although aqueous activity was evident in the early-mid Noachian, water activity on the surface of Mars reduced significantly in the late Noachian/early Hesperian, likely indicating a climatic shift. Observations in support of the change in water availability are, for example, the cessation of upland valley network formation at the end of the Noachian (Fassett and Head, 2008) and a transition from smecite-bearing units to allophane and imogolite at Mawrth Vallis (Bishop and Rampe, 2016).

This climate shift has also partly been attributed to a drop in atmospheric pressure caused by the formation of carbonates from atmospheric CO_2, which has been suggested to have formed a global cryosphere (Fairén *et al.*, 2010).

This period of Mars' history is characterised by a sustained high rate of volcanism, that formed extensive lava plains, including Hesperia Planum and Malea Planum (Geological Survey (US) *et al.*, 1987), low rates of valley formation in comparison to the Noachian, and a cessation of rock alteration forming phyllosilicates (Carr and Head, 2010).

1.1.1.4 Amazonian (3.0 – present)

The rates of erosion and volcanism slowed significantly over time at the end of the Hesperian and into the Amazonian until the present day where recently dated surface features at the poles are most likely to have been formed by the movement of ice (Burr

et al., 2005). However, evidence of Amazonian aged fluvial channels may indicate a slower transition toward the present cold and arid climate (Adeli *et al.*, 2016).

Current understanding of the variety of potentially habitable past environments on Mars, as elucidated by *in situ* analysis at Mawrth Vallis and Gale Crater, for example, allow for both acidic and neutral aqueous environments to have existed on past Mars (Battler *et al.*, 2013; Grotzinger *et al.*, 2014). The existence of environments on Mars with environmental conditions that appear to be suitable for both terrestrial extremophiles, and perhaps mesophiles since circumneutral pH, low salinity and above zero temperatures have been identified (e.g. (Grotzinger *et al.*, 2014) , provides a wide range of potential targets for habitability assessment and/or life detection missions.

This variation in the type of potentially habitable environment on Mars means that research efforts must be focused toward specific locations. Gale Crater, for example, has been shown to have low-salinity, circumneutral aqueous conditions, with bioessential element availability congruent with terrestrial life (see Chapter 2) (Grotzinger *et al.*, 2014; Vaniman *et al.*, 2014).

To investigate the viability of this ancient lacustrine environment as a potential habitable region requires a comprehensive environmental and geochemical characterisation and an understanding of biological analogues that may thrive in those conditions.

1.1.2. Present

1.1.2.1. Atmosphere

Since the Noachian, Mars' atmosphere has depleted significantly (see Figure 1.1). It was originally thought to have been a 2 bar CO_2 atmosphere, required by the presence of large quantities of liquid water on the surface, and a fainter young sun (Chassefière *et al.*, 2007) (Figure 1.1). However, Bristow *et al.'s* (2017) findings limit atmospheric CO_2 during the Noachian and Early Hesperian to 100s mbar, well below the levels required to maintain temperatures above 273 K. The present day martian atmosphere is relatively thin (approximately 1% of the thickness of Earth's; Hassler *et al.*, 2014) and is thought to be only 6.1 mbar (Mckay *et al.*, 1996) at the martian equivalent of terrestrial sea level. More recent analysis by the MSL Curiosity rover has demonstrated a fluctuation in atmospheric pressure at the surface between approximately 7 and 8 mbar (Harri *et al.*, 2014). It is thought be composed of ~96 % CO_2, ~2 % argon-40 and ~2 % nitrogen (Mahaffy *et al.*, 2013).
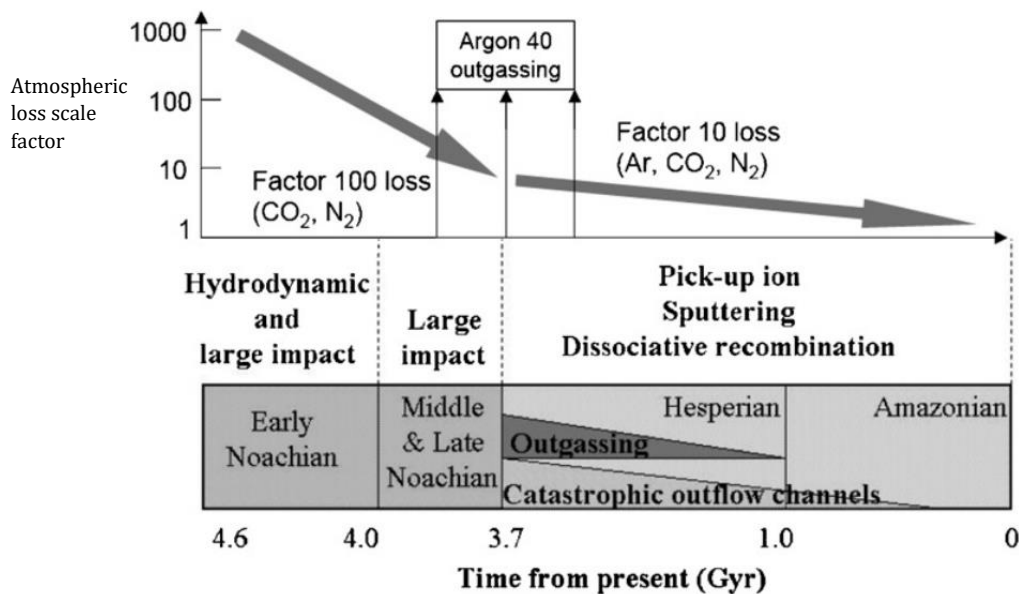
Figure 1.1. Shows a schematic chronology of the atmospheric escape on Mars, taken from (Chassefière, Leblanc and Langlais, 2007).

1.1.2.2. Water

Recent evidence of contemporary liquid brines, known as Recurring Slope Lineae (RSL), has changed our understanding of potential habitability on the surface, or in the shallow subsurface of Mars (McEwen *et al.*, 2013; Ojha *et al.*, 2015). These features, first identified by the Mars Reconnaissance Orbiter, show up as dark streaks on low albedo surfaces in the equatorial region of Mars, are seen to extend through the warmer seasons and recur over Mars year scales. Observations made by McEwen *et al.* (2013) suggest that RSL are likely to be intermittent flows of briny water, since the surface temperature during the warm seasons in Mars' equatorial region is 250-300 K, and (as mentioned above) fluctuations in surface atmospheric pressure (Harri *et al.*, 2014) will have an effect on the melting/freezing of any surface water.

In addition, recent spectroscopic evidence of the hydrated salts, magnesium and sodium perchlorate, and magnesium chlorate at four different RSL sites, has bolstered the idea that these phenomena represent contemporary aqueous activity on Mars (Ojha *et al.*, 2015), although no direct evidence of liquid water or hydrated salts has been found (Ojha *et al.*, 2013).

This transient flowing surface water, while likely to have a water activity too low to support terrestrial life (Ojha *et al.*, 2015), is still of astrobiological importance because of the seemingly universal requirement of liquid water for life, and the known existence of extremophiles that colonise extremely harsh environments. Hence, these contemporary aqueous systems are likely to be the target of future life-detection efforts on Mars.

The evidence for significant circumneutral, aqueous activity on early Mars and contemporaneous water on the martian surface widen the environmental parameters that one could use to identify a terrestrial analogue of potentially habitable martian

environments. It is now possible to include fluvial, lacustrine and perhaps marine and coastal environments in these considerations.

1.1.2.3. Radiation environment

Most notable to the possibility of life on or near the martian surface is its radiation environment. The Mars Global Surveyor mission found no evidence of a significant planetary magnetic field on Mars (Acuña et al 1998), unlike on Earth where the magnetic field provides protection from harmful radiation. Thus, potential life on the martian surface would be constrained by a significant radiation flux. Interestingly, large magnetic anomalies have been detected in the southern highlands, however these are considered an remnant of Mars' early magnetic field (Zuber, 2000; Carr and Head, 2010; Hassler *et al.*, 2014).

This lack of a magnetic field on a global scale, and the relatively thin atmosphere (approximately 1 % of the thickness of Earth's; Hassler *et al.*, 2014) creates a radiation environment similar to space. UV radiation, as shown by de la Torre et al (2010), while only penetrating a few µm into the subsurface (Parnell *et al.*, 2007), effectively rules out the possibility of unsheltered, habitable environments on the martian surface. In addition, solar radiation and galactic cosmic rays (GCR) are able to penetrate ~1-2 m, with GCR able to destroy large (~500 amu) organic molecules to a depth of 5 cm (Pavlov *et al.*, 2012; Wray, 2012; Hassler *et al.*, 2014).

Furthermore, organisms at the surface are likely to be in a dormant state because of the freezing conditions. Only actively metabolising organisms are able to repair the damage caused by ionising radiation and thus even for radioresistant organisms a lethal dose may occur. For example, a dormant community of the radioresistant organism *D. radiodurans,* at a depth of 2 m, would experience a million-fold cull in

450,000 years (Dartnell, Desorgher, Ward and a. J. Coates, 2007). However, Dartnell et al (2007) also suggested that some microorganisms may survive on the surface in cryptoendolith niches.

It is notable, however, that fluctuations in planetary obliquity may provide periodic relief from the harsh radiation environment and allow dormant communities to resume metabolic activity and thus combat the potentially lethal accumulation of cell damage (Hassler *et al.*, 2014).

In light of the radiation environment, the current hypotheses regarding the possibility of martian life are mainly focused on the subsurface where putative martian organisms would, at least, be shielded from UV radiation (Westall *et al.*, 2013). Therefore, contemporary martian life in the subsurface, especially below the ~ 2 m penetration of GCR, appears significantly more likely than at the surface. However, the limited aqueous activity in the present day constrains the possibility of current life significantly.

Past life in the subsurface during the Noachian, especially where aqueous systems thrived, could have been feasible (Grotzinger *et al.*, 2014), and some have proposed that subsurface niches colonised during the Noachian could potentially still host microorganisms (des Marais, 2010; Ehlmann *et al.*, 2011; Michalski *et al.*, 2013; Charles S Cockell, 2014).

1.2. Terrestrial vs martian life

1.2.1. Origins of life on Earth

The earliest evidence for life on Earth is dated to approximately 3.8-3.4 Gya (Mojzsis *et al.*, 1996; Martin and Russell, 2003; Martin *et al.*, 2008; Russell *et al.*, 2014). Origin of

life theories are abundant and varied but they all conform to the idea of a prokaryotic pioneer organism straddling the line the between the abiotic and biotic worlds (Bada, 2004; Orgel, 2004; Wächtershäuser, 2006; Pross and Pascal, 2013), i.e. biological life arising from a purely chemical ancestor. The transitionary phase between chemistry and biology has been suggested to be a simple, compartmentalised system with the ability to synthesise chemicals using energy collected from the environment (e.g. Kee and Monnard, 2016).

In addition, the idea of a chemoautotrophic rather than a photosynthetic origin of life has received much support since the early terrestrial atmosphere lacked the oxygen necessary for photosynthesis (Vargas *et al.*, 1998; Konhauser, 2003; Martin and Russell, 2003; Brasier *et al.*, 2006; Ferry and House, 2006; Wächtershäuser, 2006; Arndt and Nisbet, 2012; Liu *et al.*, 2012; Ramakrishnan, 2013).

Furthermore, it has been shown that the deeply rooted lineages within the 16S rRNA subunit correspond to organisms that utilise chemolithoautotrophic or chemoheterotrophic energy acquisition mechanisms (Konhauser, 2003). For example, evidence suggests that methanogenesis predates 3.4 Gya, long before the oxygenation of the terrestrial atmosphere (Battistuzzi *et al.*, 2004; Ueno *et al.*, 2006) and phylogenetic evidence implies that the complexity for photosynthetic life precludes it from being considered the earliest form of terrestrial life (Nisbet and Sleep, 2001).

1.2.2. Potential origins of life on Mars

1.2.2.1. Independent abiogenesis

The environmental conditions on early Mars are thought to be very similar to those on the early Earth (Pross and Pascal, 2013). Evidence suggests the presence of sustained

circumneutral aqueous surface conditions and of bioessential elements such as carbon (Freissinet *et al.*, 2015) and nitrogen (Stern *et al.*, 2015), on early Mars (Westall *et al.*, 2015a). Therefore, it is plausible that an independent abiogenesis occurred on Mars.

1.2.2.2. Shared ancestry

An alternative theory is that abiogenesis occurred on Earth or Mars and was transferred from one planet to another (Davis and McKay, 1996). This hypothesis, lithopanspermia, involves microorganisms being safely entrapped and protected inside meteorites after ejection from the planet's surface, then transported between planetary bodies (Meot-Ner and Matloff, 1979). Experiments on the Biopan facility of the European Space Agency show that lichen were able to survive a short (10 day) exposure to space conditions, with communities of cyanobacteria showing much less resistance (de la Torre *et al.*, 2010), and Horneck *et al* (2008) showed that impact ejecta from a Mars-like planet could yield viable microorganisms. However, re-entry remains a challenge, with evidence suggesting that lichen and cyanobacterial cells would likely fail to survive re-entry into Earth's atmosphere (de la Torre *et al.,* 2010).

The environmental limits within which abiogenesis can occur are significantly more constraining than those for the maintenance of established life (Westall *et al.*, 2015a). Therefore, some environmental constraints on the potential origin of life on Mars are avoided by hypothesising a lithopanspermia event for Mars rather than *in situ* abiogenesis.

1.3. Habitability

1.3.1. The requirements of life

Martin and Russell (2007) postulated that in order for primitive cells to form, a conducive environment must have prevailed for hundreds of thousands to millions of years. These conditions are different to those that are required to sustain life (Westall *et al.*, 2013), because once life has evolved, its adaptability allows it to overcome some environmental stressors. If life did emerge on Mars, it is therefore likely that this occurred during its early history, in the Pre-Noachian and Noachian periods when environmental conditions were less extreme (Westall *et al.*, 2015a).

It is possible to discuss habitability in terms of three main requirements for the emergence and sustenance of life:

- A suitable solvent
- Bioessential elements
- An energy source

1.3.1.1. A suitable solvent

Presently, the only evidence of contemporary liquid water activity on the martian surface comes in the form of Recurring Slope Lineae (RSL) (McEwen *et al.*, 2013; Ojha *et al.*, 2015) and gullies formed by surface run-off (Malin and Edgett, 2000). There is still uncertainty regarding the composition of the surface water responsible for the RSL, with some evidence suggesting a freshwater subsurface flow source (Grimm *et al.*, 2014; Stillman *et al.*, 2014) and others suggesting a briny aquifer (Mitchell and Christensen, 2016; Stillman *et al.*, 2016) or an atmospheric process, e.g. deliquescence (Wang, 2014; Chojnacki *et al.*, 2016). Source notwithstanding, water budgets have been

used to show that a significant amount of near-surface water might be present (Chojnacki *et al.*, 2016).

In the presence of water (and favourable physicochemical environmental factors, and bioessential elements) it is expected that life would grow and survive to produce biologically characteristic metabolic end products, physical features like microbial mats and biofilm and, over time, their fossilised counterparts (Hoehler and Westall, 2010; Röling *et al.*, 2015). Since the past and present focus of Mars missions has been *in situ* analyses, and not sample return, the first identification of contemporary martian life will likely be undertaken *via in situ* analyses. Therefore, detection of such life will require the design of specific instruments capable of detecting microbial cell structures, or definitively biological metabolic products.

1.3.1.2. Bioessential elements

Perhaps equally important to the presence of a suitable solvent is the presence of bioessential elements that are necessary for life. These are carbon, hydrogen, nitrogen, oxygen, phosphorus and sulfur (Pontefract *et al.*, 2012). In addition to this, there are several further elements, including chlorine, potassium, sodium, calcium, magnesium, selenium, zinc, iron, manganese, copper, cobalt, nickel and molybdenum, that are essential for major cellular processes, e.g. DNA synthesis and cell membrane stability (Wackett *et al.*, 2004; Pontefract *et al.*, 2012). The bioavailability of these elements will be a key constraint on potential martian life and the cellular metabolisms available to it.

1.3.1.2.1. Carbon

Terrestrial life is carbon-based (Board, 2007) and, as such, carbon has a multitude of essential functions within terrestrial organisms. Life based on other elements has been suggested, e.g. silicon, but this has been refuted (Jacob, 2016) and, as yet, there is no mainstream acceptance of the feasibility of life based on elements other than carbon. As such, the availability of carbon is key to the potential habitability of any martian environment.

For organisms capable of fixing atmospheric carbon dioxide, the martian atmosphere would be a viable source of carbon (Mahaffy *et al.*, 2013). In addition to the atmosphere, exogenous organic material, contained in interplanetary dust particles, micrometeorites and meteorites is thought to provide an influx of abiotic organic material to the martian surface (Zent and McKay, 1994; Flynn, 1996; Westall *et al.*, 2015a), which could support microbial growth.

Organic molecules have been detected in martian meteorites (Wright *et al.*, 1989; Jull *et al.*, 2000; Sephton *et al.*, 2002; Sephton and Botta, 2006; Thomas-Keprta *et al.*, 2015). Pyrolysis of martian meteorites EET A79001 and Nakhla suggest that the organic material contained within them is similar to that found in carbonaceous chondrites (Sephton *et al.*, 2002). The carbonaceous component, within carbonaceous chondrites, contains soluble and insoluble organic matter (Pearson *et al.*, 2006), including polyaromatic hydrocarbons (PAHs) (Hahn *et al.*, 1988), and, biologically-relevant molecules such as, amino acids, carboxylic acids and sugars (Botta and Bada, 2002; Sephton, 2002; Sephton *et al.*, 2004), which could be delivered to the martian surface.

Flynn (1996) suggested that the total present day incident mass of micrometeoroids at Mars is 1.2×10^{10} g year^{-1}. However, this figure was scaled for consistency with data from the Long-Duration Exposure Facility (Love and Brownlee, 1993) and was

recalculated to be 6.8×10^9 g year^{-1}. Within this infall, based on compositional similarities between IDPs, micrometeorites and chondritic meteorites, it is thought that 5.4×10^9 g year^{-1} corresponds to a carbonaceous component (Genge, 2008; Taylor *et al.*, 2012).

With regard to ancient Mars, during the peak 50 Ma of the LHB, it is thought that Mars received 1.1×10^{13} g year^{-1} carbonaceous micrometeoroids. The flux of organic material to the martian surface however, is different to that of the upper atmosphere. Court and Sephton (2014) suggest that 20 % of the infalling carbonaceous material experiences significantly reduced thermal degradation, thus potentially providing a source of organic matter to the surface.

Further, volatiles can be injected into the atmosphere *via* the ablation of micrometeorites during the LHB may have contributed to the production and maintenance of habitable environments on Noachian/Pre-Noachian Mars (Court and Sephton, 2014).

Organic molecules have been difficult to detect on Mars' surface (Westall *et al.*, 2015a), not only because of their likely low abundances, but because of photochemical destruction of volatile organic material (Summons *et al.*, 2011) and the presence of oxidants, e.g. perchlorates, which oxidise organic matter during analysis (Atreya *et al.*, 2006, 2011).

Methane has been detected by the MSL Curiosity rover (Webster *et al.*, 2015), along with chlorobenzene, C_2 and C_4 dichloroalkanes, and it has been suggested that the total organic carbon content at Gale crater is 2400 ppm (B. Sutter *et al.*, 2016). Furthermore, non-chlorinated organic matter has also been detected at Yellowknife Bay, Gale Crater (Freissinet *et al.*, 2015, 2016). However, there is doubt on the origin of these organic molecules, with terrestrial contamination possible (Summons *et al.*, 2014).

1.3.1.2.2. Other elements

The MSL Curiosity Rover has measured hydrogen, oxygen, sulfur, nitrogen and phosphorus in minerals at Gale Crater (Ming *et al.*, 2014; S. McLennan *et al.*, 2014; Vaniman *et al.*, 2014). Nitrogen and sulfur were detected within the John Klein and Cumberland drill holes, Gale Crater, in the form of nitrate, as well as the sulfur minerals, pyrite and pyrrhotite (Bridges *et al.*, 2015; Stern *et al.*, 2015). With the carbon sources discussed above, this accounts for the six main elements essential for life. In addition to these, Curiosity has also detected many of the metals listed above including Fe, Mg, Mn, Na, K and Ca (Bish *et al.*, 2013; Lanza *et al.*, 2014; Ming *et al.*, 2014; S. McLennan *et al.*, 2014; Vaniman *et al.*, 2014; Bridges *et al.*, 2015; Stern *et al.*, 2015). Sources of chlorine have been discovered within organic material (Freissinet *et al.*, 2015) as previously mentioned, and within perchlorate salts (Leshin, Mahaffy, Webster, Cabane, Coll, Conrad, Archer, Atreya, Brunner, a Buch, *et al.*, 2013; Ming *et al.*, 2014).

The exact bioavailable concentration of these elements is unclear. However, biologically mediated elemental release, often focused on specific minerals based on their bioessential element composition (Rogers and Bennett, 2004), may serve to increase bioavailability and therefore increase the survivability of microbial martian life, e.g. (Welch and Ullman, 1993; Welch *et al*,. 2002).

1.3.1.3. Energy sources

Electron donors and acceptors are essential components of metabolism because energy is released when electrons are passed from donor to acceptor, through the electron transport chain. For example, during methanogenesis, hydrogen is the

electron donor and carbon dioxide, the acceptor (Westall *et al.*, 2015b). The carbon dioxide is reduced, and methane is formed, releasing energy for use by the microorganism.

Currently, the general consensus is that aerobic respiration on Earth was preceded by anaerobic respiration during the early stages of life's evolution (Martin and Sousa, 2016). On Mars it appears that the availability of electron acceptors is the limiting factor in whether potential metabolic pathways are utilised (Westall *et al.*, 2015a).

Excluding the possibility of phototrophic life, since phototrophy is likely precluded by the radiation environment of the surface, Table 1.1 displays potential energy sources for chemotrophic life on Mars. Many of the electron donors shown in Table 1.1 have been detected on Mars. Hydrogen is available through several processes including serpentinisation (Nealson *et al.,* 2005) and potentially microbially mediated mineral alteration (Parkes *et al.*, 2011). In addition, abiotic methane (Webster *et al.*, 2015) and Fe^{2+} produced by the alteration of magmatic rocks mean that sources of electron donors are plentiful on Mars. As mentioned above, sourcing electron acceptors can be problematic, however, several of those mentioned in Table 1.1 have been discovered on Mars. For example, CO_2 is in the atmosphere (Mahaffy *et al.*, 2013), sulfate has been found in bassanite and anhydrite minerals (Aubrey *et al.*, 2006; Bish *et al.*, 2013; Bridges *et al.*, 2015; Rapin *et al.*, 2016), H_2O is likely in RSL and elsewhere (McEwen *et al.,* 2013; Grotzinger *et al.*, 2014; Ojha *et al.*, 2015; Pajola *et al.*, 2015; Mitchell and Christensen, 2016), Fe^{3+} has been detected in haematite, jarosite and goethite (Morris *et al.*, 2004; Bibring *et al.*, 2007) and organic material may be present (Leshin, Mahaffy, Webster, Cabane, Coll, Conrad, Archer, Atreya, Brunner, a Buch, *et al.*, 2013; Freissinet *et al.*, 2015, 2016).

1.4. Putative martian life

Electron donor	Electron acceptor	Metabolism	Comment
Chemolithotrophy			
H_2	CO_2	Methanogenesis; acetogenesis; C fixation via Wood-Ljundahl pathway	Hydrogen from hydrothermal alteration of mafic/ultramafic minerals and microbial mediation of H_2 from mineral alteration
H_2	Fe^{3+}	Iron reduction	Hydrogen from sources mentioned above
H_2	SO_4^{2-}, S^0	Sulfate reduction	Hydrogen from sources mentioned above
H_2	O_2	Hydrogen oxidation	
H_2	ClO_4^-	Perchlorate reduction	
CH_4	(Mn^{4+}, Mn^{3+})	Bimessite reduction	
CH_4	Fe^{3+}	Ferrihydrite reduction	
CH_4	NO_3^-	Anaerobic methane oxidation	
CH_4	SO_4^{2-}	Sulfate reduction	
CO	H_2O	Carbon monoxide oxidation	
CO	O_2	Aerobic carbon monoxide oxidation	"CO oxidisers" are bacteria capable of growing with CO as a sole carbon and energy source
CO	NO_3^-	Aerobic methane oxidation	
CO	SO_4^{2-}	Sulfidogenesis	
CO	CO_2	Methanogenesis; acetogenesis	
Fe^{2+}	CO_2	Carbon dioxide reduction	
Fe^{2+} (basalt glass)	O_2, NO_3^-	Iron oxidation	Not confirmed for terrestrial microorganisms
Fe^{2+} (aqueous)	O_2, NO_3^-	Microaerobic iron oxidation	
Fe^{2+} (biotite)	O_2, NO_3^-	Aerobic iron oxidation	
Fe^{2+} , Fe^{3+} (magnetite)	NO_3^-	Aerobic iron oxidation	
FeS_2	MnO_2, NO_3^-	Aerobic pyrite oxidation	
S^{2-}	(Mn^{4+}, Mn^{3+})	Anaerobic sulfides oxidation	
HS^- (aqueous)	O_2, NO_3^-		
S^0	NO_3^-	Sulfur oxidation	
S^0	Fe^{3+}	Anaerobic sulfur oxidation	Occurs in acid conditions
S^0	MnO_2	Sulfur oxidation	
H_2S, HS^-, S^0, $S_2O_3^{2-}$	O_2	Oxidation of reduced sulfur compound	
NH_3	O_2	Oxidation of ammonia	Part of the nitrification process
NO_2^-	H_2O	Oxidation of nitrite	Part of the nitrification process
NH_4^+	NO_2^-	Anammox	
Chemoorganotrophy			
Organics	Fe^{3+}	Iron reduction	Carbon from abiotic/prebiotic sources as well as biogenic; hydrogen from sources mentioned above
Organics	SO_4^{2-}	Sulfate reduction	Carbon and hydrogen sources as above
Organics	Perchlorates		
Organics	Organics	Fermentation	Carbon sources as above

1.4.1. Potential characteristics of martian life

The environmental conditions on early Mars, and the discontinuity of habitable environments, indicate a relatively small window in which life could have appeared. Thus, putative martian life would have remained in a primitive state of evolution, unlikely to achieve anaerobic photosynthesis because of its relative complexity (Westall *et al.*, 2013, 2015a). With this in mind, chemolithoautotrophy is a likely metabolic pathway for putative martian life (Grotzinger *et al.*, 2014; Röling *et al.*, 2015). Chemolithoautotrophs are believed to be the first terrestrial primary producers (Nealson *et al.*, 2005; Martin and Sousa, 2016) and the multitude of possible chemotrophic metabolic pathways available on Mars (see Table 1.1) means it is prudent to conclude that martian life would be in the form of chemotrophic microorganisms.

Additionally, the subsurface is likely to be oligotrophic (Westall *et al.*, 2015a), and therefore subsurface microbial communities, especially those founded from surface dwelling communities when conditions became unfavourable (Charles S Cockell, 2014), would be constrained by the adaptability to nutrient-poor conditions.

During the pre-Noachian and Noachian conditions favourable to the emergence of life on Mars would have been present, but were likely to have been geographically separated (Westall *et al.*, 2013). Interestingly, this presents a situation where life could have emerged separately and independently in multiple habitats (Davies and Lineweaver, 2005), making the classification of martian life problematic. However, if future life detection efforts were to identify geographically isolated populations on Mars, testing of their respective origins, and potential relatedness, would require the construction of rigorous hypotheses, built around models for the emergence of life.

1.4.2. Potential analogue microorganisms

1.4.2.1. Chemolithotrophic microorganisms

The immense adaptability of terrestrial microorganisms to even extreme environments is uncontested, and microorganisms have been shown to utilise a variety of energy yielding redox couples (Nealson, 1997)(Figure 1.2). While most terrestrial life thrives in Earth's oxygen rich atmosphere, some microorganisms have become well adapted to environments with little or no oxygen at all and are known as anaerobes.

Anaerobic chemolithotrophs use inorganic chemicals from their surroundings to generate energy using the redox couples shown in Figure 1.2. With the exception of chemolithoautotrophs, which are able to convert inorganic carbon (CO_2) to organic carbon (Nealson, 1997; Stevens, 1997), most chemolithotrophs rely on an organic carbon source. Chemolithotrophs therefore have great potential as martian analogue organisms because of their suitability to the metabolic and environmental conditions discussed above.
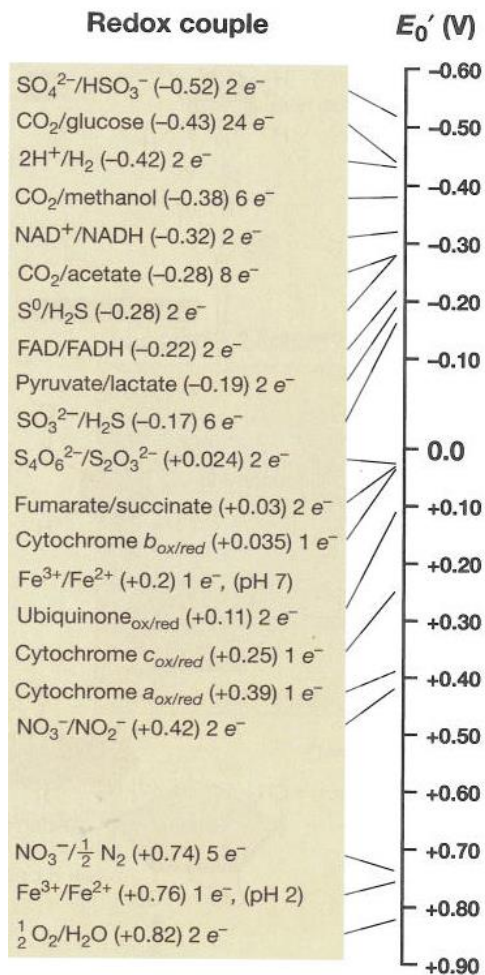
Figure 1.2. The redox tower. Redox couples are ordered from strongest reductants at the top to strongest oxidants at the bottom.(Weber et al., 2006).

There are several groups of anaerobic microorganisms that are relevant to potentially habitable martian environments (Table 1.1). These include:

1.4.2.1.1. Methanogens

A group of Archaea that produce energy almost exclusively through methanogenesis whereby CH_4 is produced *via* the use of H_2 and CO_2. Thus, conditions on Mars, where it has been suggested that H_2 can be produced during serpentinisation reactions in the subsurface (Hellevang *et al.*, 2011), and the abundance of CO_2 in the atmosphere is high, means such metabolism could be sustained. Methanogens are obligate anaerobes and will not survive unless strict anaerobic conditions prevail (Liu *et al.*, 2012).

1.4.2.1.2. Sulfate reducing bacteria (SRB)

These are also obligate anaerobes and uniquely carry out dissimilatory sulfate reduction whereby sulfur, in its oxidised state, is fully reduced to facilitate the dissimilation of organic matter (Postgate, 1979). The discovery of sulfate-rich evaporite assemblages on the martian surface supports the idea that SRBs are of relevance to martian habitability (Bridges and Grady, 2000).

1.4.2.1.3. Iron reducing bacteria (IRB)

These oxidise hydrogen and organic substrates while reducing iron from its ferric to ferrous forms to release energy (Fredrickson and Gorby, 1996). They are generally split into two groups: (1) obligate anaerobic clostridia and (2) a heterogeneous mix of facultative anaerobes (Ottow and Glathe, 1971). Compared to terrestrial basalts, martian basalts have high iron concentrations as evidenced by analysis of meteorites (Stolper and McSween Jr, 1979), the MER rovers (Squyres *et al.*, 2006), and by the MSL Curiosity Rover (Schmidt, Campbell, Gellert, Perrett, a. H. Treiman, *et al.*, 2014). Therefore, their applicability and suitability to potential martian habitats is likely significant, with iron reduction a plausible metabolism for putative martian life.

Evidently, useful biological analogues of potential martian life are available to facilitate life-detection efforts. However, the significance of their impact on research is limited unless they are investigated within Mars-like environmental conditions.

1.5. Life detection missions

1.5.1. Past and present

There have been a plethora of Mars missions since the Viking programme in 1976 (Klein *et al.*, 1976; Nier and Mcelroy, 1977; Klein, 1978, 1979), but Viking 1 and 2 remain the only life detection missions to successfully land and operate on Mars (Houtkooper and Schulze-makuch, 2007; Schulze-Makuch *et al.*, 2015). Though the Beagle 2 lander (Sims *et al.*, 1999; Wright *et al.*, 2003), part of the Mars Express mission (Chicarro *et al.*, 2004), was a dedicated life detection mission it was unable to collect data because of technical faults. In 2020 the ExoMars mission will be the first dedicated life detection mission on Mars since the Viking missions, and will have the capability to investigate the subsurface as well as the surface (Baglioni *et al.*, 2006).

There has been much debate regarding the findings of the Viking missions, with some contesting that evidence for life was obtained, while others cite uncertainty in the findings, based on issues of contamination or attribution of the findings to abiotic phenomena (Quinn and Zent, 1999; Benner *et al.*, 2000; Levin, 2015). In addition, Glavin et al. (2001) showed that the instruments on-board the Viking landers would have been unable to detect the degradation products of even several million bacterial cells per gram of martian soil, thus casting further doubt over the findings from the Viking mission. At present, the consensus is that the Viking missions detected oxidising and reactive surface chemistry, but not life (Klein, 1999; Benner, 2010).

Since the Viking missions, there have been several missions to Mars that have employed remote sensing instruments to analyse the surface and atmospheric environment, for example the Mars Global Surveyor (MGS) (Cunningham, 1996; Albee *et al.*, 1998) and the Mars Reconnaissance Orbiter (MRO) (Lock *et al.*, 2005; Zurek and Smrekar, 2007). Also, rover missions on the surface of Mars have carried out *in situ*

investigations of potentially habitable environments, characterising physical and chemical conditions, however so far none of these missions have carried instruments designed to detect life (Webster and Mahaffy, 2011; Grotzinger *et al.*, 2012). The first were the Mars Exploration Rovers, Spirit and Opportunity, which successfully landed on the surface of Mars in 2004 and worked for 2555 sols (Spirit) and over 4500 sols (Opportunity), investigating Gusev Crater and Meridiani Planum respectively (NASA JPL, 2003a, 2003b; Arvidson *et al.*, 2006; Squyres *et al.*, 2006). More recently, the Mars Science Laboratory (MSL) Curiosity Rover has been analysing Gale Crater, a highly important site for investigations into habitability on the martian surface (Grotzinger *et al.*, 2012) since the 6th of August 2012 and continues to operate (1654 martian sols on 1st April 2017 (NASA, 2017a)).

1.5.2. Assumptions of life detection

It is prudent to note that life detection efforts, and missions planned for the future, are subject to a significant assumption about extra-terrestrial life. That is, life would at least resemble terrestrial life in terms of chemistry, arrangement into cells and being composed of analogous structures with analogous associated functions (Prieto-Ballesteros *et al.*, 2011; Sims *et al.*, 2012). It is, of course, possible that extra-terrestrial life would not resemble terrestrial life in any aspect and thus would not be similar enough to be recognised by reliable scientific methods (Conrad and Nealson, 2001). Presently, there have been few efforts to account for this assumption (e.g. Kounaves *et al.*, 2002), though Conrad and Nealson (2001) proposed that defining life by measurable characteristics only, e.g. high fidelity replication and metabolic production, as well as providing that a combination of measurable characteristics would be

necessary for a definitive conclusion, has provided a framework from which future life detection efforts could be informed.

Accurate characterisation of life is therefore a problematic exercise. Researchers are limited by the availability of only a single sample from which to characterise life: Earth's life. The search for extra-terrestrial life is therefore prefaced by a series of assumptions made about the nature of life elsewhere. In 2007 the National Research Council (NRC) Committee on an Astrobiology Strategy for the Exploration of Mars set out these assumptions and described the basic characteristics of hypothetical martian life. They are set out as follows (Hoehler and Westall, 2010):

- martian life is based on carbon, hydrogen, oxygen, nitrogen, phosphorus, sulfur, and the bio-essential metals discussed below

- martian life requires water

- martian life possesses structures reminiscent of terrestrial microorganisms, i.e. it exists in the form of self-contained, cell-like entities

- martian life has morphological and gross metabolic characteristics that are determined by the same physical, chemical, and thermodynamic factors that determine the respective characteristics of terrestrial organisms

- martian life utilises complex organic molecules in biochemical roles analogous to those of terrestrial organisms

Given a sample size of only one (Earth) it could be suggested that basing our extra-terrestrial life detection efforts on a fundamental similarity to terrestrial life could be unwise. However, the alternative entails an infinite list of possibilities, whereby controlled and informed experimental methodology would be nearly impossible. Therefore, the work contained within this thesis will be firmly based on this

fundamental assumption that if life existed elsewhere in the Solar System it would be similar to that of Earth.

1.5.3. Martian analogues

1.5.3.1. Biological

Martian analogue experiments are most relevant if the model microorganisms used are relevant to the specific martian environment in which they represent putative martian life. The search for microorganisms for use in martian analogue experiments has almost solely focused on extremophiles, e.g. acidophiles, psychrophiles and halophiles (Landis, 2001; Fernández-Remolar *et al.*, 2004; Amils *et al.*, 2007; Direito *et al.*, 2011), such that processes within extreme environments could be investigated. For example, Gómez Felipe *et al.*, (2010) investigated the survival of chemolithotrophic bacteria in present day martian surface conditions. Yet, even where the microorganisms were sampled from an environment not typically regarded as extreme, the microorganisms were found to have strong resistance to one or more factors that could severely limit microbial growth. For example, several strains of cyanobacteria were sampled from the marine high-intertidal zone and exposed to low earth orbit (LEO)(Olsson-Francis *et al.,* 2010). Among the surviving species were osmophilic, halophilic and UV resistant species (Mancinelli *et al.,* 2004*;* Olsson-Francis *et al.,* 2010; Olsson-Francis *et al.,* 2013).

1.5.3.2. Environmental

Life detection efforts are most commonly informed by the aid of a terrestrial analogue (Preston and Dartnell, 2014) that provides an opportunity to investigate planetary

processes on Earth before extrapolating the findings to analogous extra-terrestrial environments (Léveillé, 2009). These terrestrial analogues have traditionally been extreme environments and vary greatly, from highly acidic environments, as in the Rio Tinto analogue site (Fernández-Remolar *et al.*, 2004; Amils *et al.*, 2007), to the extremely cold Antarctic Dry Valley (Wynn-Williams and Edwards, 2000) and the hyper arid environment of the Atacama Desert (Navarro-Gonzalez, 2003). However, recent work has focused on the mild conditions of ancient Mars, especially ancient palaeolakes (Treiman *et al.*, 2014; Cousins, 2015; Marshall and Cestari, 2015). Similarly, useful geological analogue sites have been established to investigate martian processes (e.g. The Golden Deposit (Battler *et al.*, 2013), The Pilbara Region (Brown *et al.*, 2005), Haughton Impact Structure (Parnell *et al.*, 2004), Antarctica, (Dickinson and Rosen, 2003)) .

Analogue sites are characterised in two ways. Initially, environmental parameters are investigated and compared to potential martian analogue sites and then investigations into biological processes at the site can be used to infer processes that may have occurred at the martian site. For example, the iron bioformations formed by iron metabolising bacteria at Rio Tinto have been studied to provide insight into biosignature production at Meridiani Planum on Mars (Amils *et al.*, 2007).

Evidence for the existence of extreme environments on Mars is commonplace, e.g. acidic environments indicated by the detection of jarosite at Meridiani Planum (Klingelhöfer *et al.*, 2004; Aubrey *et al.*, 2006).

At Gale Crater the MSL Curiosity Rover has characterised the mineralogy such that largely unknown characteristics of the aqueous environment, e.g. pH and salinity, can now be ascertained and interpreted. This has shaped the contemporary understanding of these environments. The Sheepbed mudstone, discovered at Yellowknife Bay, gave

strong indications of circumneutral water activity because of the prevalence of clay minerals, and apparent sediment transport mechanisms also suggested low salinity (Bridges *et al.*, 2015; Schwenzer *et al.*, 2016). Therefore, *in situ* analyses carried out by the MSL Curiosity Rover has provided a wealth of evidence toward an ancient environment at Gale Crater with moderate conditions in which many mesophilic terrestrial organisms could survive and grow. Circumneutral pH conditions have also been inferred through orbital observations for other locations on Mars, for example the Terby and Jezero craters (Bethany L. Ehlmann *et al.*, 2008; Ansan *et al.*, 2011; Schon *et al.*, 2012).

Currently, terrestrial analogues for such environments have ranged in specificity from general environmental comparisons, e.g. fluvial-lacustrine environments in Iceland (Cousins, 2015), to geological comparisons (e.g. the Green River Formation) (Marshall and Cestari, 2015)), to mineral specific analogies, e.g. a clay mineral analogue used for the specific palaeolacustrine environment at Yellowknife Bay (Treiman *et al.*, 2014). As yet, there has been no investigation of anaerobic microbial communities from mesophilic intertidal environments and their application to studies of putative martian life. Further to the above examples, the investigation of further analogue sites is necessary to fully explore potential microbial processes in the potentially habitable martian palaeoenvironments.

The subsurface environment of an inter-tidal zone is one possible environmental analogue for the ancient martian lake system at Gale Crater, and furthermore, work by Olsson-Francis *et al.* (2009) showed that microorganisms isolated from intertidal zones can survive the simulated martian conditions. Environmental conditions in those environments are similar to those evidenced by findings at Gale Crater. For example, the subsurface is isolated from the oxygenated terrestrial atmosphere, creating an anaerobic environment comparable to that of the martian atmosphere. Further, the

terrestrial marine environment is analogous to the 1-2 % chloride salt concentration likely present in the ancient lake system (S. McLennan *et al.*, 2014; Vaniman *et al.*, 2014). This potential analogue site is characterised and compared to Gale Crater in Chapter 2.

1.6. Biosignatures

The definition of a biosignature, is a "measurable indicator of a specific biological state" (Rifai *et al.,* 2006). Therefore, in terms of using biosignatures to identify whether life exists or existed on Mars, a biosignature is an indicator of the presence of a biological organism. Investigation into specific biological states would only be considered once definitive proof of life has been discovered.

Thus, the detection of biosignatures on Mars must be supported by an understanding of which type of biosignature would be most likely, since experiments and instruments would have to be developed accordingly.

1.6.1. Inorganic/geochemical

Inorganic or geochemical biosignatures range from the physical alteration of a substrate via microorganism-induced dissolution or weathering (Rogers and Bennett, 2004; Wu *et al.*, 2007b; L. Wu *et al.*, 2008), to the enrichment or variation in abundance of certain elements or isotopes (Chela-Flores and Negro, 2005). Additionally, physical features ranging from fossilised cells, biofilms and microbial mats are also considered potential biosignatures (Hoehler and Westall, 2010). For detection, biosignatures are required to persist long enough to be of use analytically, preserved in the geological record.

1.6.1.1. Bioweathering processes

Bacteria have repeatedly been shown to increase dissolution rates and even cause etching beneath biofilms when grown with a rock substrate (Ehrlich, 1996; Brantley *et al.*, 1999; Edwards and Goebel, 1999; Aouad *et al.*, 2006; Wu *et al.*, 2007b; L. Wu *et al.*, 2008; Olsson-Francis *et al.*, 2012). Furthermore, as well as dissolution, microorganisms have also been shown to cause secondary mineralisation (Fortin *et al.*, 1996).

Microbes are thought to preferentially increase dissolution of bioessential minerals, e.g. iron and phosphorus, from rocks to compensate for a lack of such elements in their environment (Rogers and Bennett, 2004; Wu *et al.*, 2007b; Lingling Wu *et al.*, 2008). Commonly used mechanisms that microbes employ to induce or facilitate dissolution are as follows:

1. Production of excess protons
2. Production of organic ligands
3. Production of extracellular polymeric substances (EPS)
4. Formation of mineral surface ion complexes

Chemolithotrophic and chemo-organotrophic microorganisms have been shown to utilise the above mechanisms, for example, leaching unwanted metals from contaminated material (Gadd, 2010). Microbial weathering of minerals has often been associated with changes in pH caused by the production of organic acids and/or excess protons during metabolism (Barman *et al.*, 1992; Welch and Ullman, 1993; Vandevivere *et al.*, 1994; Barker *et al.*, 1998; Rogers, Bennett and Choi, 1998; Liermann *et al.*, 2000; Bennett *et al.*, 2001; Uroz, Calvaruso, Turpault and Frey-Klett, 2009). These changes in pH are usually localised and often occur where microbial extracellular polymeric substances (EPS) form a biofilm. Variability in pH of up to 1.1 pH units has been measured across a biofilm (Liermann *et al.*, 2000). However, the dissolution rate

of some aluminosilicate minerals has been shown to be pH independent, in the range of pH 5-8 (Brady and Walther, 1989, 1990). Therefore, dissolution rates will depend on mineral composition.

In addition to altering the pH, organic acids can increase dissolution by complexing and chelating essential nutrients from mineral surfaces and from solution (Wanner and Egli, 1990; Barker *et al.*, 1998; Kalinowski *et al.*, 2000; Bennett *et al.*, 2001; Uroz, Calvaruso, Turpault and Frey-Klett, 2009; Gadd, 2010). However, differentiating between pH-dependent and pH-independent processes has been problematic (Vandevivere *et al.*, 1994) and it has been shown that organic acids can enhance dissolution at distance from the microbial cell, independent of microbial attachment (Vandevivere *et al.*, 1994; Hersman *et al.*, 1995). Siderophores are an example of compounds with this ability and are the largest group known to bind and transport iron back to the microbial cell (Gadd, 2010).

Microbial attachment also contributes to weathering, often preferentially promoting dissolution of specific minerals based on the nutrient content (Bennett *et al.*, 2001). For example, microorganisms have been shown to colonise feldspars and preferentially scavenge phosphorus from apatite inclusions while destroying the silicate matrix (Rogers, Bennett and Choi, 1998; Rogers, Bennett and Ullman, 1998). Also, Gleeson *et al.*, (2006) showed that the diversity of the bacterial communities colonising granite varied according to the type of mineral inclusion, with different communities inhabiting muscovite, plagioclase, K-feldspar and quartz, respectively.

Microbial action, while often leading to increased dissolution both locally, at colonisation sites, and generally through the production of organic acids and chelating agents, has also been shown to be involved in secondary mineralisation processes (Uroz, Calvaruso, Turpault and Frey-Klett, 2009). These processes have been split into

two groups: biologically induced mineralisation (BIM) and biologically controlled mineralisation (BCM)(Gadd, 2010).

BIM occurs as a result of biological modification of the environment and leads to precipitation of mineral phases (Gadd, 2010), e.g. calcium carbonate (Chafetz and Buczynski, 1992), whereas BCM is based on microbial attachment and the alteration of the microenvironment around the cell. Often the microbial cell and the EPS serve as nucleation sites for secondary mineralisation (Barker and Banfield, 1998; Barker *et al.*, 1998).

However, it is important to note that other research suggests that, under some circumstances, microorganisms can inhibit or reduce dissolution by the formation of an amorphous leached layer (Benzerara *et al.*, 2004; Wu *et al.*, 2007b). Benzerara et al. (2004) found that Mg/Si ratios were similar in abiotic and biotic samples, and a <10 nm thick amorphous layer, chemically indistinguishable from the bulk rock, prevented dissolution. Furthermore, even where it has been shown that microbes specifically sought out Fe and P bearing minerals, there were no significant increases in the bulk fluid compositions of the growth media (Rogers and Bennett, 2004).

Thus, the broad hypothesis to be tested in this thesis is that the presence of the microbial community, and their metabolic products, would increase dissolution of bioessential minerals from the rock substrate and potentially cause the formation of secondary minerals not observed in abiotic controls. These secondary minerals could be the target of future life-detection missions.

Distinguishing between biotic and abiotic weathering processes is essential to identify past microbe-rock interaction in the martian subsurface, though this can be difficult (Uroz, Calvaruso, Turpault and Frey-Klett, 2009) since target minerals can undergo several episodes of chemical and physical weathering creating a complex history of

surface alteration. This can obscure or debilitate efforts to identify purely microbe-based weathering features (Bennett *et al.*, 2001). These uniquely biotic weathering processes can generate geochemical biosignatures that may be useful in future life detection efforts.

The implications of this work are therefore both general, in that there are strong implications for astrobiological research, and highly specific, with regard to processes involved in microbial weathering and identification of potential biosignatures.

1.6.2. Organic

Organic biosignatures represent the biological molecules and materials produced by organisms usually through their metabolism. For example, fatty acid biosignatures have been identified in gypsum-hosted endoevaporitic microbial communities on Earth. Fossil residues of these lipids have been used to provide an insight into palaeoenvironments and community compositions on Earth, and bacterially synthesised alkanes have also been considered as useful organic biosignatures (Jahnke *et al.*, 2014). Lipid carbon skeletons of microorganisms associated with hydrothermal systems have been suggested as being broadly characteristic of all aqueous-based life, and thus potentially applicable to early Mars (Jahnke *et al.*, 2016).

Until the recent focus on mineralogy and geochemistry by the MSL Curiosity Rover, organic biosignatures had been seen as a key means of identifying life on Mars. For the identification of organic molecules on Mars has been a focus of much research (Biemann *et al.*, 1976; Benner *et al.*, 2000; Glavin *et al.*, 2001; Quinn *et al.*, 2005). More recently the MSL Curiosity Rover has gathered data on martian organics, although, crucially, without a focus on biosignatures (e.g. (Freissinet *et al.*, 2015)).

Key target molecules, nucleobases and amino acids are considered useful organic biosignatures for life-detection on Mars, with planned missions carrying instruments to detect them, for example, the Mars Organic Analyzer proposed for NASA's Mars 2020 rover (Skelley *et al.*, 2005, 2006; Millan *et al.*, 2014).

Further efforts to detect organic biosignatures on Mars focused on methane, considered to be a reliable indicator of biological activity, and significant quantities were detected in the martian atmosphere (Krasnopolsky *et al.*, 2004; Krasnopolsky, 2006; Atreya *et al.*, 2011; Webster and Mahaffy, 2011; Webster *et al.*, 2014; Yung and Chen, 2015). Presently, the prevailing view is that there is insufficient evidence to support a biogenic origin of methane on Mars, with abiotic sources, e.g. serpentinisation (Oze and Sharma, 2005), considered more likely.

The identification of organic biosignatures is beyond the scope of this report as their accumulation and persistence, at the surface and shallow subsurface at least, is highly unlikely (Fairén *et al.*, 2010).

1.6.3. The limitations of biosignatures

In the absence of the direct discovery of microbial life it is thought that biosignatures could provide a definitive means of identifying life. However, as with the controversy surrounding the purported evidence of fossil life in the ALH84001 meteorite (McKay *et al.*, 1996), and the presence of organic molecules in Viking data, the discovery of a biosignature in an extra-terrestrial habitat would be met with fierce critique because of the impact of such a finding on societal and scientific paradigms. Such a discovery would require incontrovertible proof that it was not the result of terrestrial contamination. It is likely that a single biosignature would never be sufficient and

multiple methods of analysis would be necessary to rule out abiogenic explanations (Banfield *et al.*, 2001).

As with the carbonate globules in the ALH84001 meteorite, true biosignatures are hard to distinguish from their abiotic counterparts. Definitively determining the biogenicity of any biosignature is likely the biggest challenge facing contemporary life detection efforts. However, biosignatures remain the best chance of discovering extinct extra-terrestrial microbial life.

1.7. Ecological processes on Mars

1.7.1. Colonisation and succession

If life had evolved on Mars in a similar way to that on Earth then ecological processes, such as those responsible for colonisation and succession, are likely to have also occurred in a similar way. Therefore, the study of potential colonisation and succession processes on Mars could help to elucidate an understanding of potential martian life.

Succession refers to the changes in an ecological community that are observed following an event that "opens up a relatively large space" (Connell and Slatyer, 1977), i.e. previously occupied niche space becomes available (primary succession), or previously uninhabited environments are colonised (secondary succession; Fierer *et al.*, 2010). This has been documented to occur during processes such as during glacial retreat (Kastovská *et al.*, 2005; Nemergut *et al.*, 2007; Schmidt *et al.*, 2008).

Recently, there has been a shift in focus toward studies of microbial succession, with Jackson (2003) amending a previous model of microbial succession (Jackson *et al.*, 2001) during biofilm formation to improve the representation of competition processes and refine vague terms. Contemporary studies of microbial succession have

utilised this model to understand microbial colonisation and succession in many areas ranging from the effects of nutrient availability (Li *et al.*, 2016) to the debate over the significance of stochastic effects in the initial stages of succession (Dini-Andreote *et al.*, 2015).

The model proposed by Jackson *et al.*, (2001) and amended by Jackson (2003) is demonstrated by Figure 1.3.
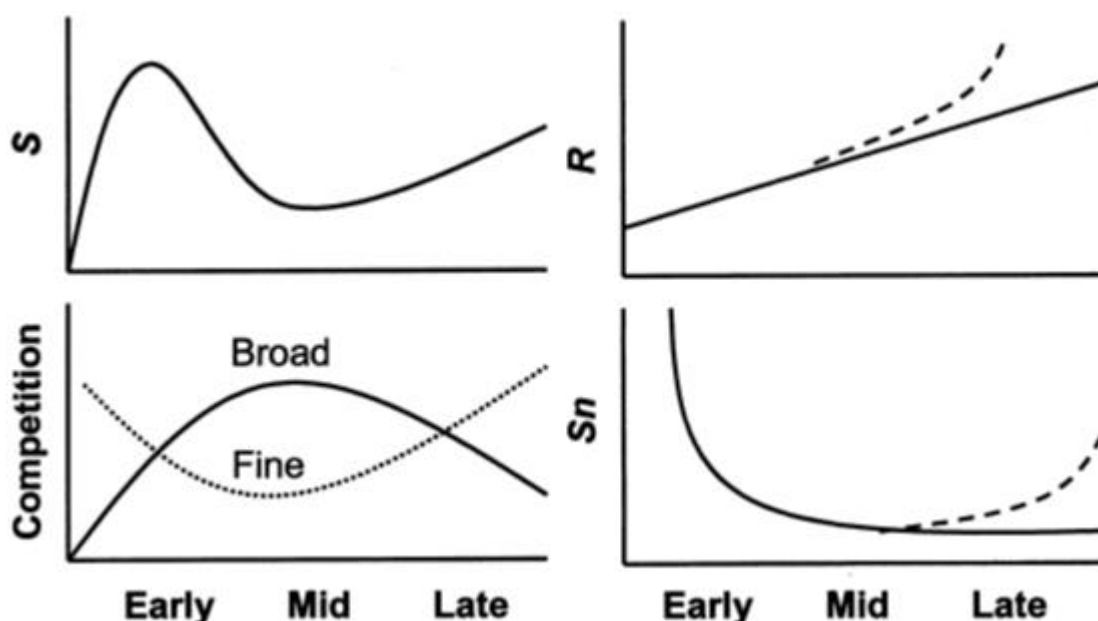
Figure 1.3. Microbial succession model proposed by (Jackson et al., 2001) and amended by (Jackson, 2003). S= Species richness, R= niche space, Sn= New species. Dashed lines denote the expected variation if R were to increase toward the exponential instead of linearly.

According to this model species richness (S) is expected to peak early on, shortly after colonisation, and represents a larger microbial diversity than the environment can support. This peak brings about a change in the type of competition, from fine scale, where competition is for resources that are only required by certain species, to broad scale competition that influences the entire microbial community. This broad scale competition causes species richness to decrease in the middle stage of the succession.

The number of ecological niches (R) increases as the biofilm develops because of the appearance of new substrates for microbial growth, and an adjustment from a two-dimensional community to a community with three-dimensional architecture, allowing for a larger area for attachment and growth (Jackson *et al.*, 2001; Jackson, 2003). With S and R both increasing with time, it is thought that the number of specialised microbial species will require specific resources, therefore increasing the prominence of fine scale competition toward the late stage of succession. As a result of this continuous increase in R, the number of previously undetected species (Sn), after an initial decline from the peak caused by initial colonisation, would either remain constant as R steadily increases, or increase if R tends toward infinity.

By the end of the process of succession, both R and S are thought to level out, producing a relatively stable population. At this stage a switch from bottom-up control through niche diversity to top-down control through trophic interactions has been suggested (Jackson, 2003).

Historically, shifts in microbial community species richness have been attributed to deterministic principles, i.e. through competition or varying environmental conditions (Bååth, 1996; Dini-Andreote *et al.*, 2015). Thus, the tracking of environmental change on Mars throughout its history could inform predictions of possible species richness in putatively habitable martian environments.

The amended model of succession proposed by Jackson (2003) places great importance on the effects of fine scale competition in the early phase, whereas Dini-Andreote *et al.*, (2015) found that stochastic effects had a greater influence than deterministic effects during the initial stages of succession. These stochastic processes include ecological drift and random probabilistic dispersal of species. Dini-Andreote *et al.*'s (2015) results are supported by indirect evidence from several other studies (e.g.

Sigler and Zeyer, 2002; Sigler *et al.*, 2002; Martiny *et al.*, 2003; Inceoğlu *et al.*, 2011; Badri *et al.*, 2013).

Simulation studies can be used to determine which model of colonisation and succession might be applicable to Mars, and in this thesis, the colonisation of a simulated martian environment by a mixed microbial community will be undertaken.

1.7.1.1. Hypothetical succession on Mars

As discussed above, putative martian life would most likely exist/have existed in the form of primitive bacteria/archaea-like organisms (Westall *et al.*, 2015a). Therefore, any potential colonisation of habitable martian environments, either by indigenous life produced by an abiogenesis event, or from some external source, would need to be discussed from a microbial point of view.

Fierer et al. (2010) noted that the categorisation of ecological succession into primary and secondary was limited when applied to microorganisms and suggested instead a three-category system based on a separation of autotrophic and heterotrophic organisms, excluding potentially complex secondary succession dynamics. Heterotrophic succession is then further divided into those with endogenous and those with exogenous carbon sources. Their three categories are:

1. Autotrophic succession – an uninhabited environment is colonised by autotrophic microorganisms, e.g. photoautotrophs and chemotrophs
2. Heterotrophic succession with an exogenous carbon source – an uninhabited environment is colonised by heterotrophs. Organic carbon is continuously provided *via* an external source

3. Heterotrophic succession with an endogenous carbon source – an uninhabited environment is colonised by heterotrophs. Organic carbon is provided *via* a single initial input

With the relatively scarce organic carbon sources available on Mars (see above) it is likely that any microbial community, and perhaps any early martian life, would be composed of, or at least founded on, autotrophy. As discussed above, variants of chemotrophy, primarily chemolithoautotrophy, appear to be the most likely metabolisms for martian life. Thus of the three types of succession described by Fierer et al (2010), autotrophic succession seems most likely. On Earth, this type of succession has been, and is most likely to be, found in volcanic deposits, glacial till and newly exposed mineral surfaces (Hoppert *et al.*, 2004; Gomez-Alvarez *et al.*, 2007; Nemergut *et al.*, 2007; Schütte *et al.*, 2009). This, however, does not preclude it from occurring in the suggested fluvio-lacustrine environment at Gale Crater. Furthermore, the martian equivalents of the three environments listed above would have significant constraints on life, e.g. the surface radiation environment, that are not found in their terrestrial counterparts.

1.8. Outline of work

This study focuses on a comprehensive environmental analysis of the subsurface environment of an intertidal zone (the River Dee estuary) as a potential analogue system for the ancient lacustrine environment at Yellowknife Bay, Gale Crater, described by Grotzinger et al. (2014). Characterisation of the River Dee microbial community facilitated investigation into the application of microorganisms within the community as biological analogues for martian life in the ancient fluvio-lacustrine environment at Gale Crater (Chapter 2).

Using a simultaneous microbial weathering/ecological succession experiment focusing on microbially mediated mineralisation and microbial community structure, carried out within a Mars analogue experimental system, the ecological succession in the potentially habitable Gale Crater environment was investigated and the microbial production of biosignatures assessed (Chapter 3).

Finally, by isolating individual species from the post-experiment microbial community of the Mars analogue weathering/succession experiment, comprehensive characterisation of the growth optima and limits for growth was ascertained for Mars-relevant environmental characteristics. Thus, individual species are presented as potential biological analogues for future investigations into putative martian life in ancient lacustrine systems (Chapter 4).

Chapter 2- The intertidal subsurface microbial community: an analogue for putative martian life

2.1. Introduction

The aims of this chapter are twofold:

1) To determine the plausibility of using a microbial community from an intertidal zone as an analogue for past life on Mars.

2) To characterise the microbial community to determine the constraints of potential habitable martian environments

This chapter builds on the recent insights into martian environments as described in Chapter 1, to identify a novel terrestrial analogue for lacustrine, potentially saline martian environments.

Terrestrial analogues provide an opportunity to investigate planetary processes on Earth before extrapolating the findings to extra-terrestrial environments (Léveillé, 2009; Preston and Dartnell, 2014). They have traditionally been extreme environments and range from highly acidic environments, as in the Rio Tinto analogue site (Fernández-Remolar *et al.*, 2004; Amils *et al.*, 2007), to the extremely cold Antarctic Dry Valley (Wynn-Williams & Edwards, 2000), and the hyper arid environment of the Atacama Desert (Navarro-Gonzalez, 2003).

There are two main aspects of analogue sites that are characterised. Initially, their environmental parameters are investigated and compared to known martian environments. Secondly, investigations into biological processes at the site can be used to infer processes that may have occurred on Mars. For example, the iron bioformations formed by iron metabolising bacteria at Rio Tinto have been studied to

provide insight into biomarker production at Meridiani Planum on Mars (Amils *et al.*, 2007).

To study the possibility of habitability on Mars, microorganisms isolated from analogue environments are commonly used in growth experiments, which mimic the expected conditions on Mars (e.g. Hansen *et al.*, 2005, 2009; Fajardo-Cavazos *et al.*, 2008; Berry *et al.*, 2010; Gómez Felipe *et al.*, 2010; de Vera *et al.*, 2013). These Mars simulated growth experiments have predominantly focused on microbes from extreme environments, such as high saline, cold temperatures and acidic environments(Landis, 2001; Fernández-Remolar *et al.*, 2004; Amils *et al.*, 2007; Direito *et al.*, 2011; Fox-Powell *et al.*, 2016). However, microorganisms isolated from environments that are not typically considered extreme have also been shown to grow in Mars simulated growth experiments. For example, several strains of cyanobacteria were sampled from the marine high-intertidal zone at Beer, UK, which is not necessarily considered extreme, and exposed to the combined environmental conditions of low Earth orbit (LEO) as part of the Biopan VI mission (Delonge, 1992). Among the surviving species were osmophilic, halophilic and UV resistant species (Mancinelli *et al.*, 2004; Olsson-Francis *et al.*, 2010, 2013).

Evidence for the existence of extreme environments on Mars is commonplace, e.g. acidic environments indicated by the detection of jarosite at Meridiani Planum (Klingelhöfer *et al.*, 2004; Aubrey *et al.*, 2006). However, in recent years, *in situ* analyses carried out by the MSL Curiosity Rover has provided a wealth of evidence toward an ancient environment at Gale Crater with moderate conditions that would be ideal for many mesophilic terrestrial organisms (see Chapter 1).

Alongside the significant evidence of past aqueous activity that the MSL Curiosity Rover has collected at Gale Crater, e.g. (Williams *et al.*, 2013; Grotzinger *et al.*, 2014; S.

McLennan *et al.*, 2014; Martín-Torres *et al.*, 2015), it has also characterised the mineralogy such that largely unknown characteristics of the aqueous environment (e.g. pH and salinity) can be ascertained and interpreted to shape the contemporary understanding of these environments, as well as understanding the presence of bioessential minerals and carbon sources (Leshin, Mahaffy, Webster, Cabane, Coll, Conrad, Archer, Atreya, Brunner, A. Buch, *et al.*, 2013; Grotzinger *et al.*, 2014; Ming *et al.*, 2014). The Sheepbed mudstone discovered at Yellowknife Bay gave strong indications of circumneutral water activity because of the prevalence of clay minerals, and apparent sediment transport mechanisms, which also suggested low salinity (Grotzinger *et al.*, 2014; Vaniman *et al.*, 2014). Circumneutral pH conditions have likewise been inferred for other locations on Mars, for example the Terby and Jezero craters (Bethany L. Ehlmann *et al.*, 2008; Ansan *et al.*, 2011; Schon *et al.*, 2012).

At present, there has been only one proposed terrestrial analogue for the processes that occurred when the lacustrine system at Gale Crater was active, and that is Watchett Bay, UK. The formation of sulfate veins at this site has been proposed to be analogous to the formation of sulfate veins in the clay sediments at Gale Crater (Schwenzer *et al.*, 2015). Since Gale Crater is the target of current analysis and likely future Mars missions, understanding the potential microbial processes in this ancient lacustrine environment is of vital importance. Therefore, there is a need for new analogue sites to be identified and characterised, such that life detection missions aimed toward investigating this ancient habitable environment can be designed effectively.

In this chapter, the subsurface environment of the inter-tidal zone at the River Dee Estuary, UK, is proposed as an ideal environmental analogue for the ancient martian lake system at Gale Crater, and perhaps other locations on Mars. Environmental conditions at the estuary are similar to those evidenced by findings at Gale Crater. For

example, the subsurface is isolated from the oxygenated terrestrial atmosphere, creating an anaerobic environment comparable to that of the martian atmosphere. As a terrestrial marine environment, it is analogous to the 1-2 % chloride concentration likely present in the ancient martian lake system (S. McLennan *et al.*, 2014; Vaniman *et al.*, 2014).

This chapter focuses on comprehensive environmental analysis of this potential analogue system and, in highlighting further similarities between it and the ancient lacustrine environment at Gale Crater, presents the microbe-environment interactions there as being relevant to potential relationships between putative martian life its environment. Understanding these processes will allow identification of biomarkers for future life detection missions, and sampling and characterisation of the River Dee microbial community will allow further investigation into how this community, and therefore an analogous martian community, could have been impacted by changing environmental conditions in the Noachian and early Hesperian (Mars, 3.95-3.7 Ga and 3.7-3.0 Ga respectively), among many other applications.

2.2. Methods

2.2.1. Sample collection

The sample site for this study was the River Dee estuary, UK (Figure 2.1) 53°21'15,40N 3°10'24,95 W, near West Kirby. The location of the sample sites (n = 12) is shown in Figure 2.1. Samples were taken in November 2013.
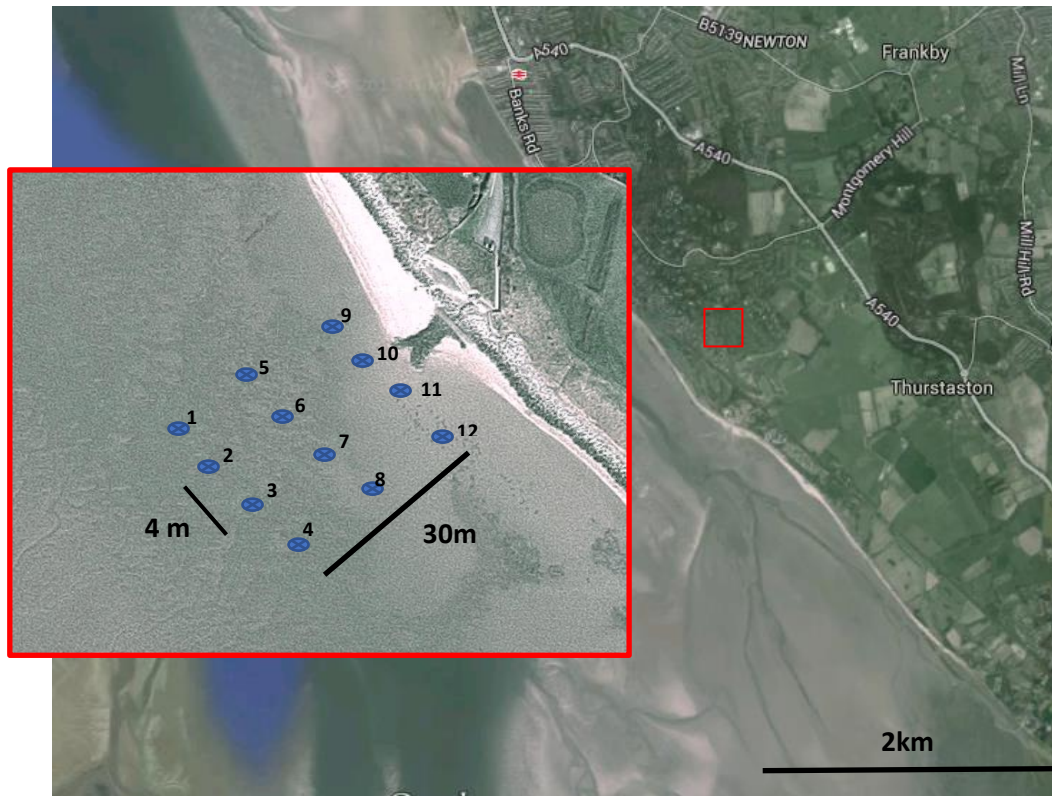
Figure 2.1. The sampling area at the River Dee estuary. The large red box denotes a magnification of the small red box permitting sufficient detail to be observed. Circular markers denote the 12 individual sampling sites.
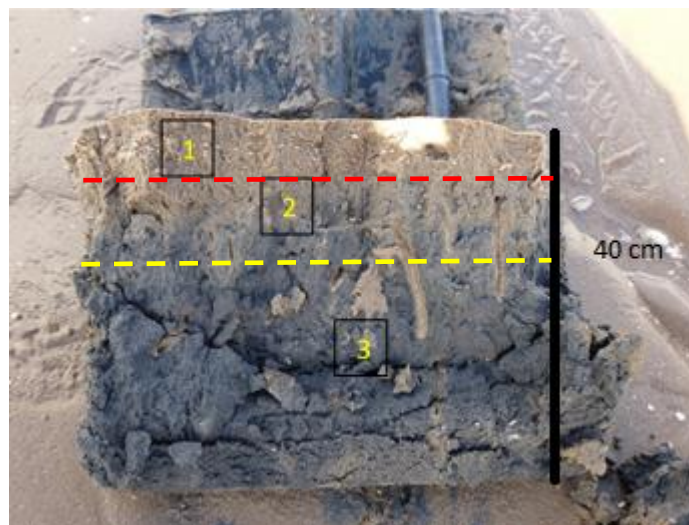
Figure 2.2. A cross section of sample site 3 of the Dee estuary showing aerobic (1), microaerophyllic (2) and anaerobic (3) zones. Dotted lines denote the borders between the three layers. The yellow dotted line represents the RPD.

Sample sites were set out in four rows (see Figure 2.1) 10 m apart, running parallel to the estuary, the closest being approximately 1 km from the water at low tide. The sample sites were situated in the inter-tidal zone, between low and high tide and were subject to diurnal flooding. Samples were collected from three sites along these rows,

at 4 m intervals. At each of the 12 sites, a cross section of the seabed was obtained using a flat headed spade (Figure 2.2). As expected, qualitative analysis of the cross sections showed that the sand was drier moving inland. Characterisation of environmental parameters was carried out *in situ* (Section 2.2.2).

From each of the sites, samples were collected from the anaerobic zone (Figure 2.2). The Redox Potential Discontinuity (RPD), the boundary between the microaerophyllic and the anerobic zone, was used as a marker to ensure samples were taken within the anaerobic zone. The RPD was identified by the apparent transition to black sulfides, indicative of a common form of anaerobic metabolism, as shown in Figure 2.2.

Ten grams of material was collected from each sample site and stored in a plastic Whirl-Pak bag for Total Organic Carbon (TOC) analysis. For culturing experiments (see Chapter 3), approximately 20 g of sample from each site was aseptically stored in 30 mL plastic screw-capped bottles, which had been autoclaved and flushed with N_2. The bottle was filled completely with the sample to minimise the presence of the oxygen-rich atmosphere, and then quickly sealed to reduce any further atmospheric exposure.

All of the samples were then stored for transit in an insulated container along with several ice packs (at approximately 4 °C) for six hours. This was carried out to prevent cell death and DNA breakdown. Upon arrival back at the laboratory, sub-samples were taken for DNA analysis and stored in a -80 ˚C freezer and all others were refrigerated at approximately 4 ˚C.

2.2.2. Environmental characterisation

The environmental conditions at the River Dee site were characterised in the following ways:

1. Temperature (*in situ*)

2. pH (*in situ*)

3. TOC

4. Mineralogy

2.2.2.1. Temperature measurements

Temperature readings were taken at approximately midday. Partial cloud cover prevented most direct sunlight from reaching the surface. Where direct sunlight did reach the sample sites, care was taken to avoid the part of the sample sites that had been warmed. However, all readings were taken 2-5 cm beneath the exposed surface and thus were unlikely to have been significantly affected by such short exposure to direct sunlight.

The sample site will have been subject to diurnal and seasonal temperature variations, possibly resulting in sub-zero temperatures in the winter and elevated temperatures in the summer. In addition, diurnal variations would have likely resulted in a significant variation in temperature.

Temperature measurements were carried out *in situ* using an RS 1327K Infrared Thermometer. The probe was inserted into the sample site, below the RDP zone, taking care to avoid any areas that were in, or had been in, direct sunlight. The probe was left in place until a steady reading was obtained.

2.2.2.2. pH measurements

pH measurements were carried out *in situ* using a Thermo Scientific Orion Three Star pH probe. The probe was calibrated using Omega Buffer solutions at pH 4 and 10. The

probe was inserted into the anaerobic zone and left in place until a steady reading was obtained. As the probe measures in real-time, adjusting to variations as they occur, once a stable figure was reached it was sufficient to record the value in place of taking repeated measurements.

2.2.2.3. Total Organic Carbon measurements

Total Organic Carbon (TOC) measurements were carried out using a Shimadzu Total Carbon Analyser (TOC-V $_{C5N}$) with an SSM-5000A solid state module. Prior to analysis, 1 g of sample (n =3), was thawed and dried for 2 hrs at 180 °C to ensure that no water entered the instrument. The samples were accurately weighed and transferred into a ceramic boat and covered with ceramic wool. The sample was heated in the TOC furnace at 900 °C until no more CO_2 was detected in the instrument.

Glucose was used as a standard, and intra-sample machine drift was accounted for by rerunning glucose standards after every 10 runs.

2.2.2.4. Mineralogy

A polished thin section of a pellet of compressed sand was mounted on an epoxy-resin block and carbon-coated using an EMITECH K950X Turbo carbon sputter coater. This was examined using a Quanta 3-D Focused Ion Beam Scanning Electron Microscope (FIB-SEM). Energy Dispersive X-ray spectroscopy (EDS), for the creation of element maps and to conduct point & ID analysis, was carried out using the attached Oxford Instrument INCA energy dispersive X-ray detector. An acceleration voltage of 20 kV and a beam current of 0.6 nA was used to acquire all images and maps.

This setup was used to identify the modal mineralogy of the anaerobic layer of the River Dee sediment. Twenty Backscattered Electron (BSE) images were captured at 427× magnification and point & ID EDS spectra (30 second exposure) were obtained. This aided the identification of the bulk composition of large grains in the images.

Using the 20 BSE images, the proportions of each mineral were calculated using ImageJ software. The percentage area of each image occupied by each mineral phase was measured. Approximately 30 % of each image was occupied by resin only. This was removed from the calculation before normalising to 100 %. A mean composition was calculated based on the proportions of each mineral in the 20 images. To ensure that the true mineralogy of the sample was being obtained, a BSE image at only 100× magnification was obtained, which selected a portion of the sample that appeared to be representative of the whole, and treated identically to the other higher magnification images.

In addition to this, the elemental composition of the minor mineral phases was examined through the construction of detailed element maps. The presence of Si, Na, K, Al, P, S, Cr, Fe, Ti, Mg, Ca and Cl were mapped to facilitate mineralogical identification.

Quantification of the elemental composition was not possible due to instrumental constraints, and the presence of some elements can be false positives where spectroscopic peaks are in close proximity or overlap. The likelihood of various minerals being present was considered with regard to the bulk mineralogy and the likelihood of minerals with such a composition existing in this environment.

2.2.3. Microbial community analysis

A molecular approach was used to characterise the microbial community at the River Dee site, including the following analyses/procedures:

- Cell enumeration

- DNA extraction

- Terminal Restriction Fragment Length Polymorphism (TRFLP)

- MiSeq DNA sequencing

2.2.3.1. Cell enumeration

Prior to storage in the -80 °C freezer, approximately 1 g portions of the anaerobic samples were aseptically weighed and suspended in 1 mL of MilliQ H_2O. The cells were stained with Sybr Green DNA stain, which fluoresces under ultra-violet light. The 1 mL suspension was homogenised by agitation and inversion. Ten μL of the homogenised sample suspension was stained in the dark for 20 min with 10 μL of a 0.25 % solution of Sybr Green on a microscope slide. Cells were enumerated by epifluorescence microscopy using the 485 nm wavelength blue light (Green *et al.*, 1997), under a Leica DM 4000 B microscope, as previously described by Summers *et al.*, (2013). Visible cells within twenty frames of view, were counted in each of the samples and the numbers of cells per gram of sample was calculated.

Where necessary, cells were differentiated from mineral fluorescence by noting the morphology of the fluorescing regions in the image with regard to common bacterial morphologies. In addition, it has been shown that unspecific binding and high background fluorescence is mostly avoided by the use of Sybr Green over other DNA

stains for epifluorescence cell enumeration (e.g. DAPI), increasing the fluorescence yield of cells by at least an order of magnitude (Weinbauer *et al.*, 1998).

This cell enumeration method does not distinguish between archaea and bacteria and so gives an absolute value for the density of the microbial population within the samples.

2.2.3.2. DNA extraction

Total nucleic acids were extracted using an adapted version of that described in Griffiths *et al.*, (2000). 0.5 g of sediment from each of the River Dee sample sites was transferred into a 2 mL MP Biomedicals Lysing Matrix tube with 0.5ml of 120 mM K_2PO_4 buffer pH 8.0 with 5 % Cetrimonium bromide (CTAB), and 0.5 ml of phenol:chloroform:isoamylalcohol in the following proportions 25:24:1. The samples were shaken at 5500 rpm for 30 s using an MP Fastprep-24 and centrifuged at 10,000 × *g* for 15 minutes at 4 ˚C. The supernatant was then removed, and 0.5 ml chloroform: isoamylalcohol was added. The DNA was precipitated overnight at 4 ˚C using PEG (1.6 M NaCl, 30 % PEG 6000). The precipitate was then centrifuged again at 14,000 × *g* and 4 ˚C for 10 min.The DNA pellet was washed with 70 % ethanol, air dried and resuspended in a solution of 10mM Tris base with 1 mM EDTA (known as TE buffer).

2.2.3.3. Terminal Restriction Fragment Length Polymorphism (TRFLP)

TRFLP analysis comprises of three steps:

1. DNA amplification *via* a PCR reaction

2. Digestion of the amplified DNA using a restriction enzyme

3. Fragment Analysis (Macrogen, S. Korea)

Restriction enzymes bind to specific sites (cleavage sites) on the amplified DNA and cut the DNA. Variation in DNA sequence between different microbial groups means that DNA fragments produced by the restriction enzyme will vary in size according to the specific microbial group. The frequency of each DNA fragment is ascertained during the final stage of TRFLP analysis and therefore it is possible to identify all bacterial groups within a sample, as well as their relative proportions.

In this study, TRFLP analysis was carried out because it provided a low cost, high-throughput means of measuring molecular diversity, which is essential in order to obtain a comprehensive understanding of the microbial community at the sample site.

The 16S rRNA gene was partially amplified for bacteria and archaea using 63f and 530r (Muyzer *et al.*, 1993; Marchesi *et al.*, 1998), and A341f and A1204r (Baker *et al.*, 2003) primers respectively. Crucially, the 63f and A341f primers were labelled with the 6FAM fluorescent dye, which facilitates the subsequent Fragment Analysis by Macrogen. Otherwise, the amplifications were carried out identically using 1 µL template DNA, 250 nM of each primer, 5 µL of 10 × Invitrogen Taq buffer, 2 mM $MgCl_2$, 0.1 mM of each Deoxynucleotide Triphosphate (dNTP), 5 µg bovine serum albumin (BSA) and 1.75U of Taq. PCR conditions were as follows:

2.2.3.3.1.Bacteria

Initial denaturation at 94 °C for 10 mins, followed by 35 cycles of: denaturing 45 sec at 94 °C, annealing 1 min at 56 °C, elongation 3 min 72 °C. Final elongation for 10 minutes at 72 °C.

2.2.3.3.2. Archaea

Initial denaturation at 94°C for five minutes, followed by 35 cycles of denaturing for one minute at 94°C, annealing at 55°C for one minute and extension at 72°C for one minute, followed by a final extension for ten minutes.

The amplified DNA was then purified using a Qiagen PCR purification kit according to manufacturer's guidelines. DNA purification, though it reduces the quantity of recoverable DNA, is essential since unreacted PCR reactants and undesired products would interfere with the Fragment Analysis. DNA concentrations were quantified using a ThermoScientific NanoDrop 100 spectrophotometer. Purified PCR products were digested using MspI restriction enzyme, which cuts at the ccgg binding site, and producing 16S DNA fragments, which can be used for TRFLP analysis. The PCR products were digested in 10 μL reaction mixtures containing X μL of purified PCR product, 0.1 μL of BSA, 1 μL of CutSmart enzyme buffer (× 10), 0.3 μL of MspI (30 U/μL), Y μL of sterile distilled water (to make up to 10 μL) and incubated at 37 °C overnight. The samples were stored at -20 °C before analysis. Values of X and Y were calculated using the DNA concentration indicated by spectrophotometry, to achieve a final DNA concentration of 10 ng/μl.

Fragment analysis was then carried out on digested samples by Macrogen, using a 3730 DNA sequencer (Applied Biosystems, CA, USA). Analysis of the electropherograms was carried out using SoftID Genemarker V2.6.4. Data quality checks were carried out and peaks below 100 bp in length (to avoid primer dimers and significant noise) and below a frequency of 50 counts were disregarded. All peaks within these parameters were assigned and applied to the data sets. Frequencies of each significant fragment in each sample could then be obtained.

2.2.3.4. MiSeq DNA sequencing

To facilitate the identification of the TRFLP fragments, MiSeq sequencing was carried out on the DNA extract of the anaerobic zone at site three. The extracted DNA was sequenced at The Research and Testing Laboratory (Texas, USA) using a MiSeq desktop sequencer. Sequencing was carried out with 28f and 530r primers for bacteria, and 340wf-534r for archaea. *In silico* digestions were carried out using TRFLPMAP software (http://nebc.nerc.ac.uk/cgi-bin/trflp0_2.cgi) using the same restriction endonuclease MspI for both bacterial and archaeal DNA sequences. Some fragment sizes can represent many different taxa, therefore different taxa, which shared the same cleavage site were grouped and the taxon that accounted for >75% of the sequences was assigned to that fragment.

2.2.3.5. Statistical analyses

Statistical analyses were carried out using the following software packages Primer 5 (Clarke, 1993; Clarke and Warwick, 2001), R version 3.2.4 (R Development Core Team, 2013) and Microsoft Excel 2016.

Primer 5 was used to carry out Analysis of Similarity (ANOSIM) and Similarity Percentages (SIMPER) analyses, and construct Principal Component Analysis (PCA) plots. These analyses were used to investigate the source of the similarity or dissimilarity in the microbial populations sampled at each of the sample sites.

ANOSIM compares the mean of ranked dissimilarities between groups to that within groups, assuming the ranges of ranked dissimilarities within groups are equal. This provides a value, usually between 0 and 1 that denotes the level of dissimilarity between groups, with 1 being absolute dissimilarity.

In contrast to this, SIMPER attempts to calculate the contribution of individual variables to the dissimilarity between objects, thus providing further resolution and potentially identifying the source of any dissimilarity suggested by ANOSIM.

R v3.2.4 was used to generate rarefaction curves that were used to determine whether the samples taken were representative of the environmental community. Rarefaction curves are a plot of the number of species as a function of the number of sampling events. Thus, when the curve plateaus and increased sampling events do not increase species richness, it is possible to infer that the environmental site has been sufficiently sampled to ensure full representation of the species present.

Microsoft Excel 2016 was used to calculate all means, standard deviations, and carry out Analysis of Variance (ANOVA) analysis. ANOVA analysis provides a quantitative means to investigate the degree of interaction between environmental characteristics, e.g. temperature of sample site and distance from the shoreline, by suggesting the probability of an observed pattern or association of variables being attributable to chance.

2.3. Results

2.3.1. Environmental characterisation

Data from the twelve sites, in terms of temperature, pH and TOC, were collated to aid further analyses (Table 2.1).

Table 2.1. Combined environmental data for the twelve sampling locations at the River Dee site

Site	Temperature (°C)	pH	TOC (%)
1	12.2	8.23	1.500
2	12.2	8.14	1.464
3	11.6	8.08	1.491
4	11.8	8.45	1.470
5	12.3	8.31	1.103
6	13.3	8.38	1.840
7	11.9	8.29	1.412
8	12.3	8.08	0.994
9	14.3	8.09	0.740
10	12.4	8.1	1.262
11	12.6	8.09	1.153
12	12.4	8.39	1.024

2.3.1.1. Temperature

Temperature measurements taken from the anaerobic zone at the 12 sample sites were split into three groups, representing the three different sampling rows (four samples in each row). Mean temperatures were calculated for each of the rows ($n=4$) and plotted against the approximate distance from the shoreline (Figure 2.4.). The temperature probe measures to an accuracy of ±2 %. Though outliers can be identified in Figure 2.3, Analysis of Variance (ANOVA) at the 95% confidence level still showed no significant differences between any of the three sampling rows ($p= 0.167$). Thus, it is possible to average all twelve data points to obtain a representative average temperature for the anaerobic zone of the locality. This value was found to be 12.4 ± 0.7 °C (one standard deviation). Outliers at 14.3 °C (1020 m) and 13.3 °C (1010 m) were

removed because of exposure to direct sunlight during measurement. This reduced the

average to 12.2 ± 0.3 °C (one standard deviation) (see Table 2.1).
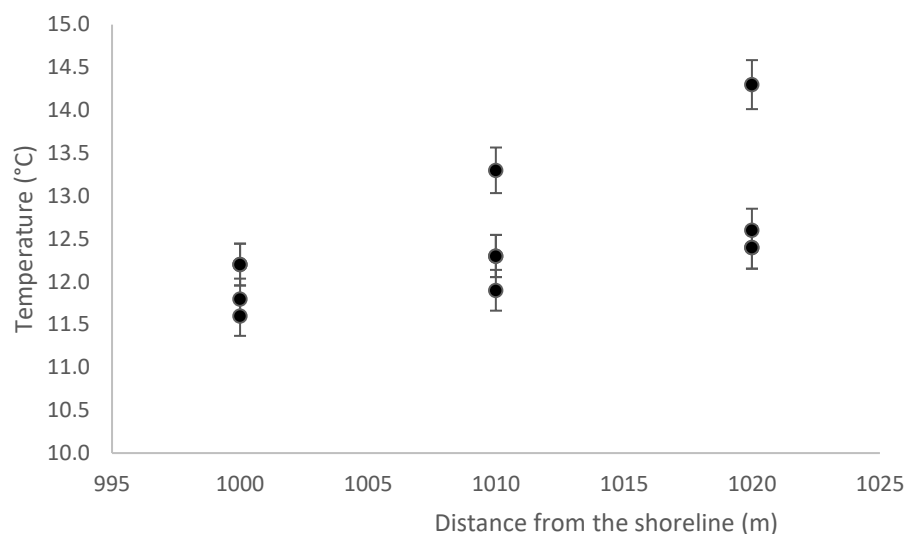
Figure 2.3. Individual temperature measurements plotted against distance from the shoreline. Error bars represent the instrument error.
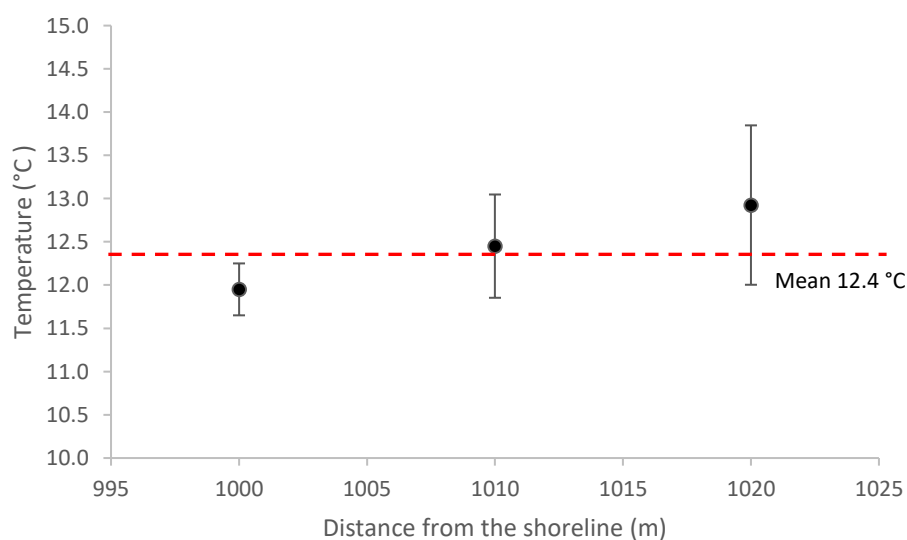
Figure 2.4. Scatter graph showing the mean temperature (without outliers) measured at each sample site plotted against the distance from the shoreline. Error bars denote one standard deviation.

2.3.1.2. pH

As above, mean pH values (*n*=4) were calculated for each of the sampling locations and

plotted against the approximate distance from the shoreline. Approximately 0.5 pH

unit variations were recorded between samples for all sampling rows (see Figure 2.6), with negligible variation accounted for by the ± 0.002 pH unit accuracy of the pH probe. ANOVA carried out at the 95% confidence level failed to show any significant differences (p = 0.655).

In the absence of any significant differences between the sampling rows, an average was taken of all twelve data points. The mean pH at the sampling locality was calculated to be 8.2 ± 0.1 (one standard deviation).
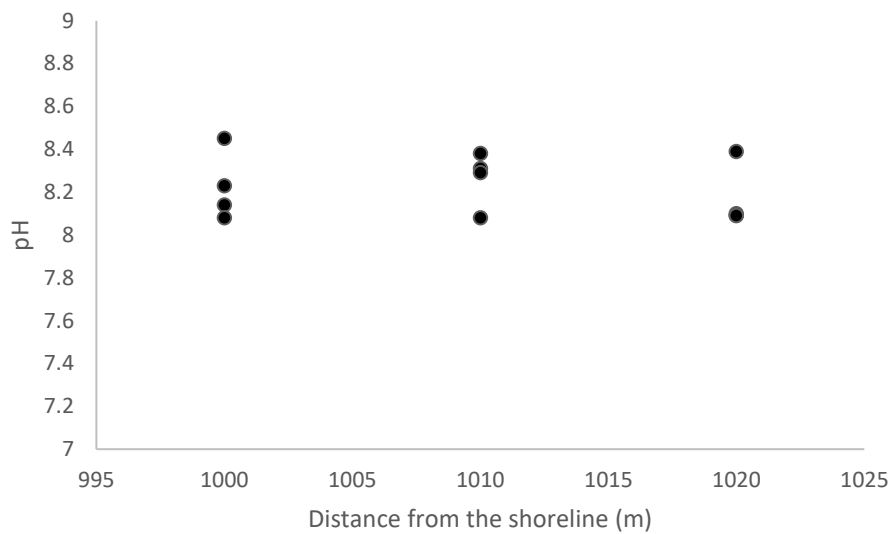
Figure 2.5. Individual pH measurements plotted against distance from the shoreline. Error bars (obscured by markers) represent the instrument error.
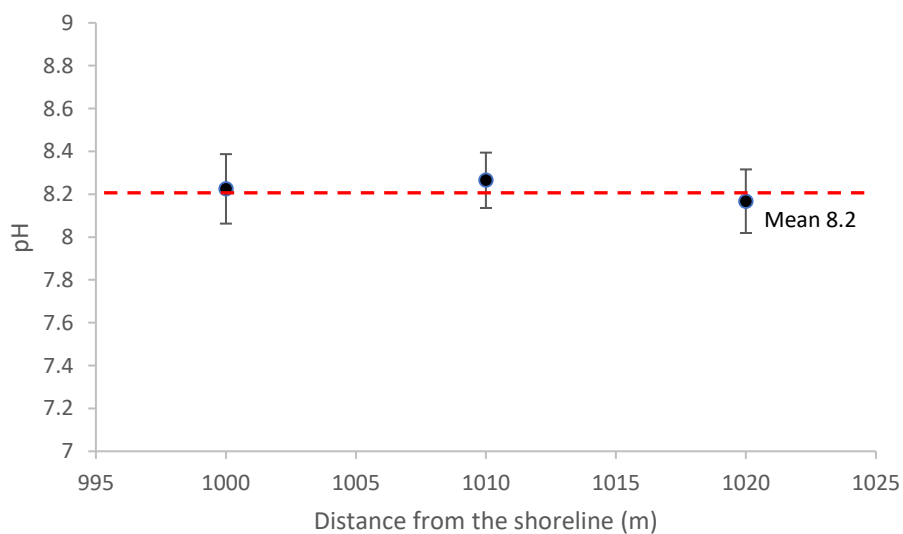
Figure 2.6. Scatter graph showing mean pH, for data collected at all three layers of sediment, at each sampling row plotted against approximate distance measured from the sea. Error bars denote one standard deviation.

2.3.1.3. Total Organic Carbon

Figure 2.7 illustrates the mean TOC results for the three sampling rows. There was only approximately 1 % observed variation in the (n=4) TOC values obtained from the four samples taken from the sampling row 1000 m from the sea. The other sampling rows (1010 m and 1020 m) had much larger variation (28 % and 21 % respectively; Figure 2.7). ANOVA was undertaken to compare the three sampling rows to each other but found no significant differences in variation at the 95% confidence level (p= 0.099). The mean TOC value for the whole data set was therefore calculated to be 1.29± 0.3 weight%.
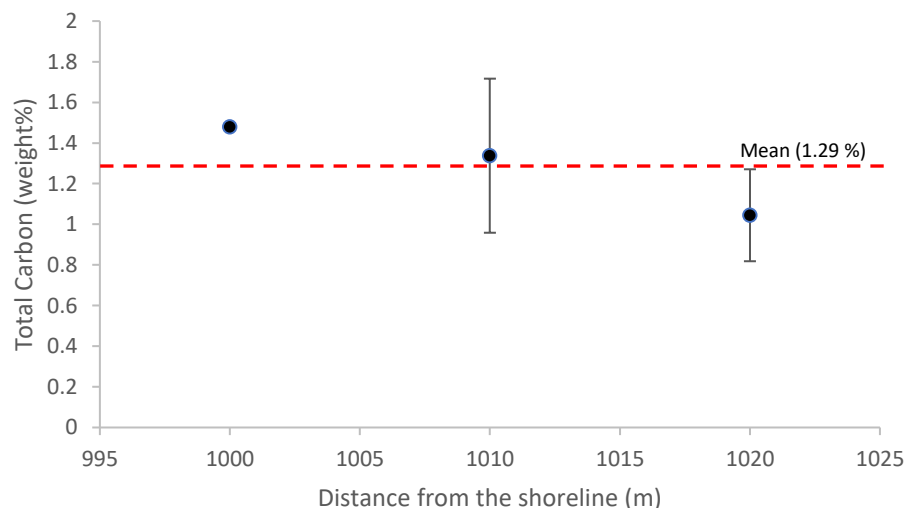
Figure 2.7. Scatter graph showing mean TOC content of samples collected from the anaerobic zone of each sampling row plotted against approximate distance measured from the sea. Error bars represent one standard deviation but are obscured by the marker in the 1000 m sampling row.

2.3.1.4. Mineralogy

The variability of the mineralogy at the River Dee site is demonstrated by the BSE image in Figure 2.8. The black areas represent the resin that the sample was mounted in, the dark grey areas are predominantly quartz grains, with lighter grey representing feldspars. EDS Point & ID was used to provide these identifications (Figure 2.9). The feldspars analysed were potassium rich and consistent with the mineral sanidine. Mineral grains that appeared bright white in the BSE images (labelled A-C in Figure

2.10) were analysed with EDS Point & ID and were mainly found to be iron-rich with some titanium. This is consistent with titanohaematite. Exceptions to this were a rare phosphate-rich grain with calcium (Figure 2.12), a single grain of zircon (Figure 2.11) and a second zircon grain with an apatite inclusion (Figure 2.10). The zircons and phosphate grains observed accounted for approximately 5-10 of approximately 400 individual grains in the mounted sample. Both the phosphate inclusion and the calcium-rich phosphate grain have been attributed to apatite. Using Figure 2.8, the approximate mineralogy of the River Dee sample was found to be ~ 67% quartz, 28 % clay (see Figure 2.9 for identification) and 5 % combined minor phases

Feldspar.

Quartz grain.

Minor fractions, mainly Titanohaematite.

Detrital material.

| HV | det | curr | WD | mag | tilt | 1 mm |
| 20.05 kV | DualBSD | 0.60 nA | 15.0 mm | 100 x | -0 ° | |
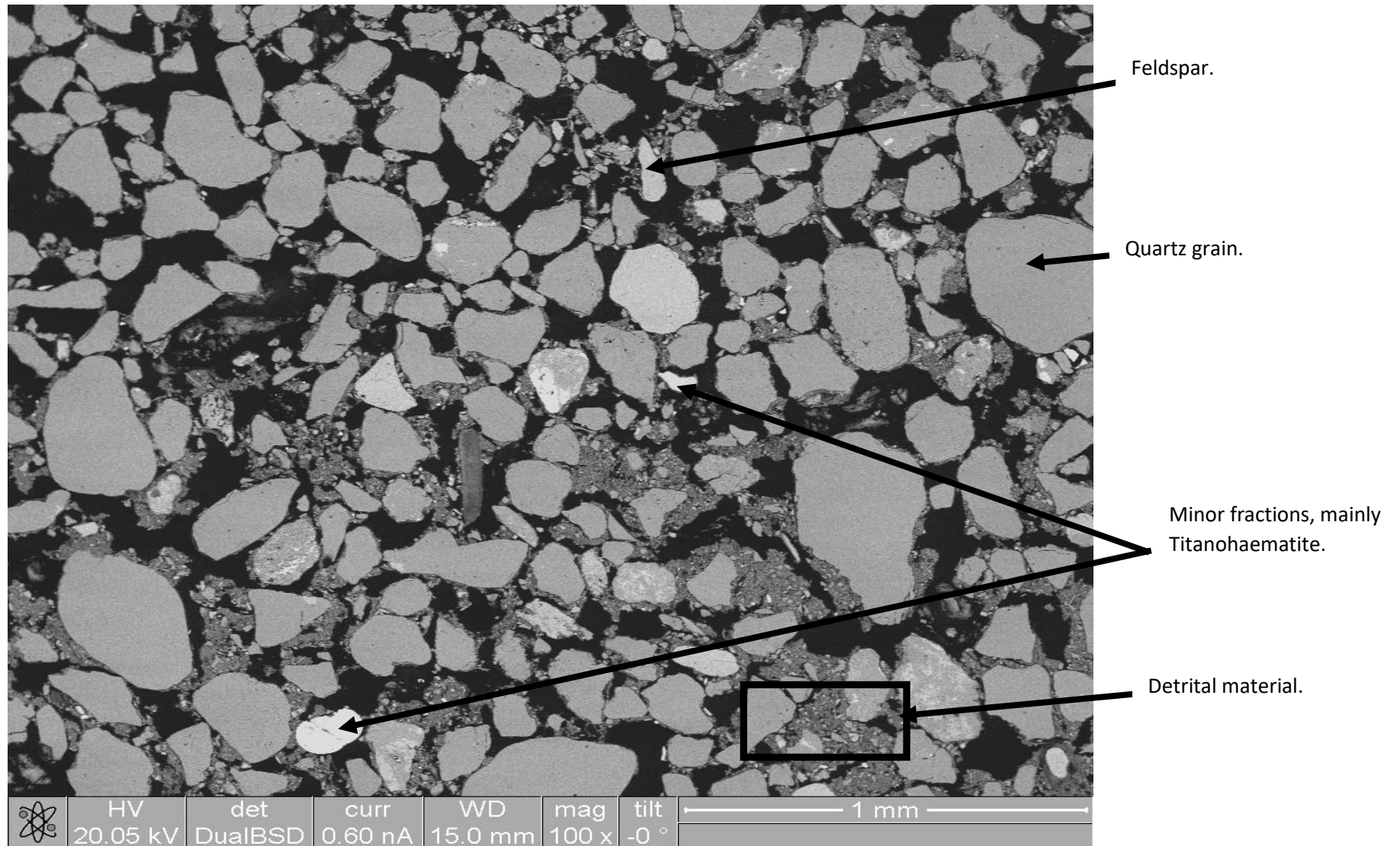
Figure 2.8. 100× magnification BSE image showing the broad mineralogical composition of the River Dee sediment.

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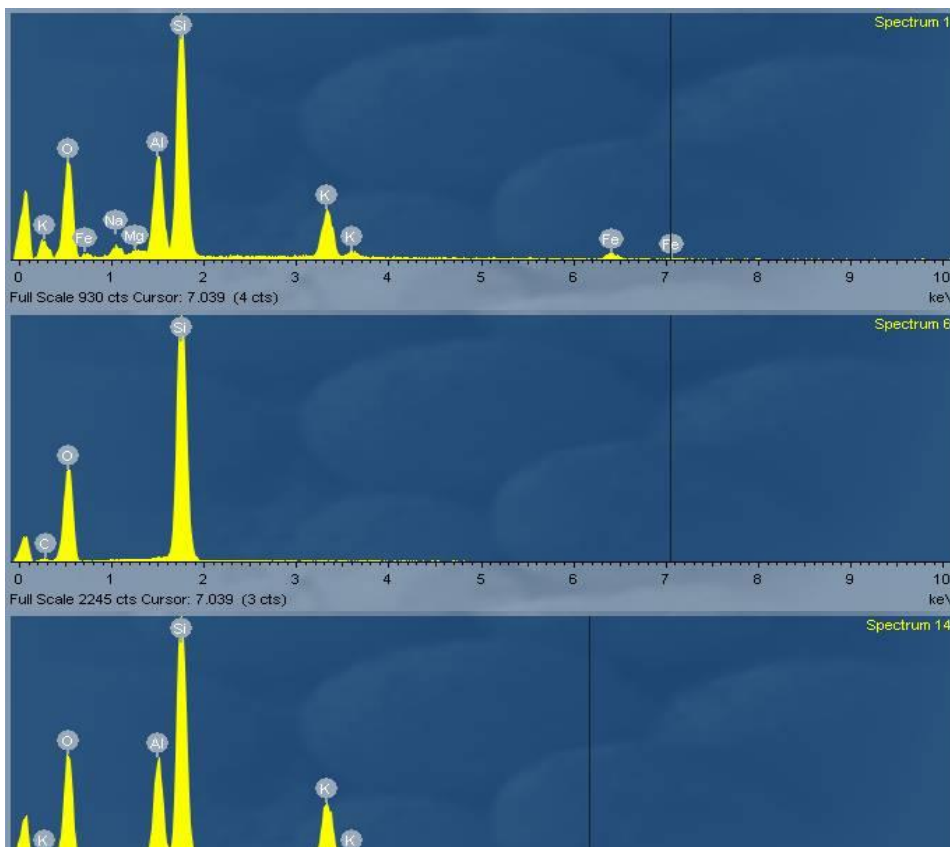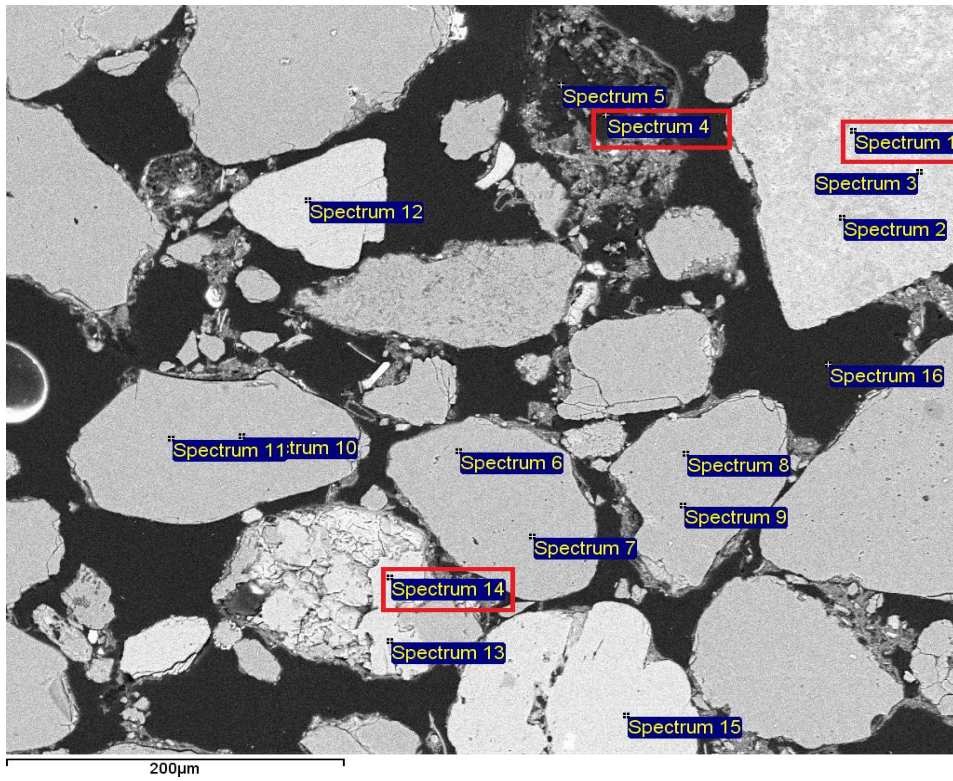
Figure 2.9. BSE image showing EDS Point & ID spectra locations with example spectra for quartz, sanidine and clay (Spectra 6, 1 and 14 respectively).

In addition, using 20 427× images the modal mineralogy was calculated. Quartz was

found to be the major constituent accounting for ~86 % of observed minerals with

sanidine and a combined minor fraction (comprising mainly titanohaematite) making up 12 % and 0.5 % respectively. Other trace phases were also present (see Figures 2.11 and 2.12). This was incongruous with the findings from the single 100× magnification image (Figure 2.8) which suggested that the 20 images were not representative of the entire sample. However, it was not possible to distinguish between quartz and feldspar minerals in the single 100× image.

Assuming a similar abundance of feldspar in the high-resolution images as in the low-resolution image (Figure 2.8), the value for quartz was adjusted to what was likely misidentified feldspar. Thus, these two incongruous modal mineralogies were combined to give a more representative modal mineralogy for the sample with 63 % quartz, 23 % clay, 9 % sanidine and 5 % minor fractions (zircons etc., see below).

A. Fe/Ti rich grain

B. Fe/Ti rich grain

C. Zircon grain with apatite inclusion

300µm
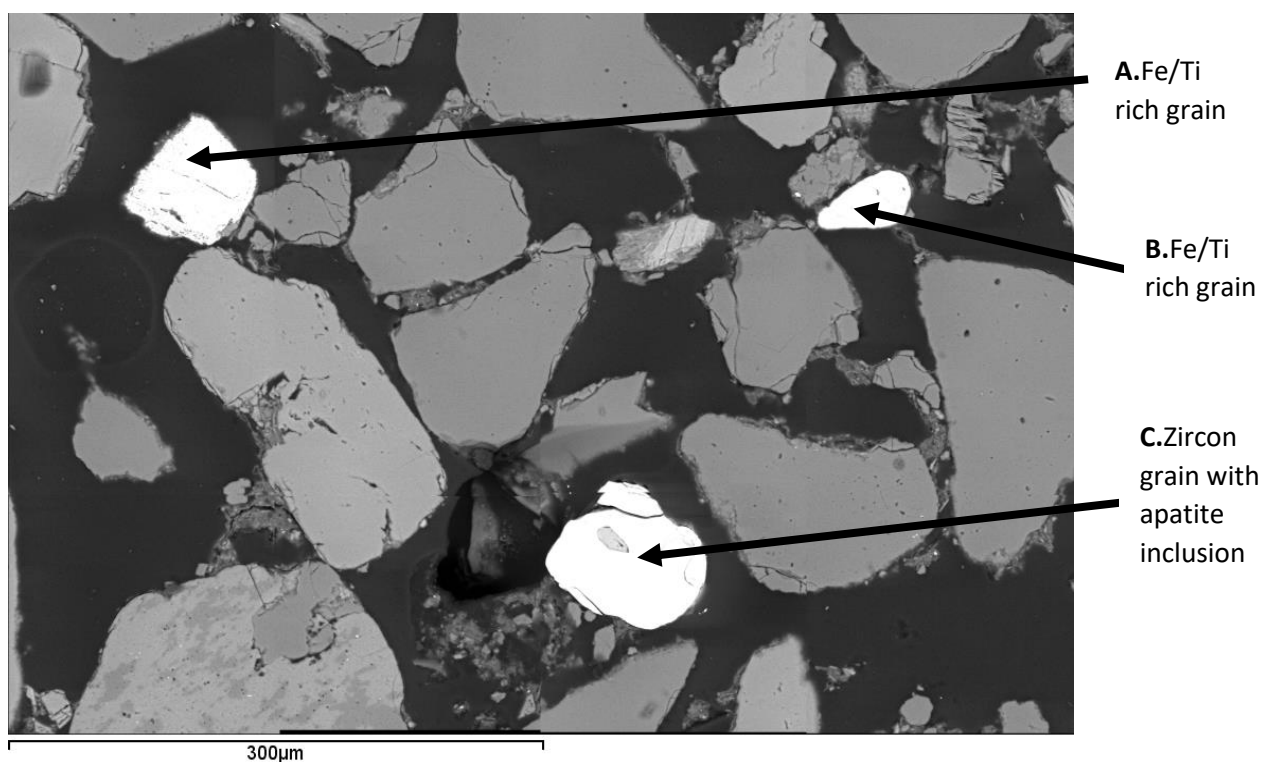
Figure 2.10. BSE image focusing on 'bright' minor phases.
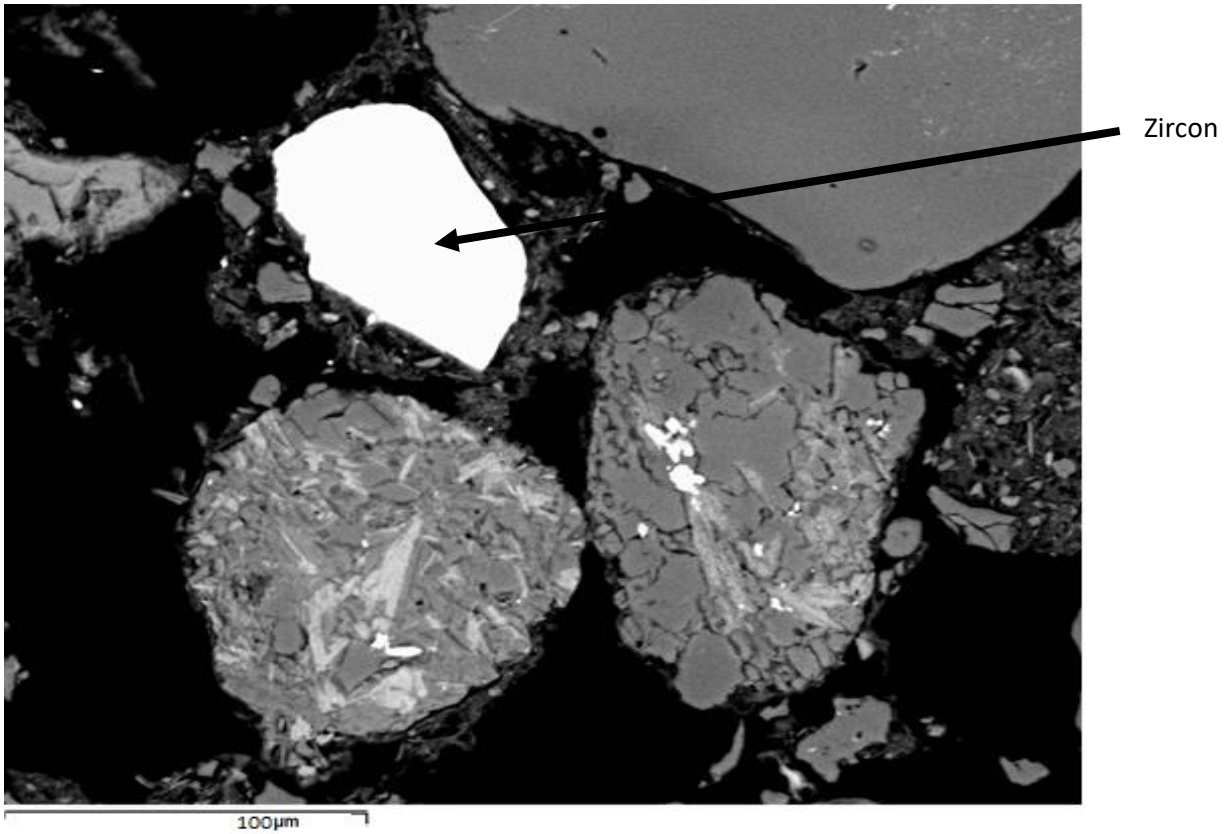
Figure 2.11. BSE image showing a zircon grain.
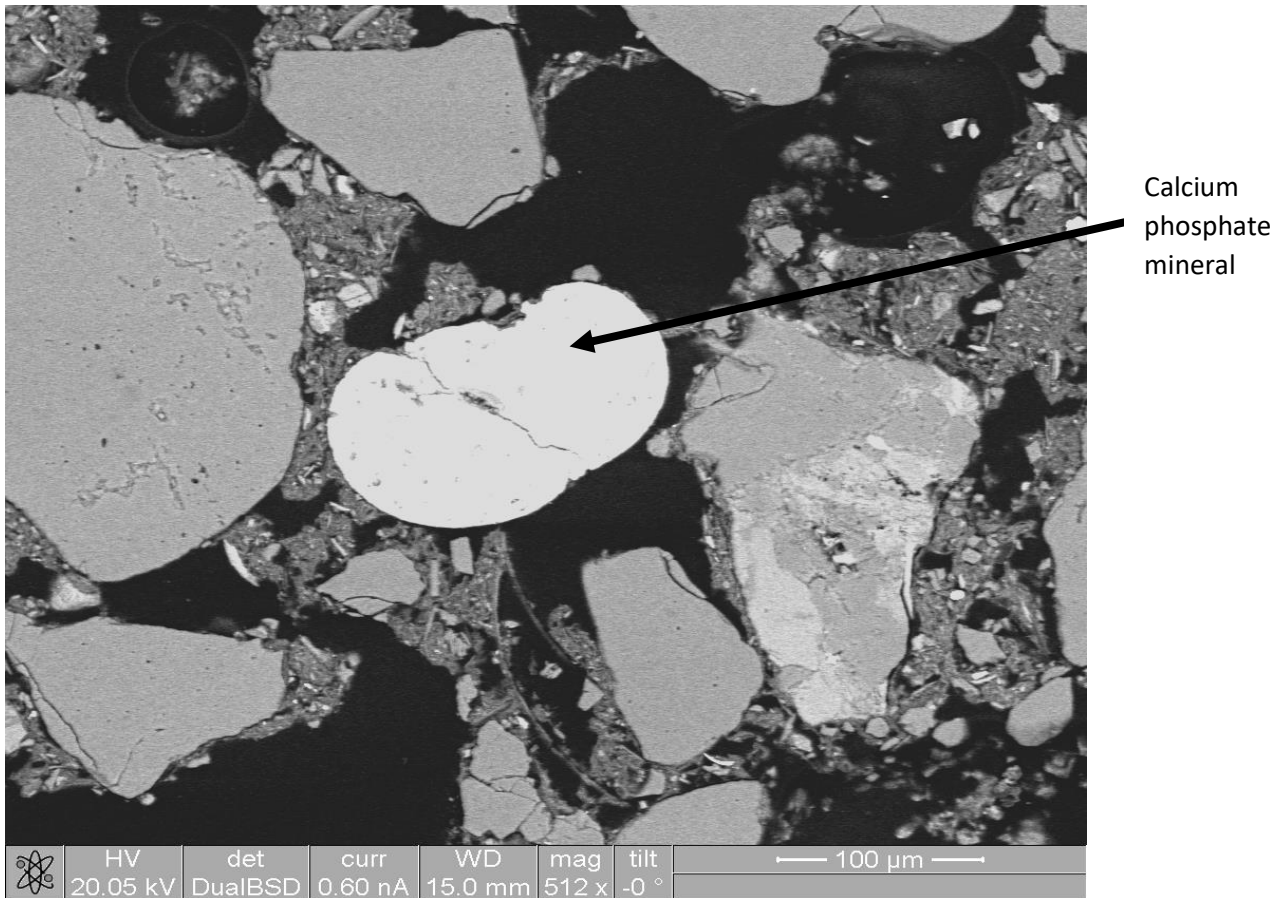
Figure 2.12. BSE image of a calcium phosphate mineral within detrital clay material.

The 427× images made it possible to analyse the detrital material with a higher resolution than Figure 2.8. BSE images and detailed false-colour element maps (e.g. Figures 2.13.-2.15) show that the detrital material was rich in Al, Ca, K, Mg, Si and O. This is consistent with phyllosilicate clay minerals. Element maps (Figures 2.14 and 2.15) show the distribution of these elements within this material.

Combining Figures 2.14. and 2.15, the main body of the detrital material is shown to be rich in Mg, K, Al and O and likely represents illite clay. Fe was also found to be present within the clay. Purple grains in Figure 2.14 were shown to be rich in both Ca and Mg, and are distinct from the matrix. Figure 2.15 shows that these grains also contain O. These purple grains are likely to be diopside. 1-5 µm crystals of calcite, rich in both Ca and O, are visible in both figures. Larger, 10-50 µm, potassium-feldspar grains were observed in the matrix. Fragments of quartz were also present.
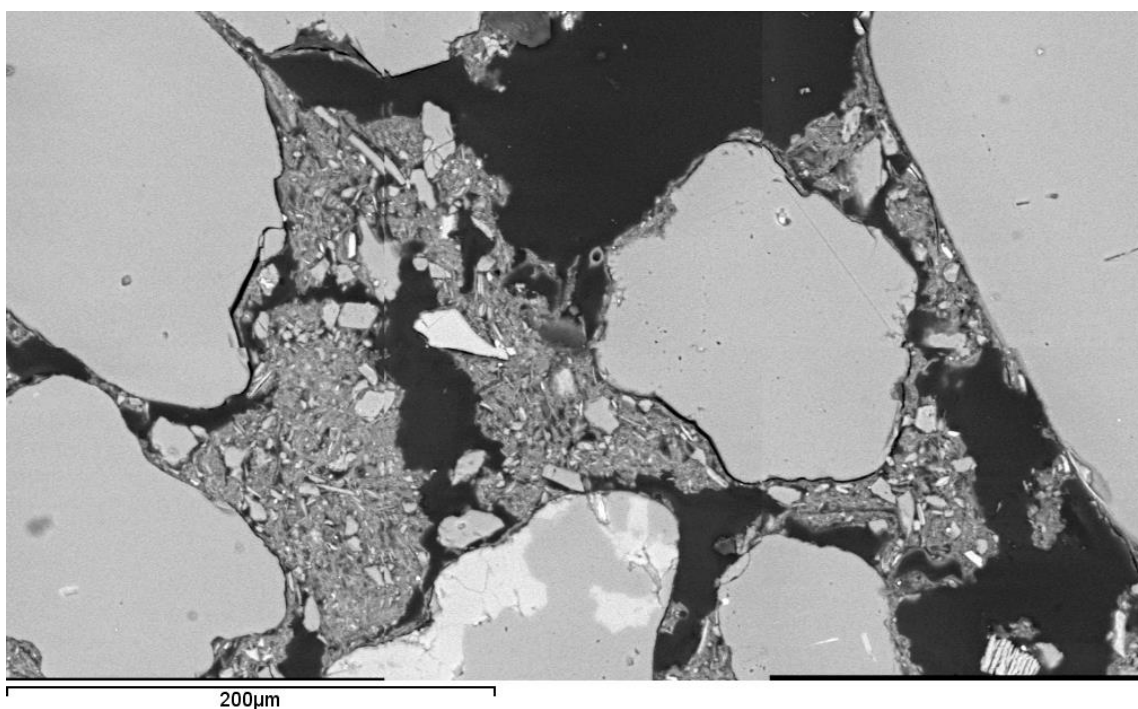
Figure 2.13. BSE from which the false-colour element maps in 2.14 and 2.15 were created.
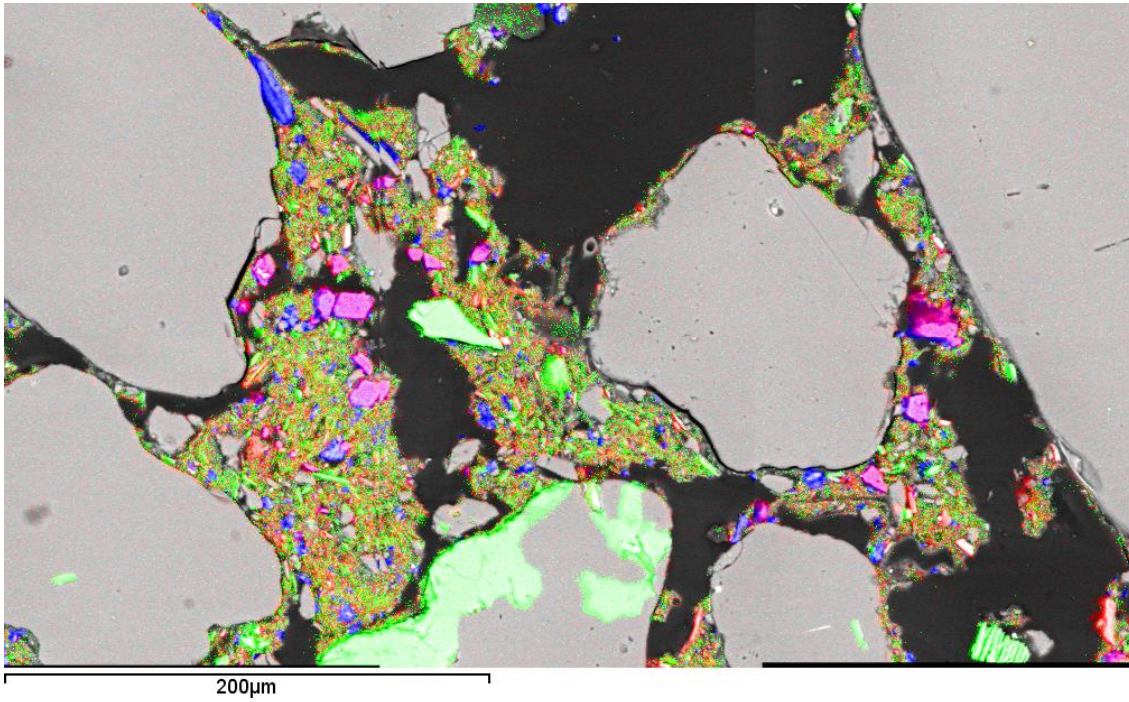
Figure 2.14. EDS spectroscopy element map with red, blue and green denoting Mg, K and Ca respectively.
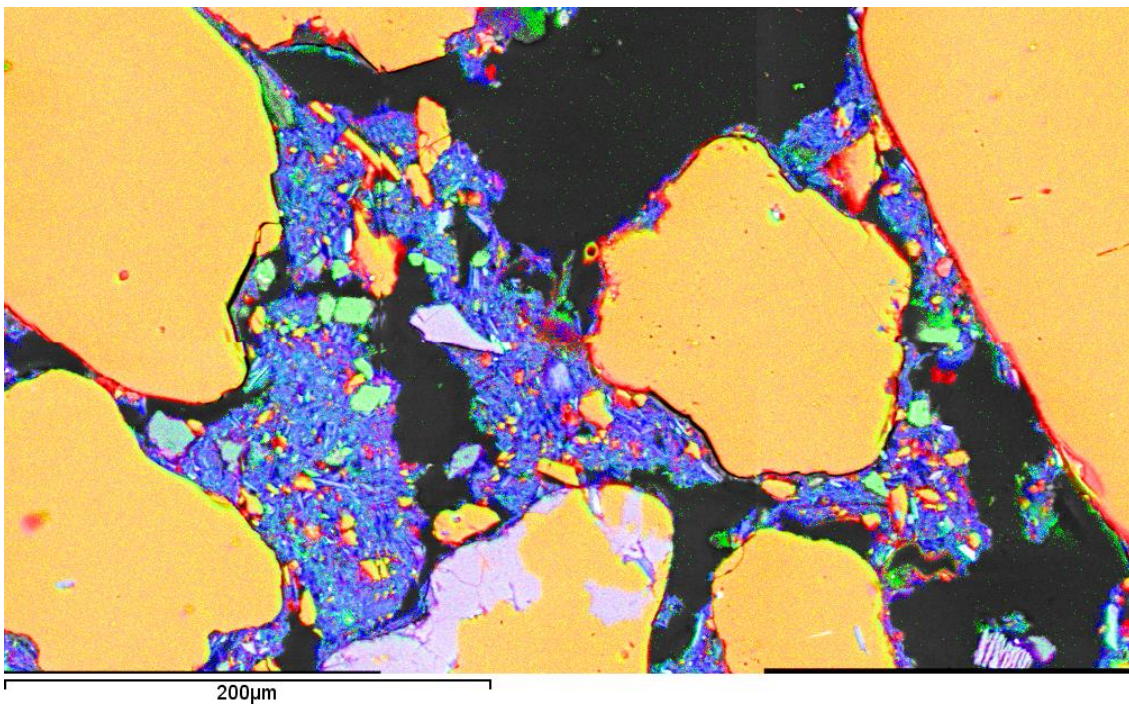
Figure 2.15. EDS spectroscopy element map with red, blue and green denoting Si, O and Al respectively.

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2.3.2. Microbial community analysis

2.3.2.1. Cell enumeration
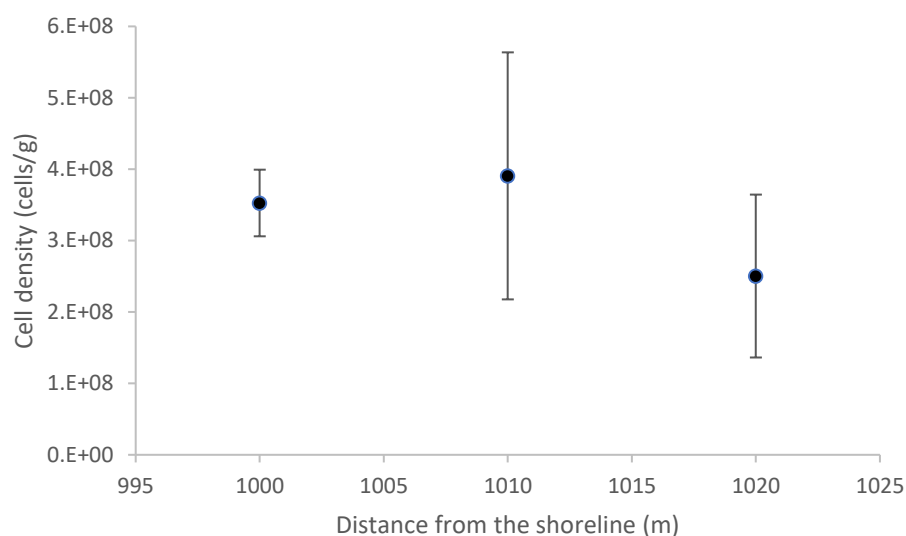
Figure 2.16. Scatter graph showing mean cell density of samples collected from the anaerobic zone of each sampling row plotted against approximate distance measured from the sea. Error bars represent one standard deviation.

Mean cell densities (n=4) for the three sampling rows were found to be approximately 3×10^8 cells mL^{-1} (Figure 2.16). However, comparison between them was problematic because of the large standard deviations in raw values stemming from up to approximately 50 % differences in observed cell density per frame of view during microscopy. ANOVA was used to elucidate any significant differences between them, but failed to find any (p=0.30). The mean cell density for the whole data set was calculated to be 3.3 ± 1.3 × 10^8 cells per gram.

2.3.2.2. TRFLP

2.3.2.2.1. Bacteria

To investigate the composition of the bacterial community and to facilitate the labelling of TRFLP fragments, MiSeq analysis was carried out on the sample from site three (Figure 2.16). This site was assumed to be roughly representative of the microbial

community at all twelve sites because environmental conditions there (pH 8.1, 11.6 °C and 1.49 % TOC) were representative of the average. Given the small sampling area it was predicted that there would be little variation in environmental conditions, and thus little variation in the microbial community. This prediction was supported by the finding that MiSeq analysis of this sample facilitated identification of all major bacterial and archaeal groups present in the twelve samples.
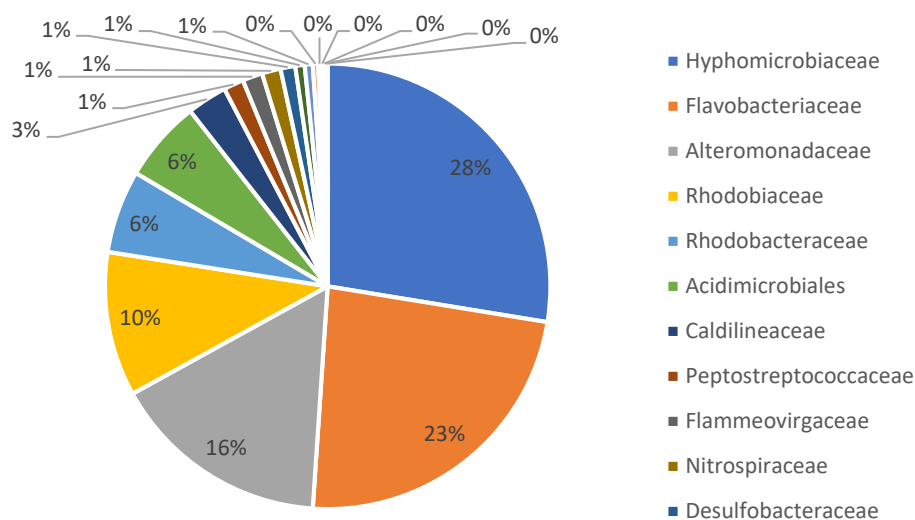
Figure 2.17. MiSeq DNA sequencing data for the anaerobic zone at sample site three, displaying the relative proportions of different bacterial families.

MiSeq sequences with a minimum of 80% confidence at family level were included in Figure 2.17 to give a reliable representation of the bacterial community in the sample from site three. Figure 2.17 shows a large diversity in bacterial families with *Hyphomicrobiaceae, Flavobacteriaceae* and *Alteromonadaceae* making up 67% of the community.

By carrying out an *in silico* digestion of the MiSeq sequences and matching up the fragments output against the TRFLP fragments, it was possible to identify the taxonomic groups that TRFLP fragment were attributable to. To account for the error in the TRFLP process, fragments detected within approximately 4 base pairs of each

other were grouped as they were likely representative of the same bacterial species. Due to the variation in confidence levels provided by the MiSeq sequencing, the resolution of identification for each fragment varies, however resolution to Order and Class levels was achieved for most fragments (see Table 2.2).

To investigate variation with distance from the shoreline, TRFLP data were grouped according to their sampling rows and an average relative frequency was calculated. This was used to construct pie charts that demonstrate the composition of the microbial community (see Figure 2.18). Evidently there was some variation in bacterial diversity between the groups, but the significance of this was subject to statistical testing. Firstly, a rarefaction curve was plotted using R version 3.2.4 (Figure 2.19). The convergence of all three sampling rows along the horizontal at y = 4 indicates that there were sufficient samples taken to be representative of the entire bacterial community.

Table 2.2. Identification of TRFLP fragments using MiSeq sequencing. * indicates where the resolution varies from the given level, e.g. Haliglobus refers to a genus level resolution.

Fragment	Taxon (Class)	Taxon (Order)
80-82	Alphaproteobacteria	-
83-87	Deltaproteoebacteria	Desulfuromonadaceae
90-91	Proteobacteria*	-
93	Alphaproteobacteria	Beijerinckiaceae
95	Acidobacteria GP21	-
96	Gammaproteobacteria	-
98	Gammaproteobacteria	-
99	Flavobacteria	Flavobacteriaceae
101	Alphaproteobacteria	Rhodobiaceae
102-103	Acidobacteria Gp9	-
105	Gammaproteobacteria	-
107-108	Caldilineae	Caldilineaceae
109-110	Alphaproteobacteria	Rhodobiaceae
125	Actinobacteria	Actinomycetales
126	Alphaproteobacteria	-
127-129	Gemmatimonadetes	Gemmatimonadaceae
130-134	Gammaproteobacteria	-
135-137	Proteobacteria*	-
138-143	Deltaproteobacteria	Nannocystineae
144-146	Clostridia	Peptostreptococcaceae
148-152	Alphaproteobacteria	Hyphomicrobiaceae
153-155	Chloroflexi* (phylum)	-
156-160	Cytophagia	-
161-163	Unknown	-
165-168	Gammaproteobacteria	-
172-176	Caldilineae	Caldilineaceae
178	Gammaproteobacteria	Granulosicoccaceae
187-189	Unknown	-
191-196	Gammaproteobacteria	Ectothiorhodospiraceae
198-202	Alphaproteobacteria	Rhizobiales*
204-207	Unknown	-
209-214	Alphaproteobacteria	Rhizobiales *
217-220	Unknown	-
221-225	Gammaproteobacteria	-
226-228	Alphaproteobacteria	-
230-231	Unknown	-
232-233	Gammaproteobacteria	-
234-237	Alphaproteobacteria	-
239-243	Acidobacteria	Gp10 *
244-246	Acidobacteria	Gp10*
244-246	Acidobacteria	Gp10*
247	Acidobacteria	-
248	Acidobacteria	Gp9*
250-253	Gammaproteobacteria	Alteromonadaceae
256	Gammaproteobacteria	-
259	Gammaproteobacteria	-
262-266	Gammaproteobacteria	Halioglobus *
276-279	Gammaproteobacteria	-
281	Unknown	-
306-308	Alphaproteobacteria	Hyphomicrobiaceae
310-315	Alphaproteobacteria	Hyphomicrobiaceae
327-329	Gammaproteobacteria	-
331-333	Gammaproteobacteria	-
423	Gammaproteobacteria	-
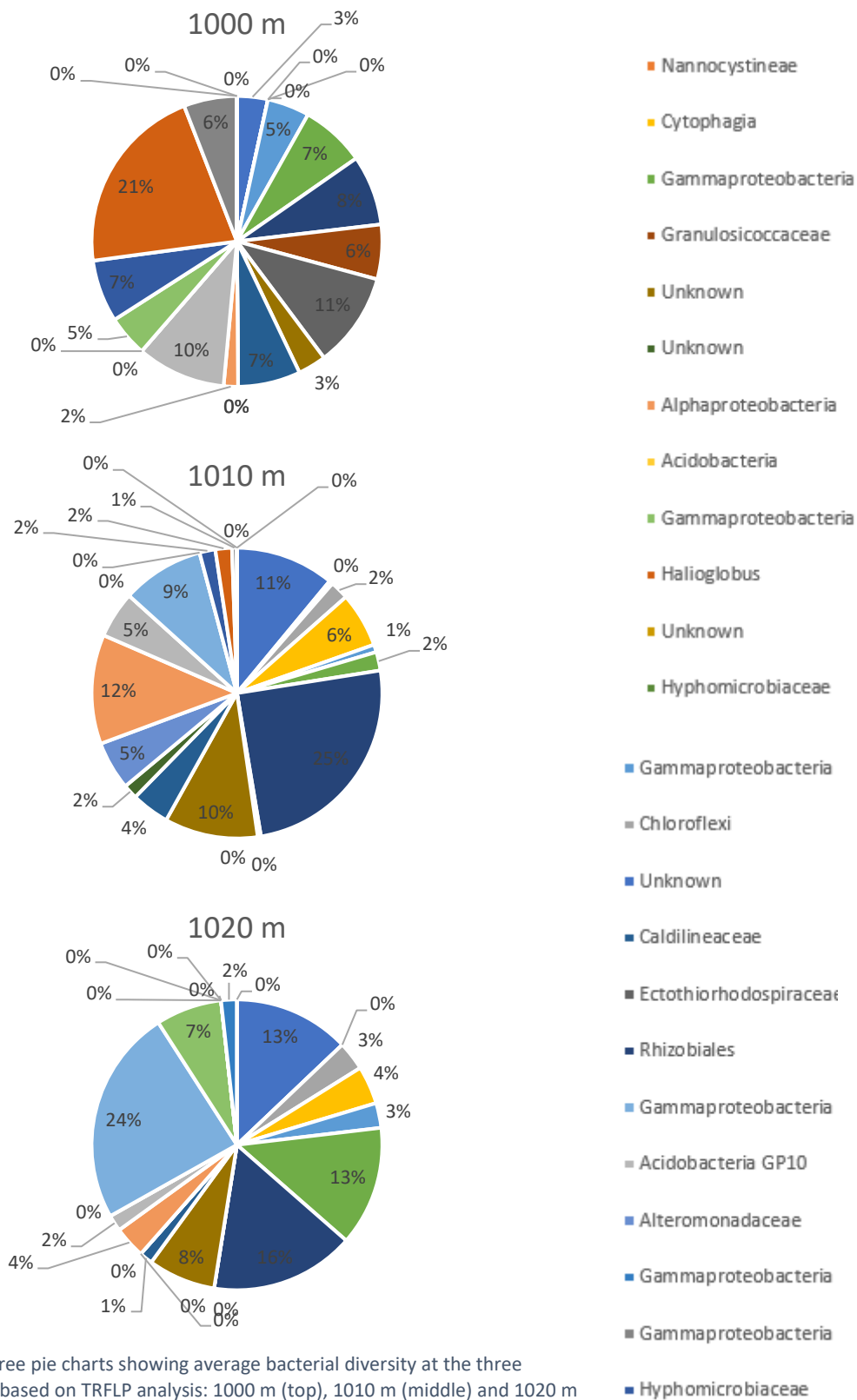
Figure 2.18. Three pie charts showing average bacterial diversity at the three sampling rows based on TRFLP analysis: 1000 m (top), 1010 m (middle) and 1020 m (bottom) from the shoreline. The legend represents the bacterial groups present, and applies to all three panels. Repetitions represent different species within the same bacterial group.
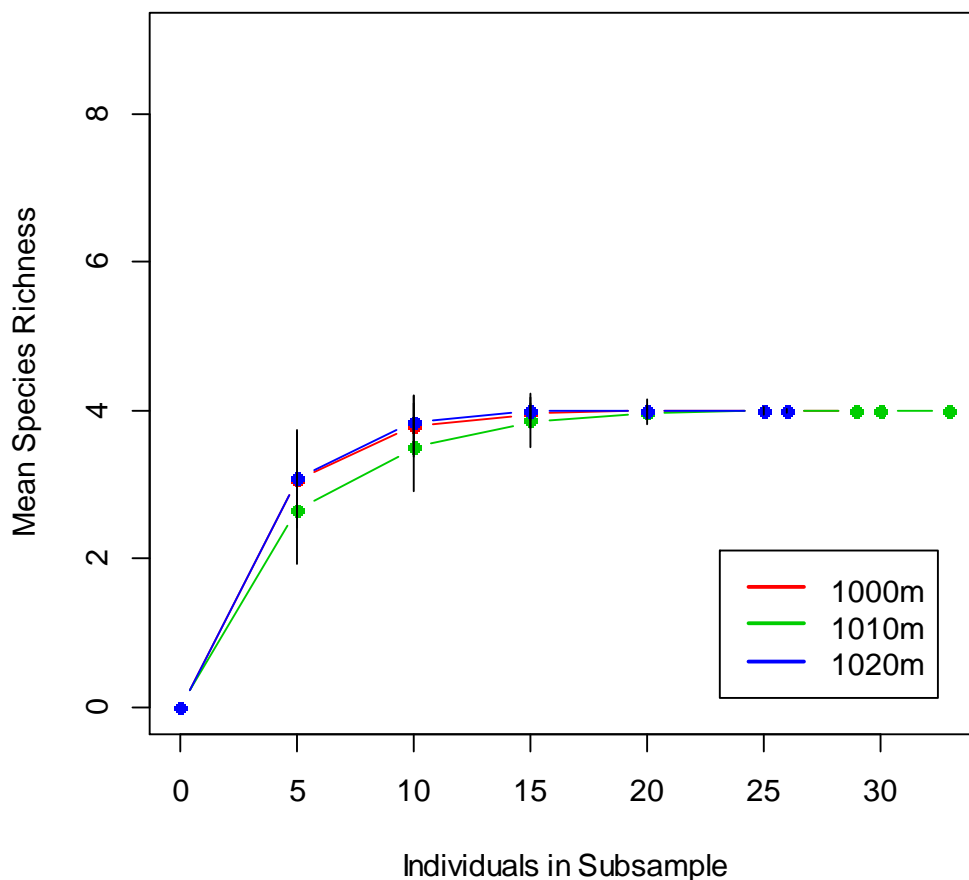
Figure 2.19. Rarefaction curves for samples taken from all three sampling rows.

To quantify similarities between the three sampling rows, ANOSIM (with a Bray-Curtis similarity matrix) was carried out using Primer 5 software. This yielded a global R value of 0.065 (p= 0.309), i.e. no significant difference. Pairwise test statistics also failed to demonstrate a significant difference between the three sampling rows. Using the same software, SIMPER analysis was carried out such that the contribution of individual TRFLP fragments to the similarity/dissimilarity of each sampling row could be quantified. Sampling rows were paired and compared to each other giving average dissimilarity scores of 81.54 %, 81.49 % and 71.31 % for 1000 m vs 1010 m, 1000 m vs 1020 m and 1010 m vs 1020 m respectively. In addition, the two largest contributing fragments to the dissimilarity between each sampling row were combinations of *Halioglobus*, *Caldilineaceae* and *Alteromonadaceae*.

With no significant trend between the three sampling rows, it was necessary to investigate differences between individual samples. For this, Principal component Analysis (PCA) analysis was carried out (see Figure 2.20).
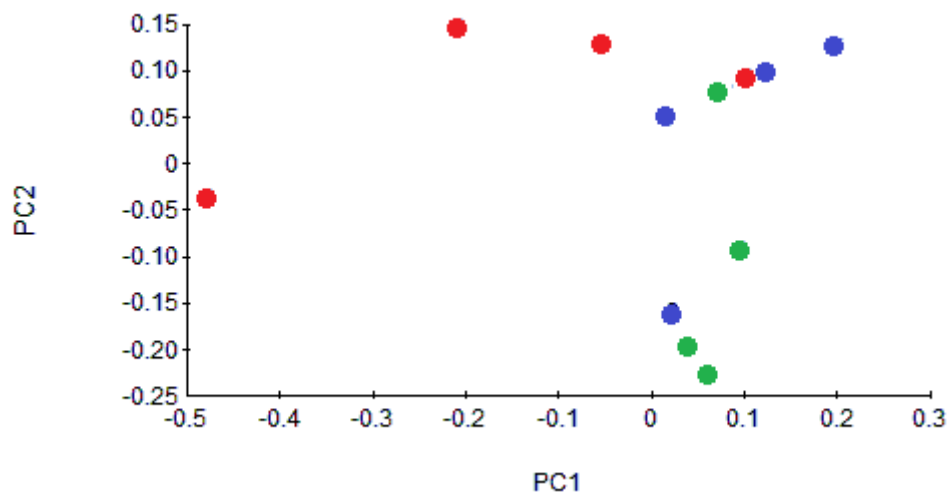
Figure 2.20. Principal component Analysis plot showing similarity of bacterial diversity between individual samples. Colours represent each of the twelve sample sites: Red- 1-4, Blue- 5-8, Green 9-12. Samples appear to cluster into two main groups, with a few outliers.

75 % of samples fell into two groups containing samples from sites 8, 9, 11 and 12, and 3, 5, 6, 7 and 10. Outliers (sample sites 1, 2 and 4) were determined based on visual inspection of Figure 2.20. After grouping these samples together based on their proximity in the PCA plot (Figure 2.20), ANOSIM was carried out yielding an R value of 0.49 (p=0.024). This indicated a significant difference in bacterial diversity between the two groups.

Figure 2.21 shows a Venn diagram detailing the differences and similarities of the bacterial diversity in each of the sampling rows. All numbers not represented on the graph occupy the middle space that indicates presence in all three sampling rows.
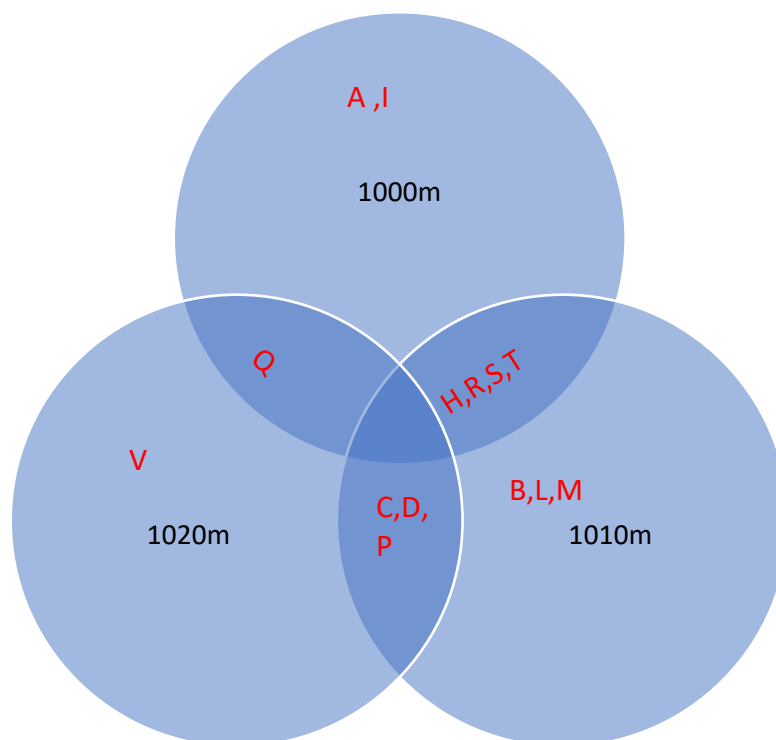
A. Gammaproteobacteria
B. Nannocystineae
C. Chloroflexi
D. Cytophagia
E. Unknown
F. Gammaproteobacteria
G. Caldilineaceae
H. Granulsicoccaceae
I. Ectothiorhodospiraceae
J. Unknown
K. Rhizobiales
L. Unknown
M. Gammaproteobacteria
N. Alphaproteobacteria
O. Acidobacteria
P. Alteromonadaceae
Q. Gammaproteobacteria
R. Gammaproteobacteria
S. Halioglobus
T. Gammaproteobacteria
U. Unknown
V. Hyphomicrobiaceae

Figure 2.21. A Venn diagram displaying the similarities and differences in the presence of bacterial groups within samples from the three sampling rows.

Figure 2.21 demonstrates that the majority of bacterial groups were not shared among all three sampling rows. However, only six of the 22 groups were unique to one row, and eight were shared between two rows.

2.3.2.2.2. Archaea

Archaeal diversity was significantly different to the observed bacterial diversity in the River Dee sediment. Much larger variation was observed in bacteria, whereas MiSeq

sequencing on the sample from site three yielded only one Archaeal group above the 80 % confidence level. All sequences above the 80 % confidence threshold were associated with the *Thaumarchaeaota* phylum, and were resolved down to the *Nitrosopumilus* genus. This represents a genus of extremely common marine archaea found in seawater.

Figure 2.22 is a comparison of TRFLP data showing archaeal diversity in samples grouped according to their distance from the shoreline. It demonstrates that 60-65 % of fragments were associated with a single group. This is in line with the MiSeq sequencing result. ANOSIM analysis based on a Bray-Curtis similarity matrix yielded no significant difference with an R value of -0.13 (p = 0.824). Because MiSeq analysis only provided one reliable sequence it was not possible to conduct *in silico* digestion and further analysis.

1000 m

Legend:
- 102-105
- 107-109
- 111-113
- 117-118
- 120-121
- 124
- 130-132
- 135
- 144-148
- 152-153

1010 m

Legend:
- 102-105
- 107-109
- 111-113
- 117-118
- 120-121
- 124
- 130-132
- 135
- 144-148
- 152-153

1020 m

Legend:
- 102-105
- 107-109
- 111-113
- 117-118
- 120-121
- 124
- 130-132
- 135
- 144-148
- 152-153
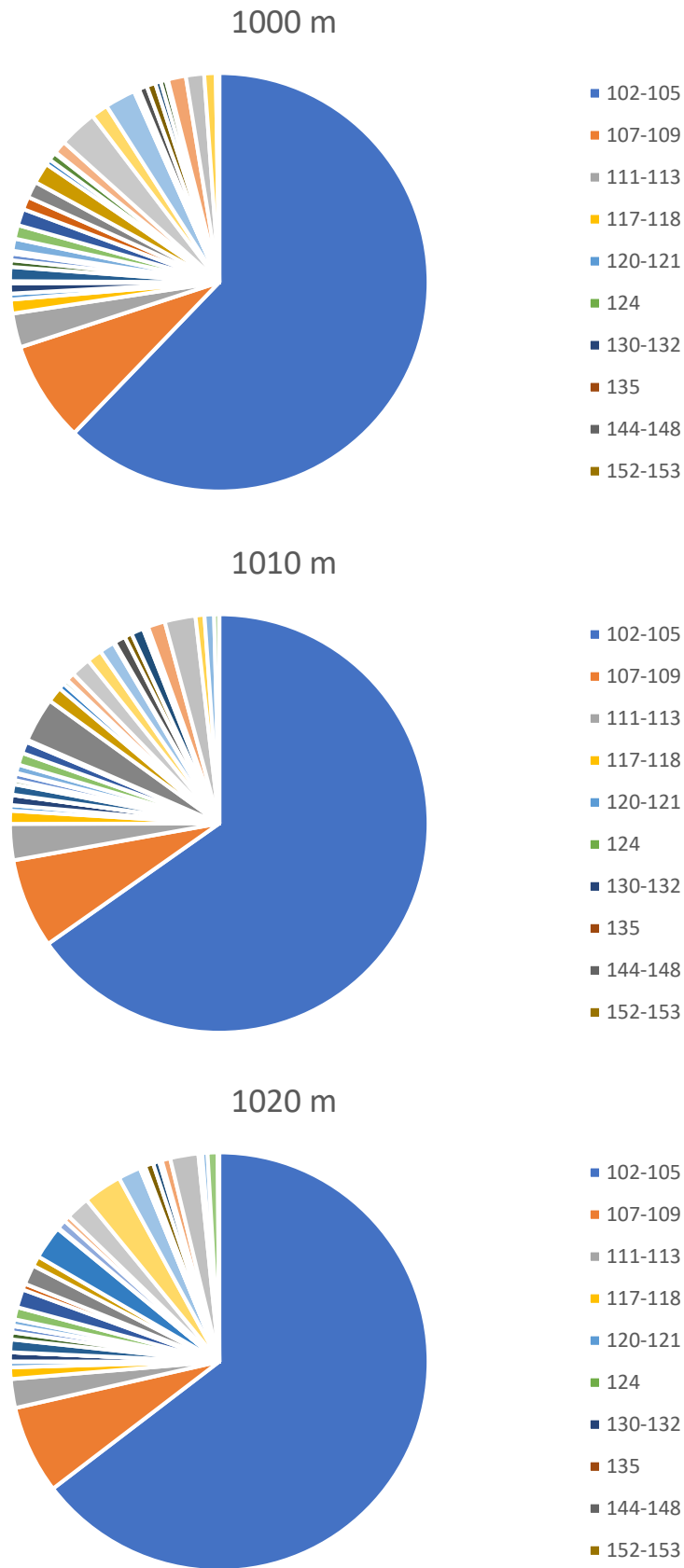
Figure 2.22. Three pie charts showing average archaeal diversity at the three sampling rows based on TRFLP analysis: 1000 m (top), 1010 m (middle) and 1020 m (bottom) from the shoreline. As it was not possible to label the individual fragments, the fragment length in base pairs is given in place of a taxonomic identification.

2.4. Discussion

2.4.1. Environmental characterisation

The focus of this study was to characterise the anaerobic layer of the River Dee estuary intertidal zone both in terms of environmental parameters and the microbial diversity present.

Statistical analysis of the environmental data collected suggests that there is no significant variation in environmental characteristics as distance from the shoreline increases. Thus, it is possible to collate all twelve samples into a single average for each parameter and present an environmental characterisation of the environment (Table 2.3).

Table 2.3. Collated means of all twelve samples for pH, temperature and TOC analyses.

Characteristic	Mean value
pH	8.2 ± 0.1
Temperature (°C)	12.2 ± 0.3
TOC (%)	1.29 ± 0.3

2.4.1.1. Comparison with Mars

Evidence from recent Mars missions has demonstrated the heterogeneity of martian environments spanning both geological time and geographic location (e.g. Squyres *et al.*, 2004; Greeley *et al.*, 2005; McCollom and Hynek, 2005; Bibring *et al.*, 2006; Wray *et al.*, 2009; Arvidson *et al.*, 2014). Thus, in attempting to compare the River Dee to environments on Mars it was necessary to focus efforts on a specific environment, particularly one that has been suggested to be potentially habitable.

With the abundance of data currently being provided by the MSL Curiosity Rover, Gale Crater is relatively well characterised and ideal for comparison with River Dee data. As mentioned above, Gale Crater is thought to have hosted a long-lasting fluvio-lacustrine system, indicating temperatures were suitable for long-standing surface water likely to have been at circumneutral pH (Grotzinger *et al.*, 2014; Vaniman *et al.*, 2014). In addition, salt concentrations are considered to have been between 1-2 %, lower than contemporary terrestrial marine environments at (approximately 2.3 %) (Lyman and Flemming, 1940; Kester *et al.*, 1967).

A comparison of the environmental data collected at Gale Crater and the River Dee is given below.

2.4.1.1.1. Redox conditions

The exact composition of the ancient martian atmosphere, with regard to its support of above-zero temperatures, is the subject of ongoing research (Forget *et al.*, 2013; Wordsworth *et al.*, 2013; Kerber *et al.*, 2015; Von Paris *et al.*, 2015). However, it is thought to have been mainly CO_2 (Phillips *et al.*, 2001). This would thus provide anaerobic conditions for potential martian microorganisms at the surface, and within the subsurface.

However, because Earth's atmosphere contains approximately 20 % oxygen (Jones, 1950), anaerobic environments on Earth must be isolated from the atmosphere. Thus, terrestrial analogues of both surface or subsurface martian environments will likely be from the terrestrial subsurface only, such as those from the River Dee estuary, beneath the RPD as characterised in this study.

Comparison of the anaerobic zone of the River Dee sediment is further applicable to the ancient lacustrine environment at Gale Crater because of recent evidence that suggests reducing conditions in the Sheepbed mudstone formation (Grotzinger *et al.*, 2014; Vaniman *et al.*, 2014). A ChemCam investigation of the John Klein (JK) and Cumberland (CB) drill holes showed that beneath the red, oxidised surface, the mudstone is grey. This suggests that a reducing environment prevailed during the deposition of the sediments (Jackson *et al.*, 2016). Therefore, it is possible, in terms of the level of oxygenation, to directly compare the subsurface environment at the River Dee to the subsurface environment of the ancient lake at Gale Crater.

2.4.1.1.2. Water availability

The River Dee Estuary is subject to tidal fluctuations in sea level. For the intertidal zone, the zone between low and high tide, this means wetting and drying on a daily basis. The microorganisms in the subsurface are therefore subjected to consequent fluctuations in water availability, leading to periods of desiccation (Garbary, 2007).

Presently, there is no evidence of diurnal tidal effects in ancient martian lake systems. The minimum duration of the lacustrine environment at Gale Crater is considered to be 1,500 years, considering only the exposed Sheepbed Mudstone member. Including the overlying strata and potential erosion of lacustrine sediments, this estimate increases to millions or tens of millions of years (Grotzinger *et al.*, 2014). However, throughout this time period, it is considered that the lacustrine system will have undergone episodic drying periods, with intermittently present lakes over approximately 30,000 Earth years (Palucis *et al.*, 2016). The periods of drying are therefore many orders of magnitude longer than that at the River Dee Estuary. However, it has been suggested that regional groundwater flow contributed significant

amounts of water to Gale Crater (Palucis *et al.*, 2016), and thus may have provided a refuge for microbial life when surface, and shallow subsurface, desiccation occurred.

2.4.1.1.3. Salinity

Salinity is an important constraint upon the habitability of a system because of its effect upon water activity. Water activity is a key factor in cellular processes and is calculated by using the following equation, where n_1 and n_2 represent the moles of water and solute respectively (Grant, 2004):

$$a_w = n_1 / (n_1 + n_2)$$

Most terrestrial organisms cannot grow below a water activity of 0.9 (Knoll and Grotzinger, 2006). Using this formula, the water activity of terrestrial seawater is approximately 0.993. Using the 2 % upper limit given above for the Sheepbed Mudstone at Gale Crater, and assuming all of the chloride salts were of sodium, a water activity of approximately 0.9934 is calculated for Gale Crater. Though Mg and Fe were found at Gale Crater, analysis of their relationship to the amount of Cl indicates that their respective chlorides do not account for Mg and Fe enrichment, and therefore the abundance of their chlorides is relatively low (Grotzinger *et al.*, 2014; S. M. McLennan *et al.*, 2014; Vaniman *et al.*, 2014).

In terrestrial systems, microorganisms can survive and grow in a range of water activities. For example, *Acetinobacter jonsonii* has been shown (see Chapter 4) to have an optimum growth rate at a water activity of 0.970, but successful growth at a water activity of 0.985. Such tolerances to variations in salinity are commonplace among microorganisms (see Chapter 4 for more examples) and thus their ranges almost

certainly extend across the relatively small difference between salinity values for the River Dee Estuary and the ancient lake at Gale Crater.

In addition, of the major bacterial groups identified in the anaerobic microbial community at the River Dee site, the type species of the *Halioglobus* genus, *Halioglobus japonicus,* requires NaCl for growth and has a salt tolerance range of 1-4 % (w/v) (Park *et al.*, 2012). *Caldilineaceae* and *Alteromonadaceae* also contain species with 0-0.5 % (w/v) and 0.5-20 % (w/v) salt tolerances respectively (Grégoire *et al.*, 2011; Ng *et al.*, 2014).

2.4.1.1.4. Carbon availability

In the anaerobic zone of the River Dee sediment, the TOC content within the sediment was found to be on average 1.29 ± 0.3 % (w/w). This supported a diverse microbial community (see Figures 2.18 and 2.22). However, to date, the tolerance of the major constituents of the River Dee microbial community to low organic carbon environments has not been quantified.

Eiler *et al.* (2003) showed that terrestrial heterotrophic bacteria can survive and grow in as little as 0.04 mM dissolved organic carbon (DOC). They did not, however, examine the limit at which growth ceased, nor did they investigate the input from other carbon sources. In Eiler *et al.'s* study they determined that the amount of bacterial biomass produced was found to increase linearly with the concentration of DOC, and the growth rate during the exponential growth phase exhibited a hyperbolic response to DOC concentration (Eiler *et al.*, 2003). Thus, it is assumed that reductions in carbon availability would reduce the rate and magnitude of bacterial growth without preventing it entirely. It may therefore be possible for microorganisms to grow, albeit slowly, with extremely low DOC availability, which could be/have been the case on

Mars. The DOC concentration in the Irish Sea (into which the River Dee runs) has been shown to be 0.09 – 0.198 mM, which would be sufficient to support microbial growth (Eiler *et al.*, 2003).

In this study, TOC of the sediment was measured, not DOC of seawater, so a direct comparison with Eiler *et al.*'s work is difficult since some organisms may be able to utilise organic material directly from the sediment. However, it could be assumed that multiple carbon sources present an increased availability of carbon for a range of organisms, supporting community diversity and growth.

To date, there is no prevailing consensus view of the total carbon content of samples from Gale Crater. Freissinet *et al.* (2016) reported that chlorobenzene has been detected at 150-300 ppbw in several Cumberland samples in the Sheepbed Mudstone. Further, the SAM instrument detected chloromethanes at the nanomole level at the Rocknest site on Gale Crater. However, these species cannot be confirmed to be of martian origin, as terrestrial contamination and instrument background remain significant challenges.

Assuming these species are not of terrestrial origin, recent work by Sutter *et al.*, (2016) suggests that the total organic carbon content at Gale Crater, as evidenced by the evolution of CO_2 and CO on heating in the SAM instrument, may be as high as 2400 ppm. <1% of this is considered enough to sustain a microbial community of 10^5 cells/g of sediment (B. Sutter *et al.*, 2016). Since terrestrial microorganisms are capable of adapting to and utilising a variety of carbon sources, it is assumed that putative martian life would be capable of the same (see Chapter 4) (Inoue *et al.*, 2002; Masurat *et al.*, 2010). The amount of organic carbon at Gale Crater is therefore likely to be sufficient to support a significant microbial population.

Additionally, atmospheric carbon dioxide (and monoxide) could be supplied to the subsurface *via* diffusion into the regolith (Weiss *et al.*, 2000; Leshin *et al.*, 2013). Methane production in the subsurface, *via* methanogenesis, or its abiotic counterpart Fischer-Tropsch Type reactions catalysed by transition metals (Yung and Chen, 2015), can also be a potential source of carbon for putative martian microbes that may rely on methanotrophy (consumption of methane to fuel metabolism) (Kalyuzhnaya *et al.*, 1999; Laskin *et al.*, 2008).

The variety and abundance of carbon sources on Mars could support a diverse microbial community, therefore suggesting that the possibility of microbial life on Mars is not limited by the availability of suitable carbon sources.

2.4.1.1.5. Bioessential elements

For comparison with the River Dee, the JK and CB drill holes and the PS aeolian deposit at Gale Crater were selected because of their relevance to the ancient lacustrine environment.

Table 2.4. A mineralogical comparison between the anaerobic zone of the River Dee sampling site and three relevant samples analysed at Gale Crater: the Portage Soil and the John Klein and Cumberland drill holes. Data taken from Bridges et al., (2015). * indicates that the value is a combination of abundances for all minor fractions. † denotes that for the River Dee sample, titanohaematite is the main mineral.

	Portage Soil	John Klein	Cumberland	River Dee
Plagioclase	40.8	44.8	41	
Fe-forsterite	22.4	5.7	1.9	
Augite	14.6	7.6	9	
Pigeonite	13.8	11.3	16	
Orthopyroxene		6.1	9	
Magnetite	2.1	7.6	9	
Anhydrite	1.5	5.3		
Bassanite		2.1	1.2	
Quartz	1.4	0.9	0.2	63
Sanidine	1.3	2.4	3.5	9
Haematite†	1.1	1.2	1.3	5*
Ilmenite	0.9		1.2	
Akaganeite		2.3	3	
Halite		0.3	0.3	
Pyrite		0.6		
Pyrrhotite		2	1.9	
Amorphous	27	28	31	
Clay		22	18	23

Most striking in the mineralogical comparison of the three is the disparity in the amount of quartz. As a terrestrial estuarine environment, the River Dee sample is mostly quartz whereas the major fraction of all three martian samples is plagioclase feldspar (see Table 2.4). Quartz provides little in terms of bioessential elements, being comprised of silicon and oxygen only. In comparison, plagioclase feldspar contains the bioessential elements Na and Ca, and through microbially-mediated weathering could be made available to microorganisms (Vandevivere *et al.*, 1994; Wackett *et al.*, 2004; Pontefract *et al.*, 2012).

Aside from the PS, where no clay was detected (Blake *et al.*, 2013), the second largest fraction within the River Dee sample were the clays, e.g. illite. The River Dee sample, along with the JK and CB drill holes, comprised approximately 20 % clay minerals. The lack of clay minerals in PS is likely because it is an aeolian sediment, representative of

Mars global soil where water was not necessarily present (Blake *et al.*, 2013), whereas the JK and CB samples are both from the Sheepbed Mudstone and representative of the lacustrine environment of Yellowknife Bay. Clay minerals are rich in Al, Mg, Fe and K, of which Mg, Fe and K are essential elements necessary for sustaining microbial life (Pontefract *et al.*, 2012). The presence of such material in both the River Dee sample and the Sheepbed Mudstone drill holes is suggestive of a similar nutritional environment.

Sanidine, present in similar levels in all three martian environments, provides a further source of K. Sanidine is a high temperature alkali feldspar and in all three samples represents a detrital mineral, formed in igneous systems elsewhere. The higher proportion detected in the River Dee sample is possibly indicative of a higher proportion of sanidine in the River Dee source rocks. The remaining trace minerals in the River Dee sample were dominated by titanohaematite, but also include phosphate minerals and zircons. These provide a source of Fe and Ti, as well as P, a major bioessential mineral (Pontefract *et al.*, 2012). The martian samples (PS, JK and CB) had lower levels of haematite, though the supply of Fe is supplemented by the Fe content of the clays.

The release of bioessential elements from minerals is often facilitated by microbial weathering (see Chapter 3), for example altering pH *via* the production of protons, organic acids, and/or chelating agents (Barker and Banfield, 1998; Barker *et al.*, 1998; Rogers and Bennett, 2004; Wu *et al.*, 2007b; Uroz, Calvaruso, Turpault and Frey-Klett, 2009; Olsson-Francis *et al.*, 2012). Silicate weathering has been shown to be increased by microbial activity (Wu *et al.*, 2007b; Lingling Wu *et al.*, 2008) and plagioclase feldspar dissolution has been shown to increase ten-fold in the presence of organic acids produced by many microorganisms as a result of their metabolism (Welch and Ullman, 1993; Welch *et al.*, 2002). In addition, silicate minerals containing bioessential

elements have been shown to be preferentially colonised by microorganisms (Rogers and Bennett, 2004). Feldspar dissolution is at a minimum at circumneutral pH (Bennett *et al.*, 2001), the pH of both the ancient lacustrine environment and the River Dee sample site (Grotzinger *et al.*, 2014; Vaniman *et al.*, 2014; Bridges *et al.*, 2015), however, localised pH changes could increase dissolution leading to the increased availability of the bioessential elements within the silicate matrix without any large-scale change in pH.

It is clear that the mineralogy of both the River Dee site and the comparable martian sediments (see Table 2.4) would provide the key bioessential elements to sustain microbial life. However, the prevalence of microorganisms within the River Dee Estuary sample site suggests that the concentration of bioessential elements is above these limits. In addition, the mineralogical and environmental similarities between the River Dee Estuary and the ancient lacustrine environment at Gale Crater suggest that bioessential element concentrations would likely have also been similar. Thus, with regard to bioessential elements, Gale Crater likely could have supported microbial life.

Furthermore, the discrepancy between the bulk mineralogy of the River Dee sample and the martian samples potentially indicates a more nutritionally favourable system in the martian samples, with the Na and Ca rich plagioclase feldspar taking the place of the relatively inert quartz in the River Dee sample. Microbially-mediated dissolution and weathering could make available those elements sequestered within the minerals. In conjunction with the other environmental parameters discussed above, this would result in a largely similar environment, albeit with potentially increased biomass in the martian environment.

2.4.1.1.6. Summary of results

In-depth characterisation of the River Dee Estuary subsurface environment carried out for this study, and comparison with the analysis carried out at Gale Crater by the MSL Curiosity Rover, indicate many similarities between the two environments. With regard to environmental conditions and the likelihood of availability of nutrients for microbial metabolism, it is possible to assert that the anaerobic zone of the River Dee Estuary is an analogue of the ancient lacustrine system discovered at Gale Crater. Thus, the microbial community that thrives there will be useful in investigations of microbial processes at Gale, and perhaps similar crater lakes, on early Mars.

Given the significant similarities, it is prudent to note the main differences, and therefore, primary limitations, of the River Dee Estuary analogue. The atmospheric pressure is significantly different between terrestrial and martian environments, with the present-day martian atmosphere being only 6 mbar. A thicker atmosphere is suggested for ancient Mars e.g. (Haberle, 1998; Forget *et al.*, 2013; Urata and Toon, 2013), however there is no consensus with regard to the atmospheric pressure, with models using between 0.5 and 7 bar CO_2 atmospheres in their calculations. Despite this uncertainty, the atmospheric pressure, interaction with the subsurface environment notwithstanding, remains a significant constraint on past or present microbial martian life.

In addition, though both analogue and martian environments experience periodic drying and wetting cycles, the scales are orders of magnitude apart. The River Dee Estuary site experiences tidal, diurnal cycles, whereas (as mentioned above) the lacustrine environment at Gale Crater likely existed for 1000s of years at a time (Palucis *et al.*, 2016). As with atmospheric pressure, the long-lasting dry periods could have

been extremely selective and thus the type of microbial life potentially surviving there could be extremely specialised.

2.4.2. Microbial Diversity

2.4.2.1. Bacteria

The bacterial diversity at the sample site was generally uniform, with the majority of taxa being represented in samples of all three sampling rows. However, ANOSIM and SIMPER analysis had somewhat inconsistent results, with ANOSIM suggesting no significant difference between the sampling rows and SIMPER indicating large percentages of dissimilarity. SIMPER analysis is based on a ranked score and so therefore does not account for the magnitude of bacterial diversities within the data, whereas ANOSIM utilises raw frequency data; this is a likely source of the discrepancy. While the magnitude of the observed dissimilarity calculated by SIMPER is potentially inaccurate, it highlights the source of the observed dissimilarity and so is still an effective statistical tool for this research. Thus, there are three major taxa to discuss: *Halioglobus*, *Caldilineaceae* and *Alteromonadaceae.*

As expected, due to the lack of significant difference calculated between the three sampling rows, the three major taxa, *Halioglobus*, *Caldilineaceae* and *Alteromonadaceae,* are not unique to any single sampling row.

Halioglobus is a genus of marine Gammaproteobacteria that is known to include two obligate aerobes, *H. japonicas* and *H. pacificus* (Park *et al.*, 2012). The presence of these species is inconsistent with the anaerobic environment and resolution to species level was not possible, so confirmation of their presence, or the presence of a facultative or anaerobic *Halioglobus* species is not possible at this time.

Caldilineaceae is a small taxonomic family comprising mainly anaerobic thermophilic bacteria but includes facultatively aerobic members, e.g. *Litorilinea* (Sekiguchi *et al.*, 2003; Grégoire *et al.*, 2011; Kale *et al.*, 2013). Though the optimum temperatures for these thermophiles ranges from 40 – 65 °C, their presence at approximately 12 °C within the intertidal zone suggests that closely related mesophilic species could thrive in the sample sites. It is possible that these thermophilic species thrive in the warmer months when air temperature is higher, and direct sunlight warms the surface more frequently. Their presence in November, during the sample collection period, is likely a remnant of their prevalence in the summer.

Alteromonadaceae are a diverse family of aerobic and anaerobic marine bacteria, with varying optimum conditions and environmental constraints. There is also a suggestion that the taxonomic classification within the group is not in keeping with recent 16S rRNA data (Ivanova *et al.*, 2004). Nevertheless, the wide ranging growth optima in the family indicate that their presence within the River Dee microbial community is expected.

The apparent prevalence of facultative anaerobes/aerobes in this environment suggest that the environmental conditions have given a selective advantage to such species. The intertidal zone, being regularly submerged and uncovered, likely results in a fluctuating availability of oxygen in the subsurface. Thus, species capable of adapting to this regular variation would likely be successful.

The bacterial community was also uniform with regard to the distance from the shoreline. This is expected as there was no significant difference in their environment moving 20 m closer. The sampling area was selected such that the mid-intertidal zone could be effectively sampled without inadvertently including high-intertidal or low-intertidal zone sediments.

At each sampling site, as in Figure 2.2, an approximately 40 cm vertical cross section of the subsurface was obtained. With the likely fluctuation in the depth of the RPD with distance from the shoreline, sampling at a constant depth could have led to inappropriate comparisons between aerobic, microaerophilic and anaerobic zones. For this study, it was imperative to sample only from the anaerobic zone (below the RPD), therefore after the layers had been identified samples were taken strictly from within the black sulfide layer identified in Figure 2.2. The exact depth of each sample was not recorded. To minimise error, these samples were taken at least 5 cm beneath the RPD, and thus, confidence in the sampling procedure is high.

In comparison to marine and freshwater sediments, intertidal zone sediments have been shown to contain various types of primary producer, which leads to an environment with concentrated and varied nutrient availability (Wang *et al.*, 2012). This nutrient availability is able to support a diverse microbial community and, compared to the other sediment types, was found by Wang *et al.* (2012) to have medium diversity. The microbial community at the River Dee Estuary, in accordance with Wang *et al.* (2012) where the intertidal zone microbial community was found to have 21 dominant bacterial groups (~99% of bacterial species), had 22 dominant bacterial species.

2.4.2.2. Archaea

The impact of the archaea on the environment is easier to discuss because of the overarching dominant, and only reliably identifiable, group *Nitrosopumilus maritimus*. This has been revealed to have unique mechanisms among Archaea, for nitrification and autotrophy, oxidising ammonia to produce nitrite while extracting carbon from inorganic sources by chemolithoautotrophy (Könneke *et al.*, 2005). Nitrogen is

essential for terrestrial life (Painter, 1970; Jetten *et al.*, 2009), and the nitrogen cycle is an extremely important nutrient cycle in terrestrial ecosystems (Hayatsu *et al.*, 2008). The action of this archaeon in increasing available nitrite could therefore be an important factor in the diversity of River Dee microbial community, since nitrite provides the substrate needed for denitrifying bacteria to produce nitrogen gas, and a metabolic source for other microorganisms (Painter, 1970). This archaeon could therefore be responsible for the bacterial diversity observed.

Overall, the River Dee environment appears to have been highly selective with regard to the archaea, with only one observed species. Low archaeal diversity has been detected in hydrocarbon-rich sedimentary environments (Grabowski *et al.*, 2005), e.g. Waldron *et al.*, (2007) identified only one archaeal group in the Antrim Shale, USA. In addition, studies of archaeal diversity in tidal flat sediments and intertidal zone coastal microbial mats identified only two and five to six archaeal groups respectively (Kim *et al.,* 2005; Bolhuis and Stal, 2011). Archaeal diversity in intertidal environments is significantly lower than bacterial diversity which is consistent with the findings of this study.

2.4.2.3. An analogue to putative martian life?

Grotzinger *et al.*, (2014) proposed a microbial community founded on chemolithoautotrophy in the ancient lacustrine system. Though chemolithoautotrophic sulfate-reducing bacteria were detected within the community by MiSeq analysis (Figure 2.17), the three main constituents of the microbial community were found not to be chemolithoautotrophic, but heterotrophic. Though the community is sequestered within the subsurface, it is likely still founded on photosynthesis (or photoautotrophy) since the products of such metabolism would

still be accessible, either as remnant organic carbon deposited within the sediments, or dissolved oxygen (Stevens and Mckinley, 1995). Therefore, the bacterial community sampled in this study, despite not representing the species responsible for primary production, may still represent the types of heterotrophic microorganisms that could survive within the environment once it had been colonised by primary producers.

Therefore, as a community it may not be suitable as a biological analogue for putative ancient martian biota. However, individual species within the community may represent more accurate analogues of putative martian microbial processes. Nevertheless, without some selectivity toward those species that thrive within specifically martian conditions, and especially those that can thrive without direct or indirect association with photosynthesis, the status as a biological analogue will remain tenuous (see Chapter 3).

2.4.3. Limitations

Though the characterisation of this environment was comprehensive, it was limited in several ways.

In total, 12 sites were analysed from different locations on the estuary floor. Though the environment was visually uniform moving parallel to the coastline, further sample sites replicating the same setup would have increased the likelihood of obtaining statistically significant differences, if existed.

In addition to sampling from more sites, a better understanding of processes at the sample site could have been obtained through seasonal and perhaps diurnal repetition of the measurements.

Further analysis identifying the amount of DOC in the River Dee seawater would provide a clearer picture of the availability of organic material for use by microbial life. Using TOC abundance in the sediment only has neglected DOC, and so presents an underestimation of the prevalence and use of carbon sources in the River Dee subsurface environment. Combining DOC and TOC into a single analysis in future may elucidate processes at the sample site more accurately. In addition, identification of the specific carbon sources used by the microbial community in the River Dee subsurface environment could help to understand metabolism in low-carbon environments and further constrain future experiments.

16S DNA sequencing of each of the 12 sample sites would have been extremely expensive and experimentally unfeasible. However, this would have enabled more detailed and comprehensive taxonomic identification of the DNA fragments detected during TRFLP analysis. In addition, 16S DNA sequencing of samples taken from the microaerophilic and aerobic zones could have been used to provide evidence that the sampling procedure was sufficient in ensuring that the anaerobic zone was accurately sampled with no contamination from the other layers. Regardless, the largely uniform nature of the microbial community of archaea and bacteria, support the identifications made.

Though comprehensive mineralogical comparisons between the River Dee sediment and comparable martian sediments were carried out, highlighting the presence of several bioessential elements, the bioavailability of these elements is uncertain at this stage. Therefore, even though a diverse microbial community is supported at the River Dee site, it is not possible to say with certainty that dissolution of the sediment provides the required bioessential elements. Future microbial growth experiments containing growth media lacking individual bioessential elements, alongside a sample of River Dee sediment, could be used to identify which, if any, are bioavailable.

2.5. Conclusions

This chapter has demonstrated the environmental similarities between the subsurface of the River Dee estuary and the ancient lacustrine environment at Yellowknife Bay, Gale Crater. Comparisons of pH, temperature, TOC content and mineralogy based on *in situ* and lab-based techniques with MSL Curiosity Rover data facilitated a comprehensive discussion of the suitability of the River Dee site as an analogue.

Characterisation of the microbial community at the River Dee produced some interesting findings, with only a single dominant archaeal group detected with a diverse bacterial community. The ability of this single archaeal group to increase nitrogen availability makes it potentially responsible for the success of the bacterial community there. This environmental conditioning by the archaeal group may be informative with regard to the development of putative microbial communities on Mars, since the bioessential element availability at the two sites is similar.

It is clear that there is merit in using the River Dee site as an environmental analogue for the ancient habitable environment at Gale Crater, and the characteristics of the microbial groups present suggest that some individual species may (through further research) provide insight into processes of growth and survivability there.

Therefore, application of this community to further investigations is also appropriate, with the aim of identifying those species most relevant to the ancient habitable environment at Gale Crater. This biological analogue of putative martian life could then be used to suggest subsurface microbial processes in these environments and inform future life detection missions on Mars.

This identification of a new terrestrial Mars analogue site has provided the possibility of further environmental analysis and potential modelling of environmental processes

on Mars, as well as providing a biological analogue for use in microbiologically focused experiments. This is undertaken in Chapter 3.

Chapter 3- The microbial weathering and ecological succession of an intertidal subsurface community under simulated martian conditions

3.1. Introduction

In Chapter 2, an intertidal subsurface microbial community was proposed as a terrestrial analogue of the palaeolake at Yellowknife Bay, Gale Crater, Mars. An environmental characterisation of the River dee intertidal region was carried out and compared to data collected by the MSL Curiosity Rover. The undeniable similarity of the two supports the suggestion that the microbial community at this analogue site could be used as an analogy to study potential life that may have inhabited the subsurface of the palaeolake at Gale Crater. Therefore, the specific aims of this chapter are:

1. To investigate community succession of the aforementioned microbial community when incubated under Mars simulated conditions (a martian regolith analogue with nutritional, atmospheric and environmental conditions similar to that of the martian subsurface (see Section 3.3 of this chapter)).

2. To identify, where possible, the microorganisms that survive and grow within the Mars analogue system so as recognise bacterial species that may function as useful biological analogues of putative martian life.

3. To observe and analyse the interactions between a terrestrial microbial community that is relevant to putative life on past Mars (see Chapter 2) and a rock substrate designed to be analogous to martian regolith.

The hypotheses for these aims are:

1. Colonisation and succession of the Mars analogue system will result in fluctuations in the composition of the microbial community, leading to a final composition that is different to that of the initial inoculum.

2. The microorganisms able to grow and survive within the Mars analogue system are ideal microorganisms for studying putative martian life.

3. When compared to an abiotic control, microbial growth within the experimental system will yield biologically characteristic geochemical biosignatures.

As discussed in Chapter 2, life on Mars, both contemporary and in its ancient past, would be heavily constrained. The UV flux on the surface of Mars, in combination with the low temperature and desiccating conditions, would be a significant selective pressure to life (Sagan and Pollack, 1974). However, the subsurface is not subject to these pressures because UV penetration is minimal (~2 mm Parnell *et al.*, 2007) (Dartnell, Desorgher, Ward and A. J. Coates, 2007; Charles S. Cockell, 2014); furthermore, temperature increases with depth (Clifford, 1993; Nimmo and Stevenson, 2001; Clifford *et al.*, 2010) in the subsurface environment and, in the absence of light, energy production would depend on chemolithoautotrophy or chemoorganotrophy.

This chapter focuses on investigating potential microbial processes (both microbe-microbe and microbe-environment interactions) in the ancient martian lakebed sediments at Gale Crater (see Chapter 2), a UV-protected, subsurface, aqueous martian environment with above zero temperatures. Potential microbial processes here are investigated using the microbial community collected from the analogue environment of the River Dee estuary, United Kingdom (see Chapter 2). Furthermore, microbial colonisation and succession processes are investigated, providing a model of putative martian microbial community dynamics.

Using a simulated martian environment should provide a means of producing a laboratory environment analogous to the ancient subsurface environment at Gale Crater and as such provide insight into potential microbial processes. Thus, it may be possible to inform our understanding of putative martian life to facilitate future life detection missions.

3.2. Designing a Mars analogue experimental system

Chapter 2 demonstrated the similarities of the River Dee estuary subsurface environment to the ancient lacustrine environment of Gale Crater, Mars and demonstrated the relevance of the River Dee microbial community to putative martian life. To investigate how such microorganisms would interact with their environment, it was necessary to try to replicate martian environments more accurately. This simulated environment must therefore fit the following characteristics:

1. Include an regolith analogue with a similar composition to the martian regolith at Gale Crater

2. Use a minimal growth medium containing only a nitrogen source, an organic carbon source, a buffer, reducing agents and sodium chloride (see Table 3.6) for details)

3. Use a Mars gas headspace

4. Include a microbial community comprised of bacteria and archaea that are analogous to putative martian life in terms of metabolism and growth conditions (as defined in Chapter 2)

5. Invoke an incubation temperature relevant to those environments on Mars with long-lasting surface/ shallow subsurface hydrous activity

3.3. Mars regolith analogue

To investigate abiotic and biotic weathering processes in a putative martian environment, a Mars analogue basalt regolith was required (McSween *et al.*, 2009; Olsson-Francis and Cockell, 2010). Several existing Mars regolith analogues were considered for use in this study, e.g. Theo's Flow and JSC Mars-1. Theo's Flow, a basalt with significant compositional similarity to the Nakhlite martian meteorites (thought to be a useful analogue of martian volcanic clinopyroxenes) was considered. However, the specificity of Theo's Flow to volcanic environments rather than lacustrine environments made it unsuitable for use in this study.

The JSC Mars-1 martian regolith analogue (Allen *et al.*, 1998) is thought to be a spectroscopic analogue of high albedo regions on Mars, and is broadly representative of the samples analysed by the Viking 1 lander (Clark *et al.*, 1982). However, it represents a weathered surface regolith and is therefore not directly relevant to subsurface environments, which are likely to be more pristine.

Problematically, general-purpose martian regolith analogues (such as JSC Mars-1, the Mojave Desert (Peters *et al.*, 2008) and Salten Skov (Hansen *et al.*, 2005) analogues) result in an oversimplification of the compositional heterogeneity of martian regolith in totality (Marlow *et al.,* 2008). By focusing this study on a single site with a known composition, i.e. the Rocknest site at Gale Crater, definition of a new regolith analogue ameliorates this risk to the relevance of the experiments.

3.3.1. Defining a regolith analogue

In this study, a martian regolith analogue was designed using terrestrial basalt as the bulk component. Terrestrial basalts are available in large quantities commercially, enabling sufficient quantities to be procured for use in planned experiments, with some contingency. A basalt from Le Cheix Puy de Dome, France was selected because it was purported to represent a typical unweathered basalt. This was purchased from Richard Tayler Minerals, Cobham, England.

However, terrestrial basalts are not true compositional equivalents to martian basalts, which are lower in iron (Longhi *et al.*, 1992). Hence the terrestrial basalt would need supplementing with an iron source that was also readily available and in approximately equivalent crystalline form (rather than free metal). Aegirine, a pyroxene, was selected because it is approximately 26 % FeO, with negligible quantities of other elements, aside from sodium and calcium which, would not interfere with the biological aspects of this study. Aegirine was also provided by Richard Tayler Minerals (Surrey, United Kingdom) and was originally sourced from Mt. Malosa, Malawi.

Prior to manufacture of the regolith analogue, and in order to ascertain the required ratio of basalt to aegirine, both were characterised using X-ray Fluorescence (XRF) *and* Electron Microprobe Analysis (EMPA). For EMPA, the constituent minerals of the basalt were analysed individually (see Appendix for compositions). Table 3.1 shows the results of this characterisation, and compares the basalt and aegirine to the Rocknest basalt (Schmidt et al., 2014).

A detailed comparison of the terrestrial basalt and the Rocknest site at Gale Crater showed that further supplementation was unnecessary (see Section 3.3.3 and Table 3.1).

Table 3.1. Comparative compositional data of bioessential elements for the terrestrial basalt used in experiments, the Rocknest site on Mars taken from Schmidt et al. (2014), EMPA data for aegirine sourced from Mt. Malosa, Malawi and the composition of the regolith analogue calculated based on a 1.75:1 ratio of Le Cheix Quarry Basalt and Aegirine.

Oxide/Element	Le Cheix Quarry Basalt (%wt)	Aegirine (%wt)	Rocknest 3 (%wt)	Regolith analogue (%wt)
SiO_2	44.70	52.35	45.98	47.48
Fe_2O_3 (T)	11.73	29.21	18.38	18.09
MnO	0.21	0.66	0.45	0.38
MgO	4.66	0.07	5.33	2.99
Na_2O	4.02	12.21	4.02	7.00
K_2O	2.37	-	1.86	1.51
P_2O_5	0.93	0.01	1.08	0.60
Cl	-	-	0.88	-
SO3	-	0.03	-	0.01
TiO2	-	0.90	-	0.33
Al_2O_3	-	0.19	-	0.07
Cr_2O_3	-	-	-	-
CaO	-	1.52	-	0.55
NiO	-	0.01	-	0.00
	(ppm)	-	(ppm)	-
Zn	94.0	-	888.0	59.82
Ni	3.3	-	364.0	2.12
Co	20.7	-	-	13.17
Cu	46.0	-	-	29.27

As expected, the most significant difference between the experimental basalt and that of Rocknest was the total Fe content, with the latter being enriched by approximately 7 %. It was thus determined that the basalt:aegerine ratio required to mimic the composition of the Rocknest site was 1.75:1.

3.3.2. Production of the regolith analogue

To produce the regolith analogue, the basalt and aegirine were crushed to a powder 100 μm in diameter using a Tema swing mill for 5 min. Sieves were used to measure the diameter of the grains. One hundred μm grain diameter was selected to maximise surface area and therefore growth (Olsson-Francis and Cockell, 2010). The relatively small grain size of the regolith substrate was selected to ensure sufficient microbial growth *via* an increased surface area for potential microbial attachment and chemical dissolution processes. However, this small grain size prevented the labelling and future location of specific sites on the grain surfaces that would have facilitated small scale geochemical analysis before and after weathering. Ostensibly representative samples had to be analysed instead. The crushed rocks were then mixed to create a Mars regolith analogue.

3.3.3. Characterisation of the regolith analogue

3.3.3.1. Surface to area ratio

The surface area of the minerals impacts abiotic dissolution rates and the size of the area available for microbial attachment. It was therefore important to quantify this characteristic of the experimental system. To measure the surface area of the rocks, BET analysis was carried out at Oxford University Begbroke Science Park using a Micromeritics Gemini V surface area analyser. Nitrogen was used as the adsorbate and the sample was degassed at 150 °C for 2 hr, followed by 2 hr at room temperature prior to analysis.

BET Surface Area was found to be 2.3881 ± 0.0144 m^2/g (See Appendix). Since the basalt and aegirine had the same grain size, it was assumed that they had equal surface

areas. Based on this, an approximate total surface area for 14 g of rock substrate is 33.4334 ± 0.2016 m^2.

3.4. Optimisation of the Mars analogue system

Several parameters were investigated before running the experiment using the analogue. This was to ensure that a microbial cell density was achieved that would be conducive to identifying biosignatures and to ensure that there were no significant fluctuations in environmental conditions throughout the experiment. Once this had been ascertained, a growth experiment was carried out to identify a relevant organic carbon source for use in the experimental system that also sustained a significant microbial population, and the effect of water/rock ratio on microbial growth was also investigated since this has been shown to impact the size of microbial populations (Olsson-Francis and Cockell, 2010). Furthermore, the impact of different factors on the pH of the system were investigated e.g. headspace gases, headspace pressure and the buffering capacity of basalt, since these are also known to impact microbial diversity and growth (Bååth, 1996).

In addition to the characteristics discussed in Section 3.2, much effort was made to optimise and characterise the experimental system before running the experiment using the analogue. Investigation into the optimum carbon source, headspace pressure, water-rock ratio and headspace gas composition provided key data on the specific conditions of the experiment. Anaerobic and medium preparation methods used in these experiments are the same as those described below (Section 3.4).

These preliminary experiments demonstrated that Na-Lactate was the best carbon source (Table 3.2), that the inclusion of a 2 bar Mars gas headspace would cause a

decrease in pH of 1.3 pH units over time (Table 3.3), that the effects on pH of a 2 bar Mars gas headspace were not considerably more severe than lower pressure atmospheres (Table 3.4) and that variations in rock ratio (medium:rock) did not have a marked effect upon the pH (Table 3.5). In addition to these, a specific growth rate of 0.071 was the maximum calculated growth rate for all of the environmental samples inoculated into rich media.

Table 3.2. A comparison of specific growth rates using different organic carbon sources in enrichment media.

Carbon source	Specific growth rate (hr^{-1})
Na-Lactate	0.0386
Glucose	0.0036
Na-Formate	0.0057

Compared to Glucose and Na-Formate, samples with Na-Lactate as their carbon source had a considerably larger specific growth rate of 0.0386 hr^{-1}. Since the weathering experiments would likely yield the most useful data with a thriving microbial community, the carbon source that stimulated the fastest growth was selected for inclusion in the simulated Mars environment.

Table 3.3. The change in pH using different headspace gas compositions.

Headspace composition	Change in pH over 150 hours
Nitrogen	+2
Mars gas	-1.3
No headspace gas (low pressure nitrogen)	+1.5

The composition of the gas headspace in the experimental system was important in constructing a simulated Mars environment. Therefore, the effect of using a Mars gas headspace, compared to nitrogen (with no specifically added headspace as a control),

on the pH of the growth medium was investigated. As expected, the medium was found to acidify in the presence of the carbon dioxide rich (approximately 96 %) gas.

Table 3.4. The pH change over time with different pressures of Mars gas headspace.

Mars gas headspace pressure (bar)	Change in pH over 150 hours
1	*-3.1*
1.5	*-3.4*
2	*-3.5*

To maintain the anaerobic conditions in the experimental system, a higher pressure is preferred. Since the atmospheric gas used in the weathering experiment is composed mostly of carbon dioxide, the acidification of the media had to be monitored. Although Table 3.4 demonstrates the increased acidification with larger pressures of Mars gas, the effect is manageable, and 2 bar remains preferable as this best prevents unintended oxidation of the anaerobic system.

Table 3.5. The pH change over time with different rock ratios.

Rock ratio (media:basalt)	Change in pH over 350 hours after initial drop
1:2	+0.36
1:4	+0.33
1:8	+0.33

The capacity of the basalt in the Mars regolith analogue was investigated to further inform predictions as to how the pH of the experimental system would change

throughout the experiment. Comparing, 1:2, 1:4 and 1:8 media:basalt ratios demonstrated that there was little to no difference.

3.5. Methods

3.5.1. Anaerobic techniques

Mars' atmosphere is approximately 96 % carbon dioxide, 1.93 % argon, 1.89 % nitrogen, 0.145 % oxygen and <0.1 % carbon monoxide (Mahaffy *et al.*, 2013), thus the conditions on Mars are anaerobic. Therefore, steps were taken to ensure that conditions within the Mars analogue system were maintained anaerobically. To ensure that anaerobic conditions were employed, the redox indicator Resazurin (Dien *et al.*, 2000) was used to monitor the redox environment throughout the experiment. In oxygenated conditions, Resazurin is light blue, changing to pink and then to colourless as partially oxygenated and anerobic conditions are reached respectively.

3.5.1.1. Media preparation

Nitrogen gas was bubbled through the medium during preparation, and oxygen traps were used on all gas lines. The ddH_2O used in media preparation was microwaved for 3 min until boiling, to remove dissolved oxygen. It was immediately transferred to a Wheaton Bottle, pre-flushed with N_2 and continually flushed throughout preparation. The chemical reagents, which are listed in Sections 3.3.2 and 3.3.4 were added as described. The reducing agents (Na-thioglycollate and ascorbic acid) were added to counteract inadvertent oxygen exposure throughout the experiment. Autoclaving at 121 °C for 15 min further contributed to the creation of an anaerobic environment. At

this stage in media preparation, the Resazurin was neither pink nor blue, indicating the successful creation of anaerobic conditions.

The key apparatus used to facilitate these efforts is shown in Figure 3.1.
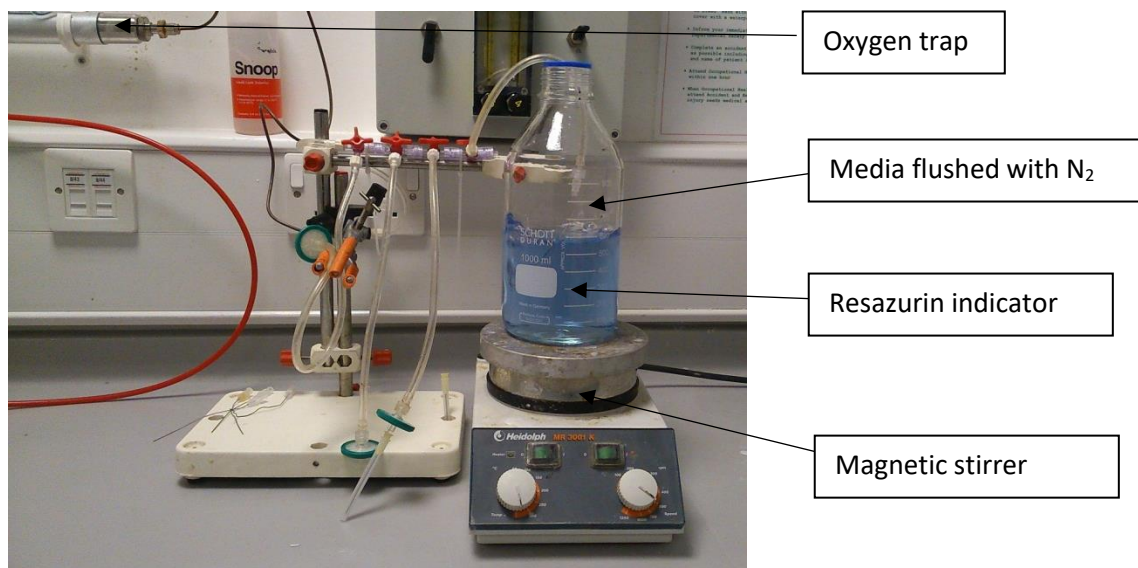
Figure 3.1. Anaerobic apparatus, which was used during media preparation.

Experiments were conducted in either 125 mL or 30 mL Wheaton Bottles. All bottles used in the preparation of media were first flushed thoroughly with N_2 to prevent oxidation of the media by any atmospheric oxygen already present in the bottles. The media were transferred to Wheaton Bottles with an automated pipette, which had been flushed with the headspace gas by operating it for 3 sec within the Wheaton Bottle but above the media surface to flush out any residual oxygen. Once the aliquot was decanted, the Wheaton Bottle was immediately sealed using a Bellco Glass 20 mm septum stopper and crimped using a Wheaton E-Z crimper.

3.5.2. Enrichment media

3.5.2.1. Preparation

The first step was to generate a stock culture, which would produce sufficient microbial cell density for the microbial weathering experiments. The enrichment medium for bacterial growth was adapted from Shuisong and Boone (1999); Haouari *et al.*, (2006) and Sungur *et al.,* (2010). These media were selected because they have previously been used to successfully culture marine chemoorganotrophic and chemolithotrophic microorganisms and therefore would ensure that the River Dee microbial community was accurately represented in the stock culture.

The enrichment medium consisted of the following (per litre): 2 g of trypticase peptone, 2 g of yeast extract, 0.3 g of KCl, 1 g of NH_4Cl, 3 g of Na_2SO_4, 23 g of NaCl, 0.5 g of Na-Lactate, 2 g of $MgCl_2$, 0.35 g of K_2HPO_4, 0.1 g of Na-thioglycollate and 0.1 g of ascorbic acid. The pH was altered to 7.75 using 5M NaOH and 1M HCl to account for the approximate 0.5-0.7 pH unit drop identified in pilot experiments as a result of headspace pressurisation.

The medium was then autoclaved at 121℃ and 15 psi for 15 min. Post autoclave, 40 µL of a 10× diluted vitamin solution comprising the following (per litre) was added aseptically: 0.05 g of thiamine hydrochloride, 0.05 g of riboflavin, 0.05 g of pyroxidine hydrochloride, 0.05 g of calcium pantothenate, 0.001 g of nicotinic acid, 0.001 g of Biotin, 0.001 g of folic acid and 0.001 g of 4-aminobenzoic acid. In addition, 100 µL of a pre-made SL10 trace element solution comprising the following (per litre unless otherwise stated) was added: 1.5 g of $FeCl_2·4H_2O$, 70 mg of $ZnCl_2$, 100 mg of $MnCl_2·4H_2O$, 6 mg of H_3BO_3, 190 mg of $CoCl·6H_2O$, 2 mg of $CuCl_2·2H_2O$, 24 mg of $NiCl_2·6H_2O$ and 36 mg of $Na_2MoO_4·2H_2O$.

5 g of sediment from River Dee sample site 3 (see Chapter 2) was aseptically and anaerobically added into Wheaton Bottles containing 100 ml of the completed medium. After autoclaving, the Wheaton Bottle was unbunged and, under microfiltered N_2, the sediment was added using a flame and ethanol sterilised spatula and resealed. Finally, as above, a 2 bar CO_2/H_2 headspace was generated within the Wheaton Bottle. This over-pressurisation was adopted to prevent oxygen seepage into the sealed bottles during the experiment.

3.5.3. Minimal medium

A nutritionally-limited minimal medium was designed such that the majority of bioessential elements, e.g. P, S, Mg, Fe (Wackett *et al.*, 2004; Pontefract *et al.*, 2012) (see Chapter 2) would be provided solely by the regolith analogue. However, a nitrogen source and a carbon source were added to the medium because they are also essential to life, yet they are not supplied by the regolith analogue. Table 3.6 explains the rationale for the minimal medium composition.

Table 3.6. Rationale for the composition of the minimal medium.

Compound added	Reason	Relevance to Mars
NH_4Cl	Source of nitrogen	Nitrogen-bearing compounds found at Gale Crater (Stern *et al.*, 2015)
Na-Lactate	Source of carbon	Organic molecules found at Gale Crater (Freissinet *et al.*, 2015, 2016)
NaCl	Relevance to martian environment	1-2 % salinity inferred for water at Gale Crater (S. M. McLennan *et al.*, 2014; Vaniman *et al.*, 2014)
Na_2CO_3	Buffer	Carbonates detected at Gale Crater (Ming *et al.*, 2014)

Certain silicate minerals have been shown to contain ammonium, either as the end-member ammonia-feldspar buddingtonite (Gulbrandsen, 1974) or where potassium ions have been replaced by ammonium ions in the feldspar structure (Rogers et al., 1998; Rogers et al., 1998). Potassium feldspars were identified in the River Dee samples (see Chapter 2). However, ammonium ion replacement of potassium has been shown to range from 6 to 196 ppm (Honma and Itihara, 1981) and Guerra et al (2010) found that the minimum nitrogen concentration that supported the first of two microbial growth phases was approximately 350 ppm. Assuming that the ammonium ions potentially present within the River Dee potassium feldspars would be completely

bioavailable it would be unlikely that silicate-bound ammonium would be sufficient to support microorganisms. Therefore, an additional nitrogen source was added.

On Mars, carbon sources available for metabolism would likely come from meteoritic organic matter (Leshin, Mahaffy, Webster, Cabane, Coll, Conrad, Archer, Atreya, Brunner, A. Buch, *et al.*, 2013; Remusat, 2014) or be scavenged from dead microorganisms. Lactate was utilised during the Labelled Release experiments carried out by the Viking lander as a potential organic carbon source for putative martian life (Klein *et al.*, 1976; Levin and Straat, 1976b; Warmflash *et al.*, 2007). Levin and Straat (1976a) believed that, since lactate is a simple Miller-Urey product (Miller and Urey, 1959; Buvet and Ponnamperuma, 1971), it might have been formed in prebiotic processes on Mars.

In addition, lactate is considered a likely electron donor for Fe- and S-reducing microorganisms, both considered to be plausible biological analogues of putative martian life (Nixon *et al.*, 2013). This study relies on similar assumptions to Levin and Straat (1976a) and though further attempts to analyse martian organics are in process, the presence of lactate on Mars is still theoretical. With these considerations, and the results of pilot experiments, Na-Lactate was added as a carbon source.

NaCl was added to accord with the original marine provenance of the microbial community and the mildly saline lacustrine systems on early Mars (e.g. Gale Crater) (Grotzinger *et al.*, 2014).

Life in the subsurface of early Mars would most likely have been an open system with movement of water on a much larger scale than the 100 mL experimental system invoked here. Thus, to attempt to align the closed experimental system with natural open systems, the microorganisms were prevented from altering the pH of the medium too dramatically by the addition of a buffer. Carbonate minerals have been discovered

on the martian surface (e.g. Bandfield *et al.*, 2003; Bibring *et al.*, 2006; Ehlmann *et al.*, 2008; Webster *et al.*, 2013; Archer Jr. *et al.*, 2014; Grotzinger *et al.*, 2014), indicating approximately 1-2 % carbonate at Rocknest (Archer Jr. *et al.*, 2014) and 2-5 wt.% in martian fines (Bandfield *et al.*, 2003). Hence, a Na_2CO_3 buffer was used.

Since Mars has an atmosphere consisting of approximately 96 % carbon dioxide (Mahaffy *et al.*, 2013), and as discussed above, to prevent oxygen seepage into the experimental system, the reducing agents Na-Thioglycollate and ascorbic acid were also used.

The minimal medium was therefore composed of (per litre): 1 g NH_4Cl , 2 g Na-Lactate, 1 g Na-thioglycollate, 1 g ascorbic acid, 37 g NaCl and 13.25 g Na_2CO_3.

3.5.4. Final experimental setup

The final experimental setup was informed by the results of the pilot experiments shown above. The following describes aspects of the method that were different to those that are described above:

To account for the acidification of the medium by the Mars gas headspace, the pH of the medium was adjusted to pH 8 by adding 1 M NaOH.

Fourteen grams (8.09 g of basalt and 5.91 g of aegirine) of Mars regolith analogue was added to N_2 filled 125 mL Wheaton Bottles and immediately sealed with Bellco Glass 20 mm septum stoppers and crimped using a Wheaton E-Z crimper. The rock was then autoclaved at 121 ℃ and 15 psi for approximately 15 min. The medium was autoclaved separately. After autoclaving, 68 mL of medium was added to the Mars regolith analogue using aseptic anaerobic technique (as described in section 3.3.1).

Using aseptic anaerobic techniques, the medium was inoculated with a 5 % inoculum from the intermediary stock (see below). Each bottle was then pressurised to 2 bar using Mars gas. All Wheaton Bottles were labelled and stored at 14.8 ˚C for the duration of the experiment. Abiotic experiments were carried out in parallel, and for each experiment there were five Wheaton Bottles.

3.5.4.1. Minimal medium intermediary stock

The nutrient limiting nature of the medium used in these experiments was essential, so an intermediate stage was carried out to prevent nutrient carry over from the enrichment medium. Using this intermediary stock, a high cell density inoculum (10^8 cells mL^{-1}) with a reduced nutrient carry-over was prepared. The excess nutrients carried over in the inoculum from the enrichment would have been diluted ×20 when transferred in to the intermediary medium. It is also likely that these nutrients were utilised by the microbial community within the intermediary stock. Figure 3.2 shows a schematic overview of this process (see Appendix for full details).
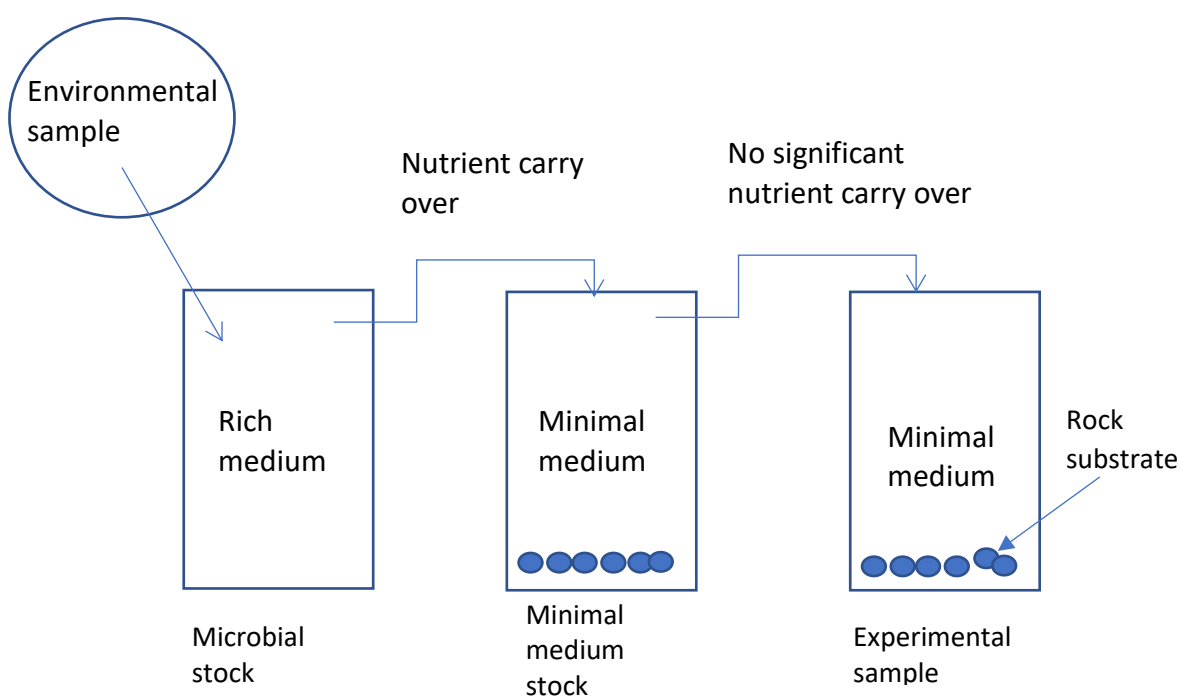
Figure 3.2. A schematic overview of the large-scale experimental stages in this study.

The inoculum for the main experiment was exponentially grown cells (they were readily replicating and well adapted to the environmental conditions). A 5 % inoculum of 4×10^8 cells were added to the medium aseptically and anaerobically, as described in Section 3.3.1.

A schematic overview of the experiment is shown in Figure 3.2 and consisted of five biotic experiments and five abiotic controls. Microbial growth was monitored until the stationary phase was reached, in intervals of approximately every 24 - 48 hr. Cell enumeration was plotted and samples were collected at each phase of growth and analysed for ICP-AES, TRFLP and pH.

Post experiment the rock substrate was dried and samples were viewed under a FEG-SEM. EDS (point) analysis was carried out to identify compositional differences between observed features and the bulk rock.

15 cm

Sealed with 20 mm butyl rubber bung

55 mL, 2bar Mars gas headspace

70 mL Minimal medium

14 g rock substrate. 8.09 g Basalt: 5.91 g Aegirine

0 cm
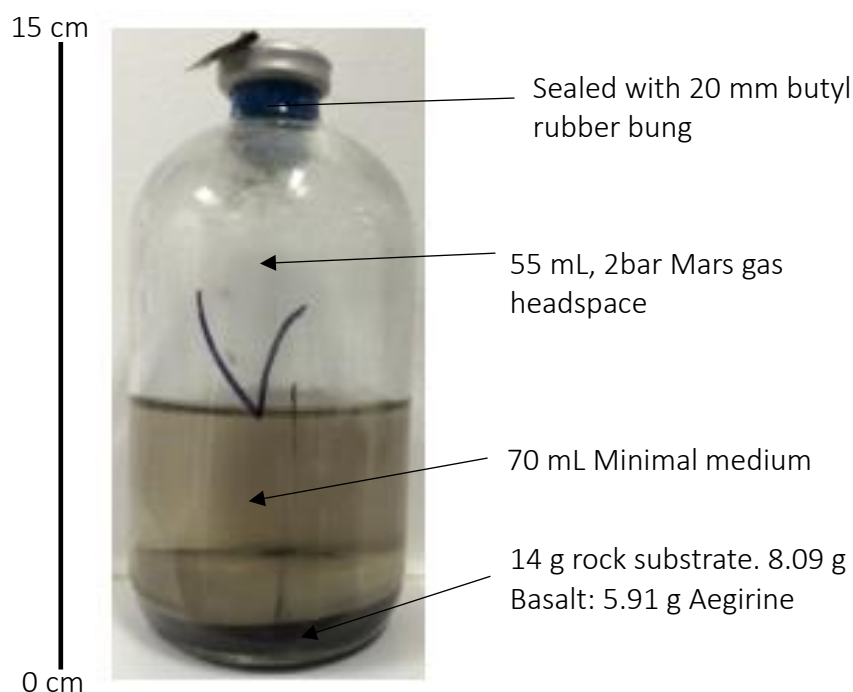
Figure 3.3. A labelled photograph of an experimental sample detailing the constituent parts of the system.

3.5.5. Analytical techniques

3.5.5.1. Cell enumeration

Cell enumeration was carried out using a modified version of the Sybr Green protocol used in Summers et al. (2013). 100 µL of a 2.5 g/L solution of Sybr Green was added to 1 mL of sample (or in respective proportions otherwise). Sybr Green dye degrades in the presence of light and so the samples were left in the dark while the dye bound to the bacterial DNA (minimum 20 min).

Once the dye had taken effect, a 100 µL portion of the stained sample was filtered to immobilise the cells on a 25 mm polycarbonate Whatman Nuclepore track-etched membrane, pore size 0.2 µm. This was then washed with 100 µL of sterile distilled water. The filter paper was then transferred onto a microscope slide, treated with glycerol (to delay the degradation of the dye) and then examined under the Leica DNRP microscope at 100× magnification. Twenty fields of view were selected to collect data evenly from all areas of the slide and the bacterial cells fluorescing green were counted. This procedure was carried out periodically and dependent on the observed trend in bacterial growth.

3.5.5.2. Microbial growth in the enrichment medium

Microbial growth in the enrichment medium was inferred by optical density (OD) increases at 660 nm. A Camspec M107 spectrophotometer was used and 1 mL aliquots of sample were measured against a distilled water blank.

3.5.5.3. Geochemical analysis

3.5.5.3.1. pH

The pH of the growth medium was measured throughout the experiment on 1 mL aliquots removed approximately every 100 hr. pH was measured using a Thermo Scientific Orion Three Star with a two point calibration using Omega Buffer solutions at pH 4 and 7.

3.5.5.3.2. Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES)

The aim was to ascertain the effects of microbial metabolism on the dissolution of the Mars regolith analogue. To achieve this, it was necessary to monitor the elemental composition of the growth medium throughout the experiment and calculate the elemental composition of the microbial cell contents to account for any cellular uptake of elements. ICP-AES was carried out on samples of the medium using a Prodigy High Dispersion ICP Atomic Emission Spectrometer.

In triplicate, 1 mL aliquots were removed aseptically from the growth medium (biotic and abiotic) using the above anaerobic techniques (Figure 3.1). To eliminate any regolith analogue from the analytical sample, the aliquots were centrifuged at 400 × g for two min and the supernatant collected. Centrifugation was repeated at 13000 × g for two min to separate the bacterial cells from the supernatant. This step was repeated in the abiotic samples to act as a control.

All samples were acidified to approximately pH 2 for storage and analysis. For this, 100 μL of 10 % (2.24 M) nitric acid was added to the tube containing the growth medium, and the tube containing the microbial cells was made up to 1 mL using 10 % nitric acid. The addition of nitric acid caused lysis of the cells, which allowed the

elemental content of the cells to be measured. Cell enumeration data were used in tandem with this to calculate accurate cell content compositions (μmol L^{-1}).

This process was repeated where the microbial growth phases were identified by cell enumeration to achieve an overview of processes throughout each phase.

3.5.5.3.3. Field Electron Gun- Scanning Electron Microscopy (FEG-SEM)

At the end of the experiment, the regolith analogue was air dried under sterile conditions in a Biomat^2 class 2 microbiological safety cabinet, and carbon coated (15-20 nm thickness) on aluminium stubs. Samples from all replicates of each experimental condition (biotic and abiotic) were analysed using a FEG-SEM (ZEISS Supra; 55-VP; Zeiss Microimaging, Gottingen, Germany) operated at an accelerating voltage of 2-15 kV and a 7-10 mm working distance. This was carried out to investigate morphological changes in the rock substrate and undertake Electron Dispersive Scattering (EDS) analysis which would provide qualitative compositional data.

3.5.5.3.4. Powder X-Ray Diffraction (XRD)

Samples of the regolith analogue from both biotic and abiotic conditions were analysed by XRD for bulk phase composition before and after the experiment to identify any potential secondary mineralisation within that may have developed during the weathering tests.

Samples were crushed and powdered for 30 sec using an automated tungsten carbide mill. The powdered samples were then sieved to <38 μm and ground by hand in an agate pestle and mortar until the powder was believed to be of sufficiently small grain size, typically between 5 and 15 μm (Schofield *et al.*, 2002). These were then top loaded

into a circular well mount, 15 mm in diameter and 1 mm deep, packed and smoothed using a spatula.

XRD data were collected using a Nonius PDS 120 powder diffraction system. A monochromatic cobalt $K\alpha_1$ X-ray beam was generated using an X-ray tube operating at 45 kV and 32 mA and a germanium 111 monochromator crystal. Slits of dimensions 0.14 × 5.0 mm were used to restrict the size of the beam. Measurements were made in reflection geometry with the sample surface at an angle of 4° to the incident beam. During data collection, the samples were spun continuously in the plane of the sample surface. A more detailed description of the experimental set-up is provided in Schofield *et al.*, (2002).

3.5.5.3.5. EMPA

EMPA was carried out to identify the elemental composition of the aegirine used in this experiment. A polished thin-section of the aegirine was created and analysed using a CAMECA SX100 electron microprobe. The analytical conditions applied were standard, i.e. 20 kV, 20 nA with a beam diameter of 10 μm.

3.5.5.3.6. XRF

To characterise the terrestrial basalt used in this experiment, XRF analysis was carried out on powdered samples using an Applied Research Laboratories 8420 + dual goniometer wavelength-dispersive X-ray spectrometer. The data were collated so that an accurate comparison to Rocknest could be carried out (Section 3.3.3).

3.5.5.4. Community analysis

3.5.5.4.1. Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis

As in Chapter 2, TRFLP analysis was used to investigate the diversity of the microbial community. Here, 1 mL aliquots were taken throughout the experiment and analysed to allow the structure of the microbial community to be monitored at all stages, and therefore potential patterns to be elucidated. The analysis was carried out almost identically to that described in Chapter 2 and thus only the differences are described here.

3.5.5.4.2. DNA extraction

For DNA extraction, 1 mL aliquots of culture were anaerobically and aseptically collected from the experimental media. Samples were stored at -20 °C until the end of the experiment when DNA extraction was carried out.

For this, the samples were thawed and 0.5 mL of culture was aliquoted into sterile 1.5 mL Eppendorf tubes. 1.5 mL of sterile 20 mM EDTA was added and the mixture was shaken at room temperature for 1 hr. This was then spun at 16,000 × g for 10 min and the supernatant was discarded. 500 μL of lysis buffer, consisting of 0.5 % SDS, 25 mM EDTA and 20 μg/mL proteinase K, was then added and mixed by inversion. This mixture was then incubated overnight at -20 °C.

The tubes were incubated in a water bath at 55 °C for 35 min, mixing by inversion every 10 min. The samples were then removed from the water bath, and 100 μL of prewarmed 5 M NaCl and 80 μL of prewarmed CTAB were added. This mixture was mixed by inversion and incubated at 65 °C for 10 min. The tubes were removed from

the water bath and 680 μL of chloroform : isoamyl alcohol 24:1 was added and shaken well. This was then centrifuged at 16,000 × g for five min.

The top aqueous layer was removed into a clean tube and 0.6 volumes of isopropanol were added and mixed by inversion. This mixture was left at room temperature for one hr before being spun at 16,000 × g for 10 min. The supernatant was discarded and the tubes containing the pellets were left to dry on a paper towel. The pellets were washed with 70 % ethanol stored in -20 °C. The ethanol was poured off and the pellets were left to dry once more before being resuspended in MilliQ water.

3.5.5.5. Analytical approach

The following is a brief summary of each analytical technique and their order of use in the experiment:

1. Cell enumeration undertaken every 24 - 48 hr

2. ICP-AES samples taken at ten time periods throughout the experiment. Sampling times selected based on cell enumeration data

3. TRFLP samples taken at the same ten time periods throughout the experiment. Sampling times selected based on cell enumeration data

4. pH measured every 48 - 72 hr (longer interval than cell enumeration used to reduce media loss)

5. FEG-SEM and XRD analysis to be used after the end of the experiment to analyse the Mars regolith analogue

It is prudent to note that compared to the 10 - 100 μL samples needed for cell enumeration, 4 mL in total was needed for ICP-AES, pH and TRFLP analyses and thus it was decided that these analyses could not be carried out with the same frequency as

cell enumeration since this was too large a proportion of the total volume of experimental medium.

3.6. Results

The succession of a microbial community in a simulated martian environment and the effect this has on the geochemistry within the system were the focus of this experiment. The results therefore fall into two categories:

1) Community succession processes

2) Geochemical analyses of substrate dissolution

All data presented in the following sections are related to the main experiment. Data from pilot experiments are provided in the appendix.

3.6.1. Microbial growth

3.6.1.1. Microbial growth

Cell density, ascertained by cell enumeration, was carried out throughout the experiment. Sampling times for subsequent analytical techniques (i.e. ICP-AES and TRFLP) were all based upon the identification of microbial growth phases, which were determined by cell enumeration values. Figure 3.4 shows the cell enumeration over time for the mean of the five biotic samples.

Unlike in the intermediary minimal medium (see Appendix), there was no initial drop in cell density. Instead there was a short lag phase, lasting approximately 24 hr (ranging from 1×10^5 cells mL^{-1} in experiment 1 to 5×10^5 cells mL^{-1} in experiment 2)

followed by a log phase that began around 25 - 50 hr after inoculation and lasted until approximately 290 hr (ranging from $4×10^6$ cells mL^{-1} in experiment 3 to $7×10^6$ cells mL^{-1} in experiment 4, at 290 hr). The log phase in experiment 1 continued until approximately 370 hr and reached a cell density of $1×10^7$ cells mL^{-1}. This was due to the fact that the microbial community had already adapted to the experimental conditions within the pilot experimental system. Stationary phase began at approximately 300 hr and the cell numbers remained relatively constant at approximately $5 × 10^6$ cells mL^{-1}.

Cell enumeration was stopped after 200 hr in the stationary phase as the death phase was considered to be unlikely to provide any meaningful results.

Cell enumeration for the abiotic controls demonstrated that no microbial contamination had occurred.
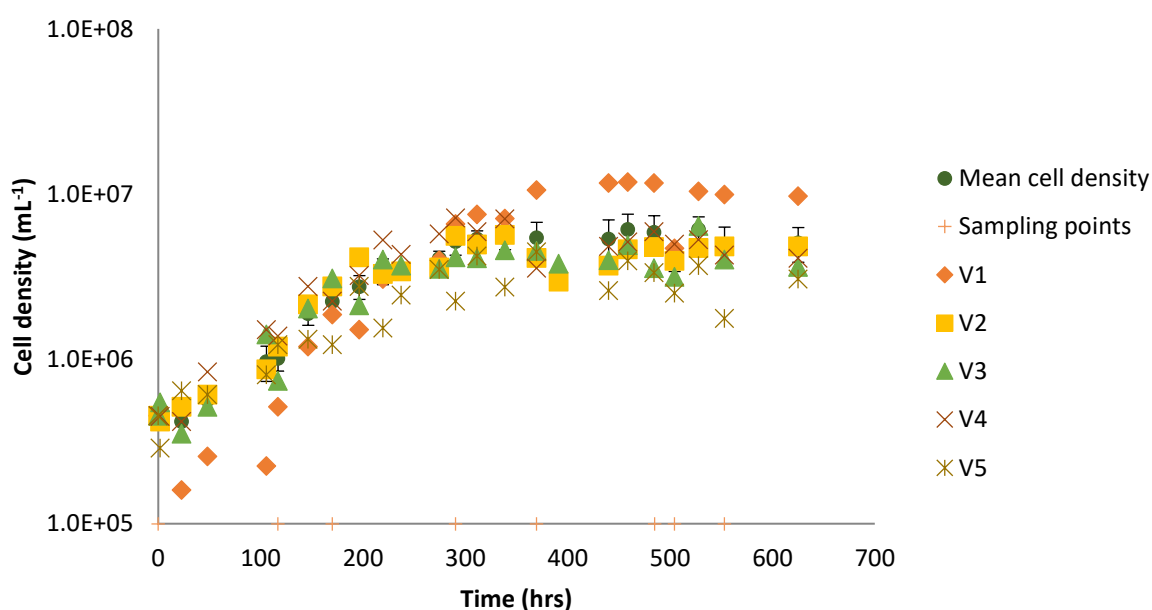
Figure 3.4. Mean cell density plotted over time for the five replicates in the analogue medium and their mean cell density. "Sampling points" denote when samples were extracted for further analyses. Error bars represent the standard deviation for the mean cell density. For samples V1-V5 error bars are hidden beneath the markers.

Marked along the x axis of Figure 3.4 are the points in time where samples were removed from the culture for further analysis. These points were taken to provide an insight into changes that occur concurrently with the transition between different microbial growth phases. To achieve this, when cell density data indicated the transition between growth phases, samples were taken. In addition, further samples were taken within each growth phase such that there were 2-3 points per phase.

3.6.2. pH

As discussed in Chapter 2, microbial metabolism has been shown to reduce pH in experimental systems by the production of protons (Rogers and Bennett, 2004; Ahmed and Holmström, 2015) and through the production of organic acids (Barman *et al.*, 1992; Welch and Ullman, 1993; Vandevivere *et al.*, 1994; Barker *et al.*, 1998; Rogers *et al.,*1998; Liermann *et al.*, 2000; Bennett *et al.*, 2001; Uroz *et al.*, 2009). However, to improve congruity with the circumneutral Noachian martian environment discussed in Chapter 2, and to address adverse effects of the 96% CO_2 atmosphere, a Na_2CO_3 buffer was added to the system. Thus, as expected, the pH for biotic and abiotic conditions showed no significant difference throughout.
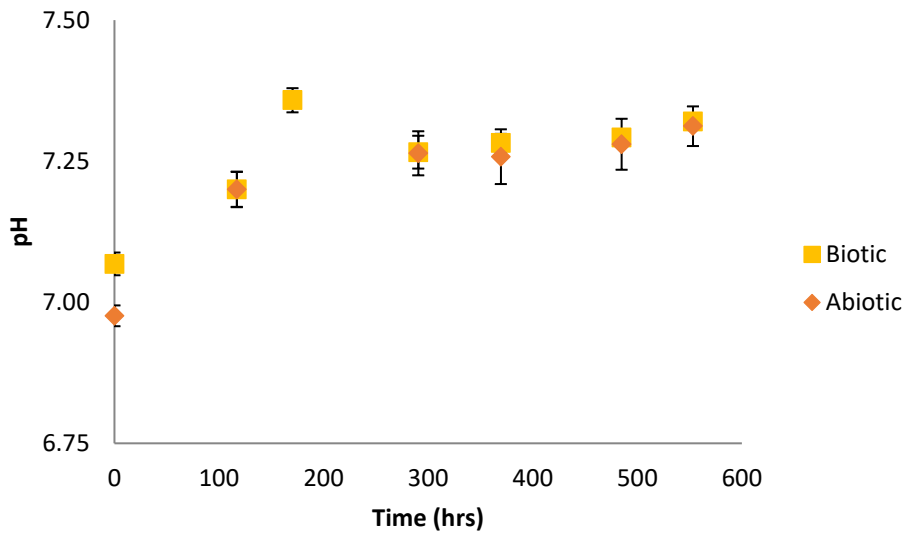
Figure 3.5. Mean pH plotted against time for both the biotic and abiotic experiments. Error bars show the standard error of the means.

The pH peaked at 170 hr with a maximum of 7.40 (Figure 3.5). The pH then dropped to 7.25 and then slowly increased to 7.30 by the end of the experiment. While there was evidently some variation in the system, this data shows that the pH was maintained between pH 6.50 and 7.50 throughout the experiment. The relatively small variation is unlikely to have had significant effects on mineral dissolution, e.g. the dissolution of aluminosilicates has been shown to be pH independent between pH 5 and 8 (Vandevivere *et al.*, 1994).

3.6.3. ICP-AES

Mineral dissolution was measured by the elemental concentration in the growth medium using ICP-AES. For this experiment, the focus was bio-essential elements, e.g. Mg, Mn, Fe, Na, Ca, and K (Wackett *et al.*, 2004; Pontefract *et al.*, 2012), Also, Si was included, because it is an important element with regard to basalt weathering (Wu *et al.*, 2007a), and Al which is intrinsically toxic to microorganisms (Piña and Cervantes, 1996). Figure 3.6 A-G shows the concentration of the above elements in the growth

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medium throughout the experiment with microbial growth plotted simultaneously for comparison.

A

B

C

D

E

F

G
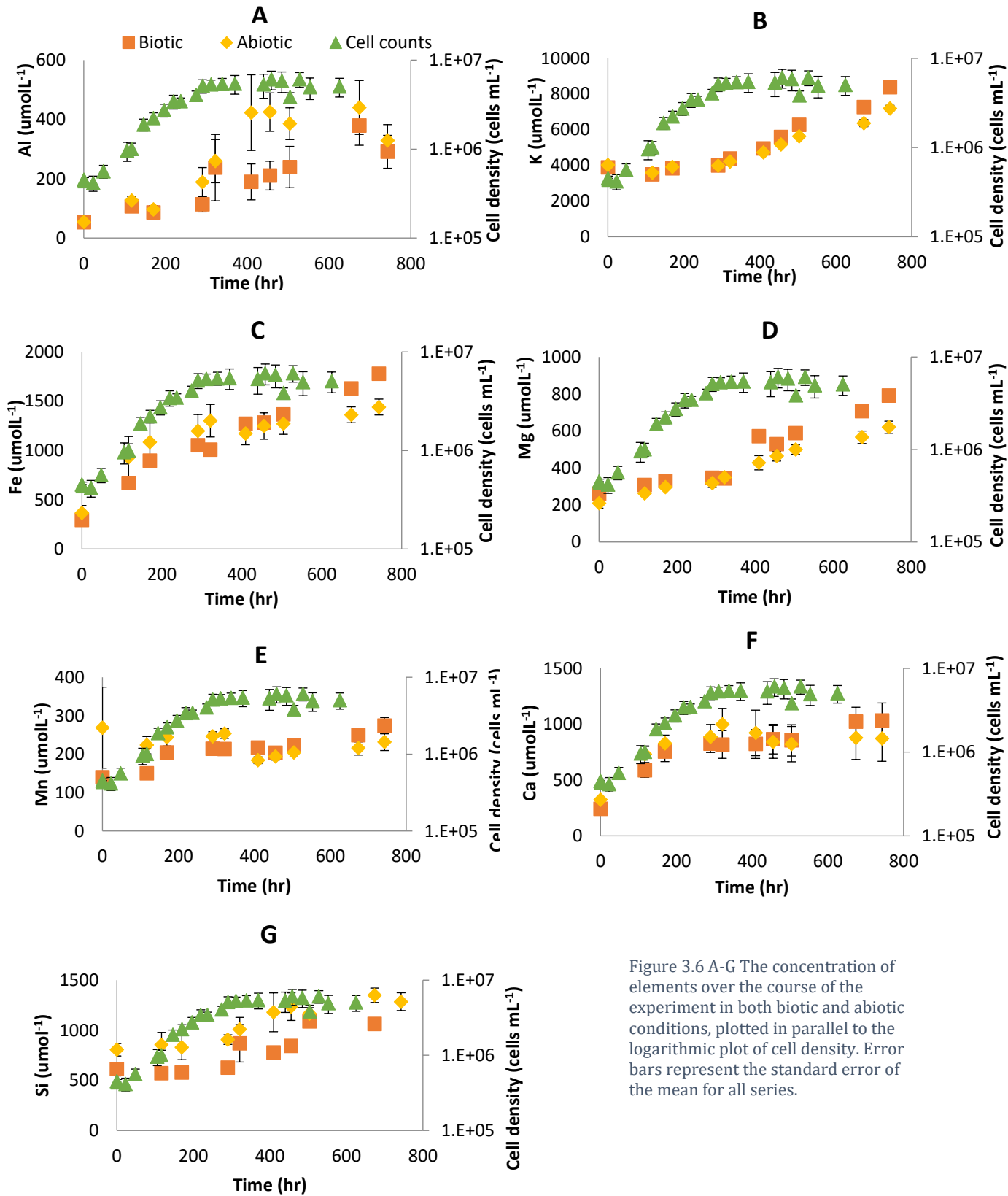
Figure 3.6 A-G The concentration of elements over the course of the experiment in both biotic and abiotic conditions, plotted in parallel to the logarithmic plot of cell density. Error bars represent the standard error of the mean for all series.

Figures 3.6B, D and E show the concentration of K, Mg and Mn. For each of these elements, the biota enhanced the amount of elemental release. For example, at 400 hr, at the beginning of the stationary phase, the concentration of Mg was ~571 μmol L^{-1} compared to ~428 μmol L^{-1} in the abiotic control.

After 400 hr the difference between the biotic and abiotic experiments increased with time, for example, at 400 hr the difference between biotic and abiotic experiments for Mg was ~143 μmol L^{-1} whereas at 734 hr the difference was ~171 μmol L^{-1}.

Although the release of Fe was enhanced by biota, the effect did not occur until the late stationary phase. For example, at 455.5 hr the difference in concentration between the biotic and abiotic experiments was only ~25 μmol L^{-1} in comparison to 743 hr where the difference was 340 μmol L^{-1}.

Initially the amount of Fe in the abiotic experiment was higher, for example, at 117 hr the concentration of Fe was ~261 μmol L^{-1} higher than the biotic experiment.

However, between 300 and 400 hr, at the beginning of the stationary phase, the concentration of Fe in the biotic experiment were higher than the abiotic experiments. This trend also coincides with the cell density reaching its maximum value.

The release of Ca, Al and Si from the regolith analogue were uniquely different. The concentration of Al within the abiotic controls began to exceed that of the biotic experiment at approximately 300 hr and continued rising relative to the biotic experiment until approximately 400 - 500 hr where it appeared to level off. The variation in the concentration of Ca in the medium was the same in biotic and abiotic samples. At around 500 hr the concentration in the abiotic controls increased above the biotic samples, leading to a peak concentration at 300 hr followed by a levelling off at approximately 900 μmol L^{-1}.

Uniquely, abiotic Si concentrations remain higher than in the biotic samples throughout. The discrepancy between this and the abiotic controls appears to increase over time, though both series follow the same general trend, reaching their respective maxima by approximately 400 - 500 hr. This trend was unexpected as microbial activity is usually considered to increase silicate dissolution rates (Rogers *et al.*, 1998; Bennet *et al.*, 2001; Rogers and Bennett, 2004; Wu *et al.*, 2007b; Wu *et al.*, 2008). The variation in the starting concentrations for biotic and abiotic samples partially accounts for the consistent difference, though the data still elude to a potential abiotic weathering mechanism or a biological inhibition of silicate dissolution.

For all of the elements, the standard error of the mean in the data for the abiotic controls is much larger than for the biotic samples.

3.6.3.1. Dissolution kinetics

Dissolution kinetics based on the linear rate law set out in Wu et al (2007) were used to calculate the linear release rate of each element in both biotic and abiotic experiments over the first 400 hr. Table 3.7. shows a comparison of these values:

Table 3.7. Linear release rates for seven bioessential elements analysed in this study.

Element	Biotic (moles $m^{-2}s^{-1}$)	Abiotic (moles $m^{-2}s^{-1}$)
Al	$2.78x10^{-8}$	$6.33x10^{-8}$
Ca	$1.35x10^{-7}$	$1.38x10^{-7}$
Fe	$1.78x10^{-7}$	$1.75x10^{-7}$
Mg	$6.95x10^{-8}$	$6.4x10^{8}$
Mn	$2.74x10^{-8}$	$2.75x10^{-8}$
Si	$1.05x10^{-7}$	$1.76x10^{-7}$
K	$7.41x10^{-7}$	$7.07x10^{-7}$

There was no significant difference in the elemental release rates of biotic and abiotic experiments, though notably, the rate of dissolution of Al is considerably higher in the abiotic than in the biotic experiments.

3.6.4. Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis

To monitor the microbial diversity throughout the experiment TRFLP analysis was used. This provided diversity information regarding the colonisation, community succession and consequent community structure of a microbial community extracted from the River Dee Mars analogue site (Chapter 2). Crucially, this community demonstrated its capability to grow and survive within an experimental system based on martian atmosphere, regolith and bio-essential element availability.

As with other analyses, the microbial diversity in the five biological replicates was, given the identical starting conditions and subsequent treatment, averaged.

3.6.4.1. Microbial diversity

3.6.4.1.1. Bacterial community structure

The TRFLP technique has a ±5 base pair (bp) error and thus data were grouped together to give a more accurate representation of the diversity in the samples e.g. the 90-91 bp fragments are considered to represent one distinct population. Though the TRFLP fragment sizes can be used to differentiate between different taxonomic groups, they do not provide a means of taxonomic identification. To address this, fragments were assigned an approximate identity based on an *in silico* digestion of MiSeq sequences obtained for the River Dee sample site (Chapter 2). All of the sequences were analysed using TRFLPMAP software (http://nebc.nerc.ac.uk/cgi-bin/trflp0_2.cgi) using the restriction endonuclease *Msp*I cleavage site. Some fragment sizes can represent many different taxa, therefore different taxa sharing the same cleavage site were grouped and the taxon that accounted for >75 % of the sequences was assigned to that fragment. Table 3.8. shows an exhaustive list of assigned TRFLP fragments.

TRFLP analysis carried out using Genemarker V.2.6.4 identified all fragments that were 50 bp or longer and that had a frequency of at least 50. This produced an unmanageably large data set so only fragments with a 1% or higher contribution to the total bacterial diversity were included in the analyses. Those that accounted for less than 1 % of the total bacterial diversity were considered unlikely to have an observable impact on the chemistry of the system.

Table 3.8. TRFLP fragments assigned to taxa based on MiSeq sequencing data and in silico digestion using MSPI restriction enzyme. * is used to show taxa that were assigned to either higher or lower resolution than Order level. "- "is used to show where resolution below class level was not possible.

Fragment	Taxon (Class)	Taxon (Order)	Fragment	Taxon (Class)	Taxon (Order)
80-82	Alphaproteobacteria	-	187-189	Unknown	-
83-87	Deltaproteoebacteria	Desulfuromonadaceae	191-196	Gammaproteobacteria	Ectothiorhodospiraceae
90-91	Proteobacteria*	-	198-202	Alphaproteobacteria	Rhizobiales*
93	Alphaproteobacteria	Beijerinckiaceae	204-207	Unknown	-
95	Acidobacteria	GP21	209-214	Alphaproteobacteria	Rhizobiales *
96	Gammaproteobacteria	-	217-220	Unknown	-
98	Gammaproteobacteria	-	221-225	Gammaproteobacteria	-
99	Flavobacteria	Flavobacteriaceae	226-228	Alphaproteobacteria	-
101	Alphaproteobacteria	Rhodobiaceae	230-231	Unknown	-
102-103	Acidobacteria	Gp9	232-233	Gammaproteobacteria	-
105	Gammaproteobacteria	-	234-237	Alphaproteobacteria	-
107-108	Caldilineae	Caldilineaceae	239-243	Acidobacteria	GP10 *
109-110	Alphaproteobacteria	Rhodobiaceae	244-246	Acidobacteria	GP10*
125	Actinobacteria	Actinomycetales	244-246	Acidobacteria	GP10*
126	Alphaproteobacteria	-	247	Acidobacteria	-
127-129	Gemmatimonadetes	Gemmatimonadaceae	248	Acidobacteria	Gp9*
130-134	Gammaproteobacteria	-	250-253	Gammaproteobacteria	Alteromonadaceae
135-137	Proteobacteria*	-	256	Gammaproteobacteria	-
138-143	Deltaproteobacteria	Nannocystineae	259	Gammaproteobacteria	-
144-146	Clostridia	Peptostreptococcaceae	262-266	Gammaproteobacteria	Halioglobus *
148-152	Alphaproteobacteria	Hyphomicrobiaceae	276-279	Gammaproteobacteria	-
153-155	Chloroflexi* (phylum)	-	281	Unknown	-
156-160	Cytophagia	-	306-308	Alphaproteobacteria	Hyphomicrobiaceae
161-163	Unknown	-	310-315	Alphaproteobacteria	Hyphomicrobiaceae
165-168	Gammaproteobacteria	-	321	Gammaproteobacteria	Alteromonadaceae
172-176	Caldilineae	Caldilineaceae	327-329	Gammaproteobacteria	-
178	Gammaproteobacteria	Granulosicoccaceae	331-333	Gammaproteobacteria	-
181-184	Gammaproteobacteria	Ectothiorhodospiraceae	423	Gammaproteobacteria	-
187-189	Unknown	-			
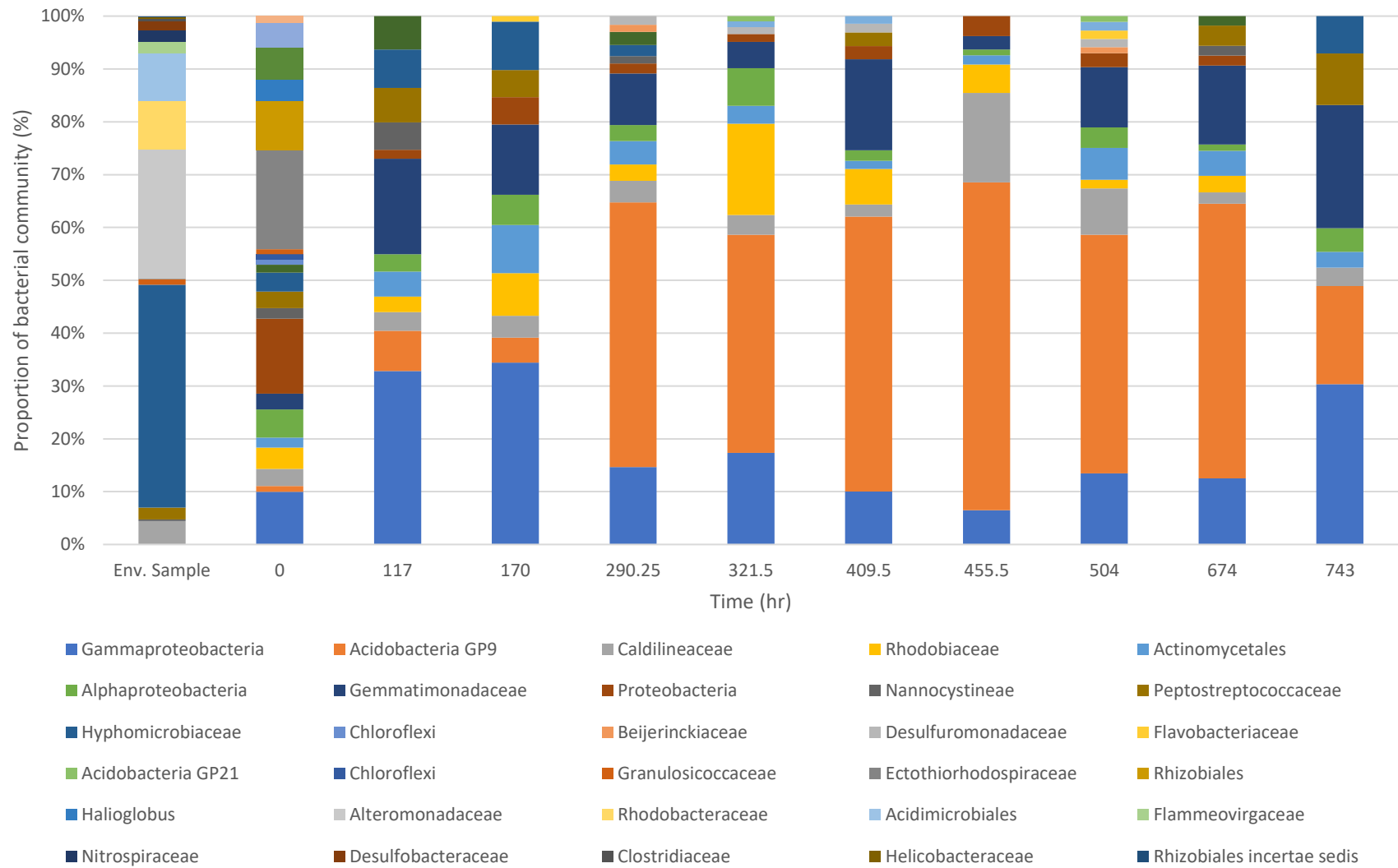
Figure 3.7. A stacked bar chart showing the mean microbial diversity based on TRFLP data for all five biological samples plotted over time, with the River Dee sample shown for comparison. Each fragment is labelled according to the family it belongs to. Otherwise, the highest resolution of taxonomic identification possible was used. Lag phase = 0 - 24 hr, Exponential phase = 25 - 290 hr, Stationary phase = 290 - 743 hr.

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Figure 3.7 displays the proportion of different microbial populations within the experimental samples throughout the experiment.

The microbial community structure of the subsurface samples from the River Dee estuary (described in Chapter 2) is considerably different to that of the community used in this experiment; several bacterial groups were not detected when grown in the minimal medium experiments. For example, the *Flammeovirgaceae*, *Alteromonadaceae* and *Nitrospiraceae* (present in the environmental sample) were below detection limits at the start of the experiment and continued at those levels throughout.

Immediately after inoculation, between 0 and 117 hr (during the lag phase) the microbial diversity decreased, and several groups (e.g. *Chloroflexi, Granulosicoccaceae, Ectothiorhodospiraceae, Rhizobiales* and *Halioglobus*) were not detected after 117 hr using TRFLP analysis, even though they were successfully detected using TRFLP analysis in Chapter 2. Between 117 and 290 hr (early log phase) the percentage of Acidobacteria Gp9 increased dramatically from approximately 1 to 43 % which remained consistent throughout most of the stationary phase. In contrast, the percentage of Rhodobiaceae increased from approximately 2 - 19 % between 290 hr and 321 hr. At 743 hr, the percentage of Acidobacteria Gp9 had decreased and was replaced by Gammaproteobacteria, Gemmatimonadaceae, Hyphomicrobiaceae and *Peptostreptococcae*.

To ensure that the findings of these analyses were representative of the actual microbial community in the experimental systems, a rarefaction curve was created using the statistical package R v.3.2.4. Figure 3.8 shows a rarefaction curve based on species richness counts of the TRFLP data and the formation of a plateau indicates that sufficient samples were taken to represent the community accurately.
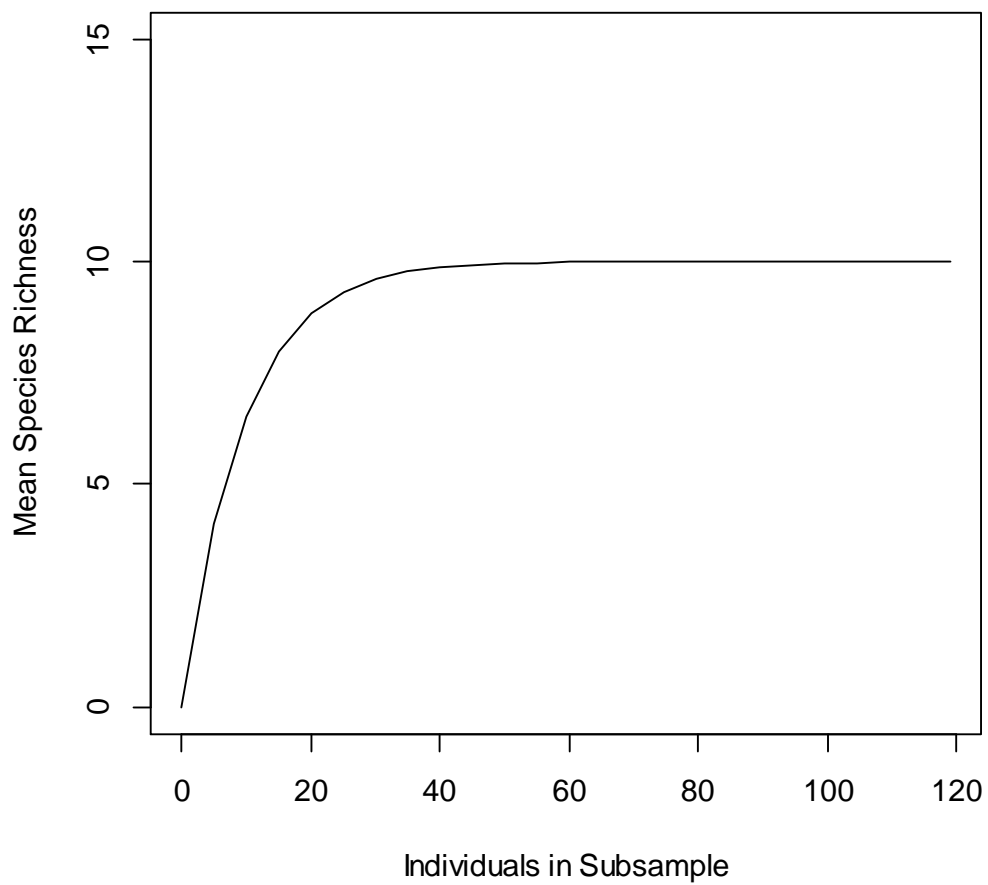
Figure 3.8. A rarefaction curve based on species richness throughout the experiment.

SIMPER analysis was also carried out using Primer 5 software to investigate and quantify the taxa that contributed most significantly to the dissimilarity between the community profile at each time period. Table 3.9 demonstrates the percentage contribution of the taxa to the overall dissimilarity between each sample.

Table 3.9. SIMPER analysis, which demonstrates individual and cumulative contributions to dissimilarity by different taxa.

Identity	Contrib.%	Cum.%
Acidobacteria Gp9	35.74	35.74
Gammaproteobacteria	22.21	57.95
Gemmatimonadaceae	14.19	72.13
Caldilineaceae	5.63	77.77
Actinomycetales	4.92	82.69
Rhodobiaceae	4.74	87.43
Alphaproteobacteria	4.39	91.82
Caldilineaceae	5.63	77.77
Actinomycetales	4.92	82.69
Rhodobiaceae	4.74	87.43
Alphaproteobacteria	4.39	91.82

In accordance with the trends observed in Figure 3.7, Table 3.9 shows that the Acidobacteria Gp9 are individually responsible for ~35 % of the dissimilarity, and therefore the variation, in the bacterial community over time. The Gammaproteobacteria and Gemmatimonadaceae contributed respectively ~22 % and ~14 % of the dissimilarity between the bacterial diversity at the different time periods. Therefore, these three taxa can be attributed to the observed variation in bacterial diversity. The remaining groups accounted for a maximum of ~5 % dissimilarity (Caldilineaceae).

Efforts to amplify and purify archaeal DNA from the experimental samples were unsuccessful. It is therefore assumed that archaea were either not present or present

in such small quantities as to be undetectable. They are therefore not considered to have been capable of having an impact on the experimental system.

3.6.5. Field Electron Gun Scanning Electron Microscopy (FEGSEM)

Post experiment, rock samples from both the biotic and abiotic experiments were examined using the FEGSEM with regard to morphology and elemental composition. To achieve this, Secondary Electron (SE) and Backscatter Electron (BSE) images were scrutinised to identify representative areas of the grains and notable features. SE imaging was used primarily to identify morphological features and BSE was used to identify compositional differences. Heavy elements backscatter electrons more strongly than light elements and appear brighter in BSE images. Therefore, visual inspection of BSE images can be used to identify general compositional differences between minerals, since minerals of different composition have different brightnesses. More detailed elemental composition data was obtained through Electron Dispersive Spectroscopy (EDS) whereby specific elements could be detected along with their approximate proportions.

The aim of FEGSEM analysis was to identify any significant differences between the biotic and abiotic conditions and therefore the data are described separately.

3.6.5.1. Abiotic samples

SE images of the abiotic control samples demonstrated relatively un-weathered mineral surfaces (e.g. Figure 3.9). Unlike the biotic samples (see below) there is little evidence of physical or chemical weathering. Figure 3.9 is representative of the features observed whilst examining all the rocks from the abiotic experiments.
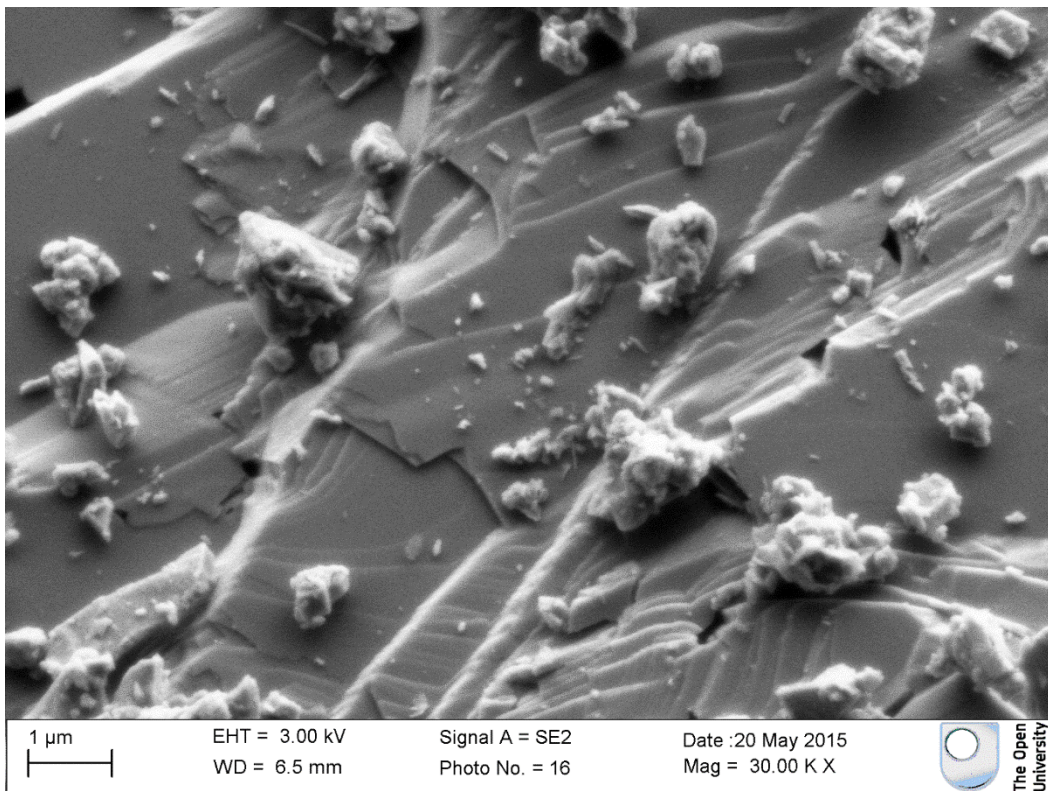
Figure 3.9. FEG-SEM SE image of a mineral surface in an abiotic control. Salt crystals are present on the mineral surface.

1 μm or smaller grains were observed on the surface of the mineral and appeared unfixed (Figure 3.9). These were hypothesised to be salts deposited during the drying procedure and their composition was investigated by EDS analysis (see Figure 3.11 and 3.12). It was expected that the EDS analysis of the salt deposit would inadvertently obtain the spectra of the underlying mineral surface therefore a comparison of the two was carried out (Figures 3.11 and 3.12).

Figure 3.10 shows a BSE image which highlights the compositional difference between the salt crystals on the surface and the underlying substrate. EDS spectra (Figures 3.11 and 3.12) showed that that the salt crystals were enriched in sodium when compared to the underlying mineral. These salt crystals were not observed in the biological samples and so were not considered to be candidates for potential biosignatures.

However, the absence of these grains in the biotic experiment may be a result of microbial action.
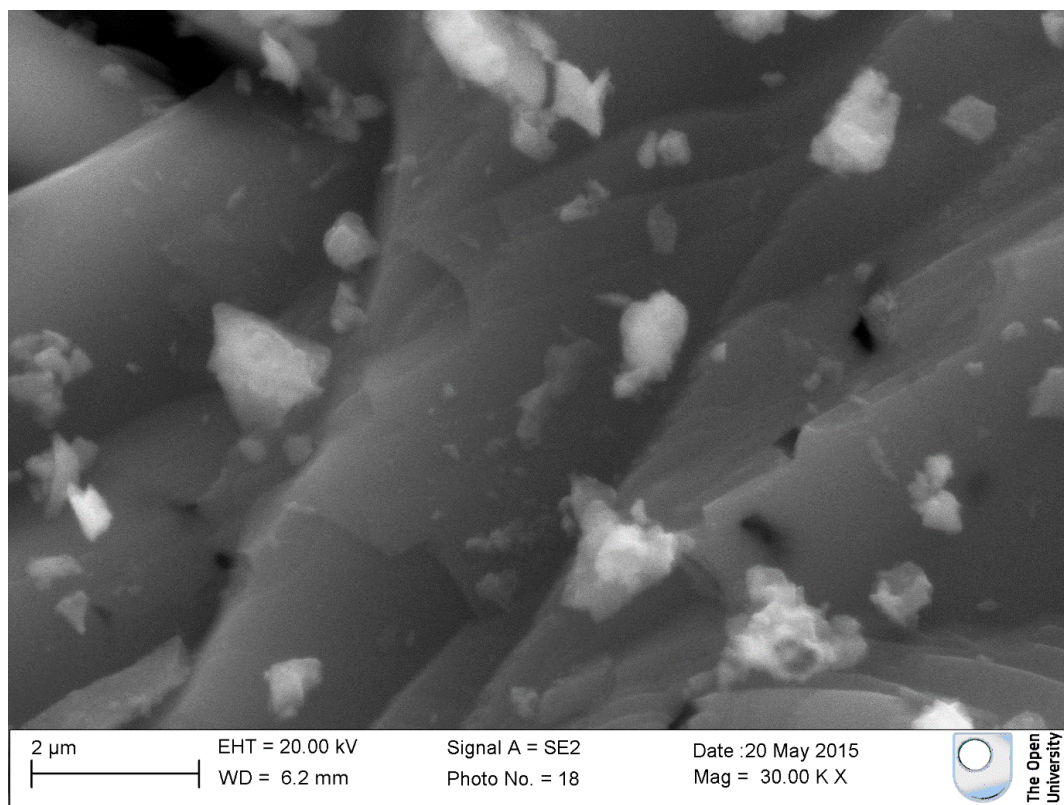
Figure 3.10. FEG-SEM BSE images of an unweathered surface in an abiotic control. Salt crystals are present on the mineral surface.
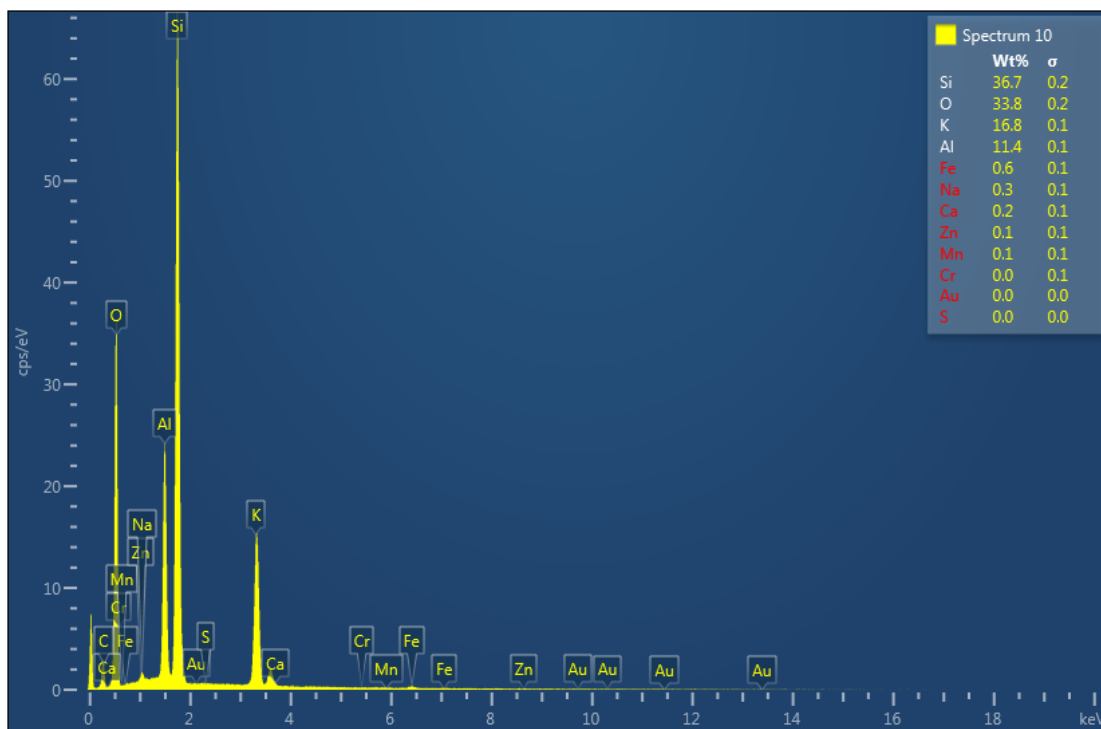
Figure 3.11. EDS spectra of the salt crystals observed in Figure 3.9 and 3.10.
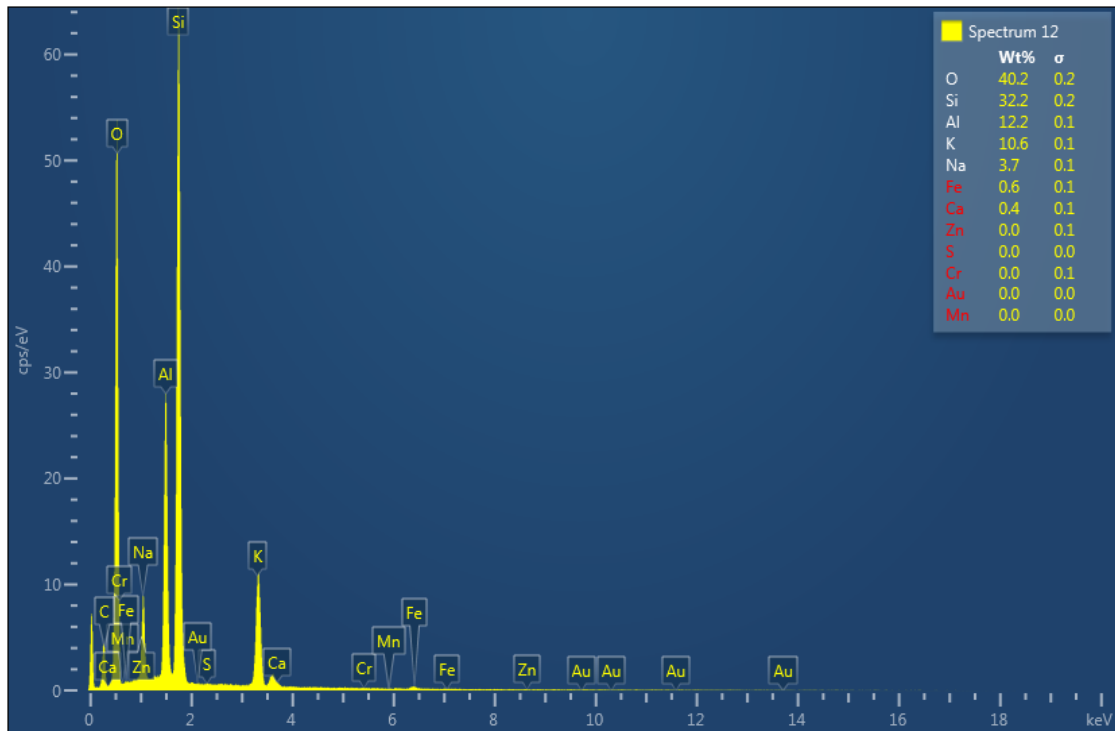
Figure 3.12. EDS spectra of the mineral surface underlying the salt crystals observed in Figures 3.09 and 3.10.

3.6.5.2. Biotic samples

FEG-SEM analysis of the regolith analogue from the biotic experiment yielded little evidence of direct microbial attachment to the mineral surface. However, it is possible that, as a result of swirling the experimental flasks to ensure homogeneous aliquots, such evidence may have been obliterated or removed. Furthermore, the preparation process, e.g. carbon coating, and the chamber vacuum conditions during analysis may have exacerbated this.

During detailed observation of the biotically weathered substrate two surface properties were observed, whereby the stepped appearance of crystal faces was assumed to be from dissolution of that crystal during the experiment. In contrast, rounded and undulate surfaces were interpreted as silica deposits (see also Figure 3.13 and 3.14). This amorphous silica deposit appeared to be unique to the biological samples. The structure was seen to overlay the bulk crystalline mineral grains,

outlining the contours on its surface. In addition, adjacent to the siliceous sediment there was evidence of weathering of the underlying mineral grain. Physical or chemical weathering of material from along the mineral cleavage plains is evident in both Figure 3.13 and 3.14.

Amorphous siliceous sediment

Zone where stripping of material is evident
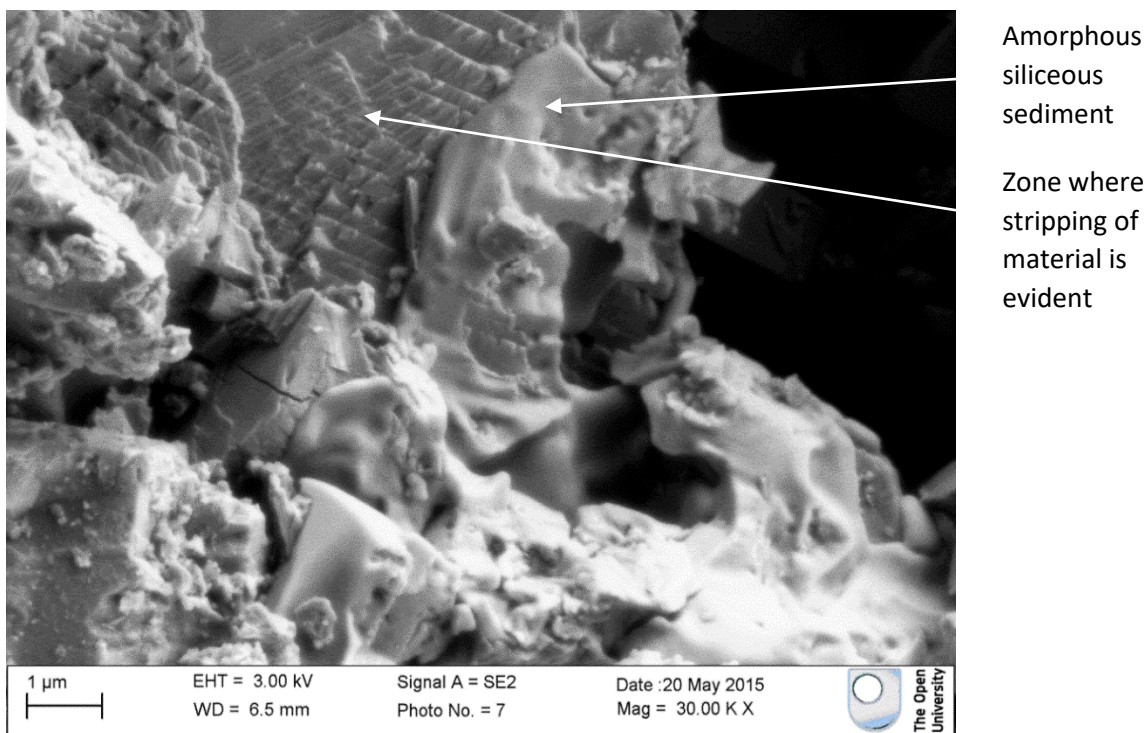
Figure 3.13. FEG-SEM SE image showing amorphous siliceous sediment structure and evidence of weathering of the bulk mineral surface.

Amorphous siliceous sediment

Evidence of material stripping

Microbial cell
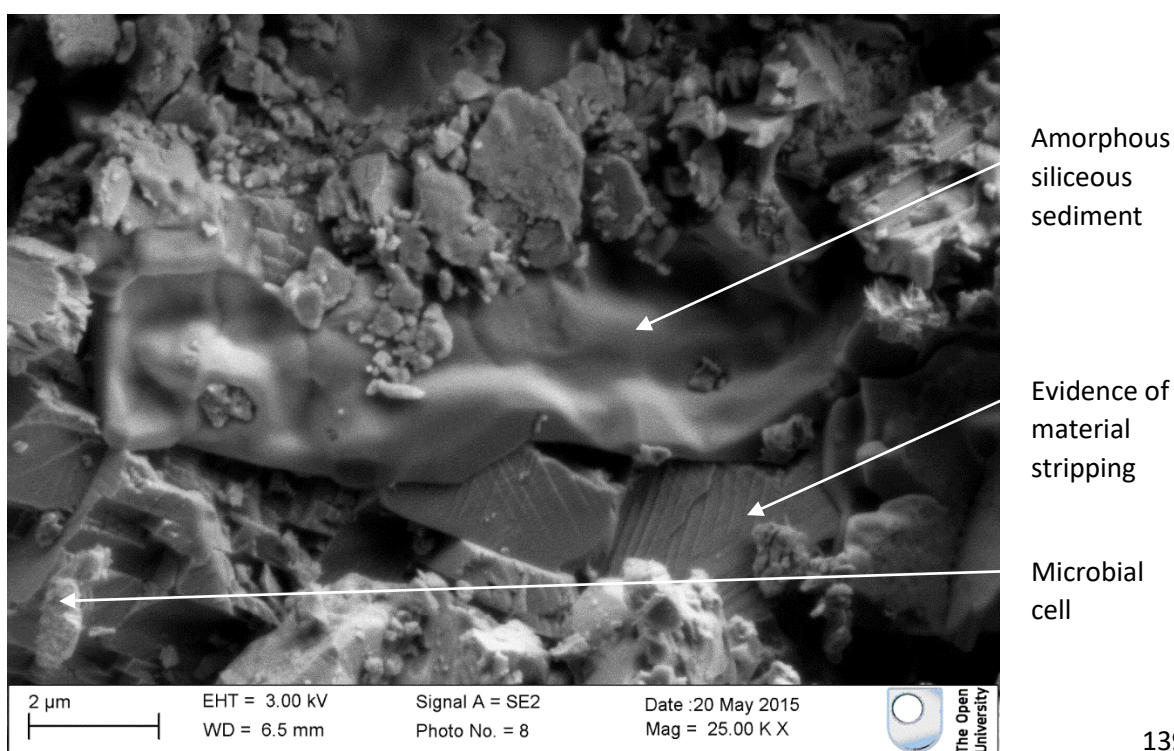
Figure 3.14. FEG-SEM SE image showing amorphous siliceous sediment structure and evidence of weathering of the bulk mineral surface.

Figure 3.15 shows a BSE image of the same area shown in Figure 3.14 with the areas analysed by EDS spectra analysis areas shown. The siliceous deposit and the underlying surface appear similar in brightness in Figure 3.15, though this does not necessarily suggest similar elemental composition.
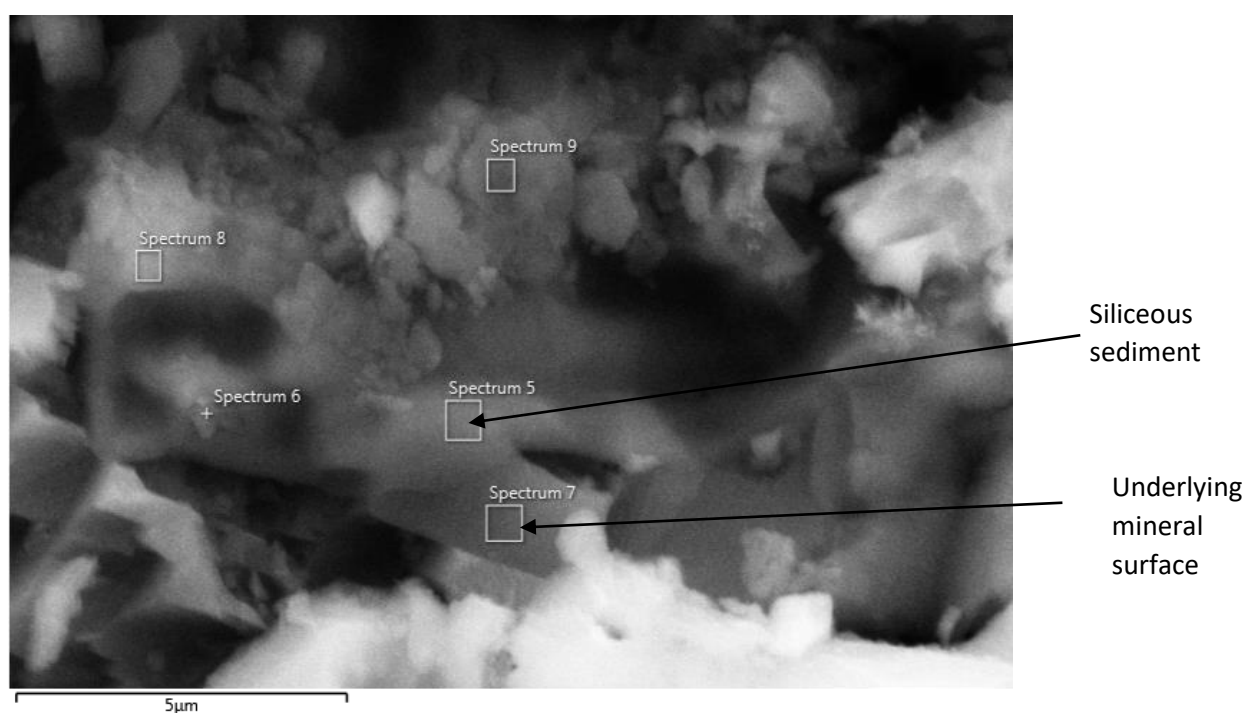
Figure 3.15. FEG-SEM BSE image showing feature observed in Figure 3.14 with EDS spectrum ⠇ained from locations displayed

areas displayed on Figure 3.15 were considered to be the most accurate representations of the true elemental composition of the amorphous siliceous sediment and the underlying mineral surface. This was because the other spectra may have resulted from beam interaction with other components (for example, see analysis zones in Figure 3.15). Sample topography, beam area and the thickness of the target mineral/structure can influence the accuracy of spectra obtained. Spectrum 5 showed the composition of the siliceous deposit and Spectrum 7 of the weathered underlying mineral grain. These were chosen to provide the best comparison between the bulk rock substrate and the siliceous sediment.
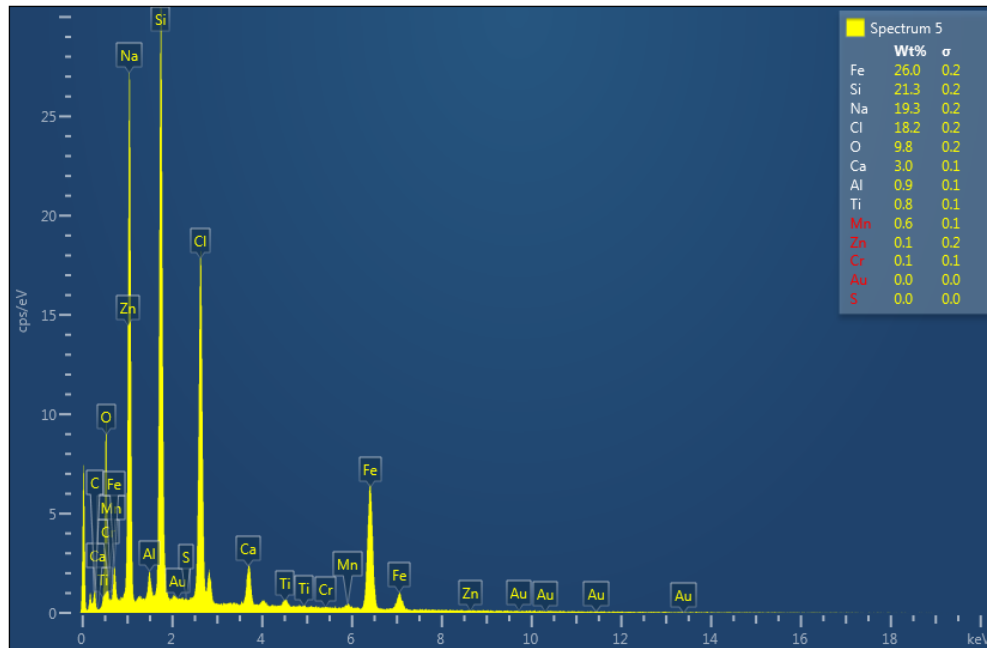
Figure 3.16. EDS spectrum 5 from Figure 3.15. The spectrum of the amorphous siliceous deposit.
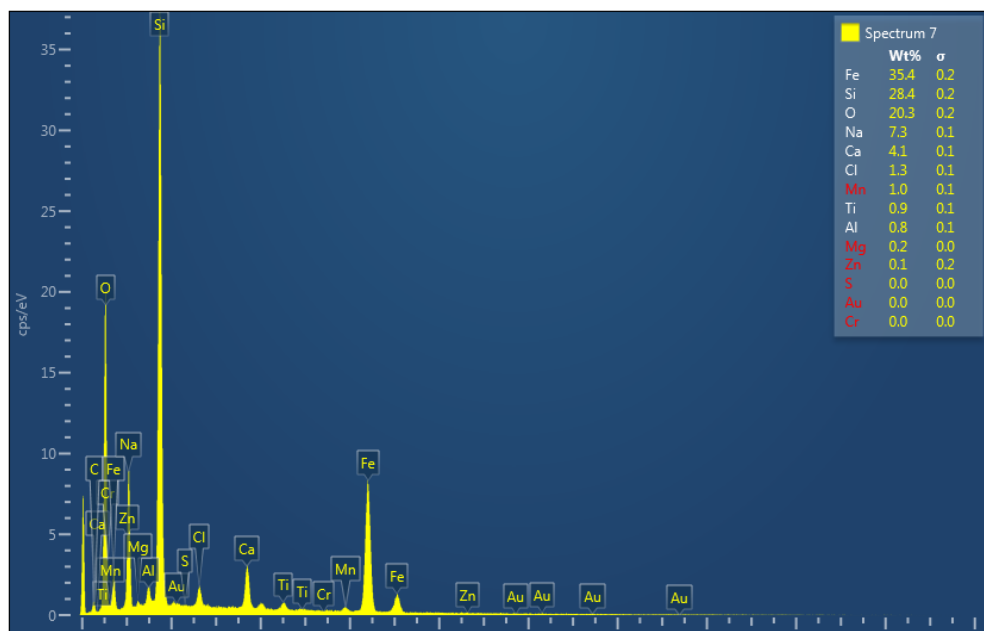
Figure 3.17. EDS spectrum 7 displayed in Figure 3.15. The spectrum of the underlying mineral surface.

The EDS spectra of the underlying mineral surface in Figure 3.15 (see Figure 3.17) showed large peaks of Si, O and Fe with Na, Ca and Cl as minor constituents. The EDS spectra of the siliceous deposit (Figure 3.16) showed similar peaks of Si, Fe and O. This either represented a siliceous deposit of similar composition to the bulk substrate or the inadvertent inclusion of the mineral surface directly below the siliceous deposit in the analysis. The only significant difference between the two spectra were the peaks of

Na and Cl in the siliceous deposit that appeared to represent equal abundances of each in the target area. It is not possible to say with certainty whether the Na and Cl represent NaCl incorporated into the deposit or its true composition.

These data were compared to the EMPA data for the individual minerals within the basalt and the aegirine. Comparisons with typical olivine composition showed enrichment in both Fe and silicon for the underlying mineral as well as the amorphous deposit. Given this, and the similarity in composition of the underlying mineral and the amorphous deposit, it is possible that the amorphous layer represents the formation of a ferrous clay at the mineral surface. If this could be replicated on a larger scale during future experiments (see Chapter 5) further analysis of the amorphous deposit using EMPA may elucidate the processes that led to its formation.

3.6.6. Powder X-Ray Diffraction

Powder XRD was used to compare the bulk phase composition of the substrate in the biotic and abiotic experiments, with the aim of identifying mineralogical differences between the two, e.g. secondary mineralisation products.Figures 3.18 and 3.19 show the powder X-ray diffraction pattern for representative biotic and abiotic weathered substrate, respectively. It was hypothesised that microbial mineral dissolution would

facilitate the production of secondary minerals that would not be present in the abiotic controls.
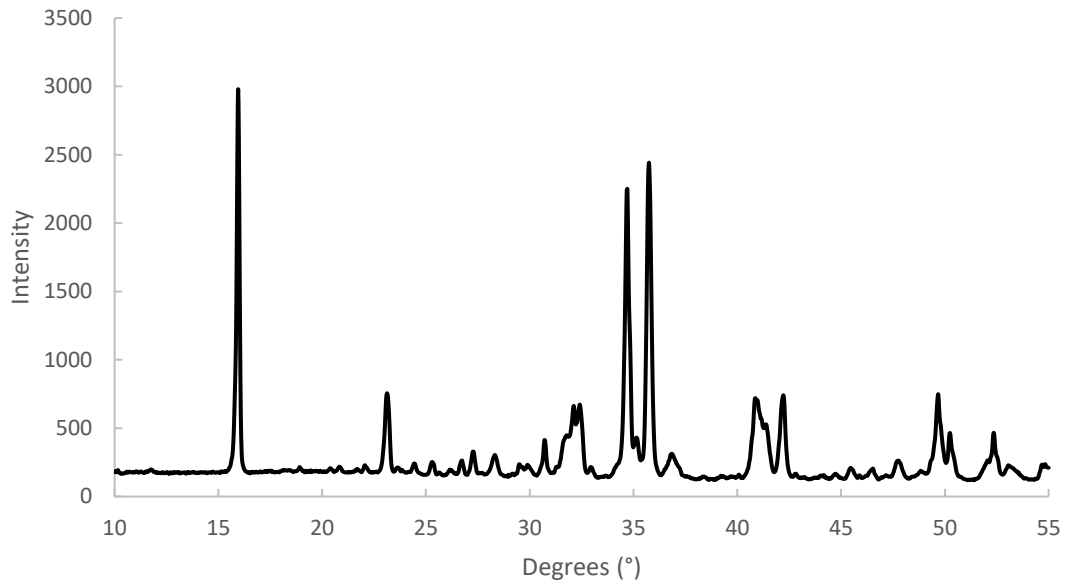
Figure 3.18 Powder XRD data from the biotic experiment

Major peaks in both, biotic and abiotic samples, were found at approximately 15 and 35 degrees. Minor peaks were also congruent between the two groups at approximately 23, 32, 41 and 49 degrees. Comparison of the two patterns did not reveal any unique peaks in the biotic samples and *vice versa*. This does not, however, completely rule out any microbially-mediated secondary mineralisation since the concentration of products of microbial action may have been too small to be measured among the bulk substrate.
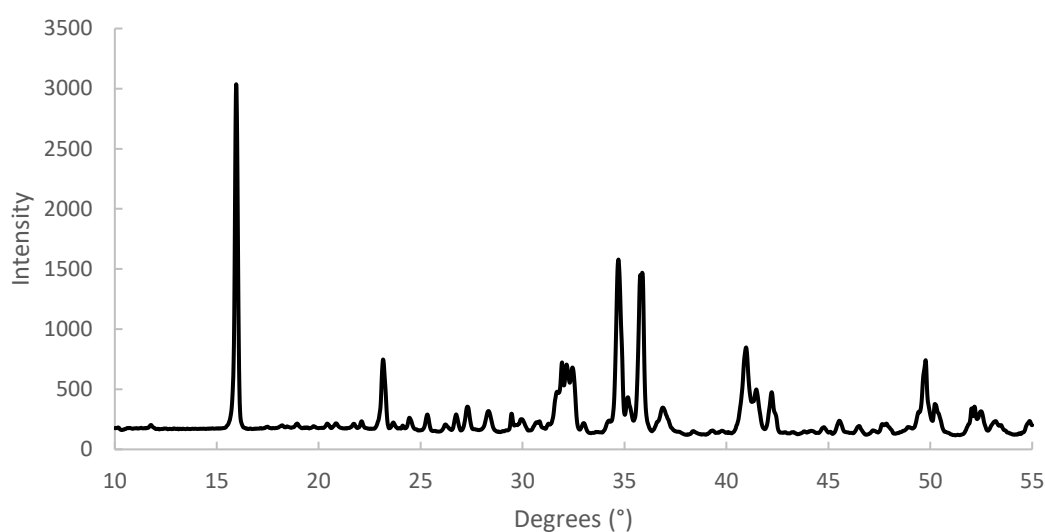
Figure 3.19. Powder XRD data from the abiotic experiment.

However, the intensity of individual peaks did vary between the biotic and abiotic experiments. For example, the two peaks at approximately 35 degrees had approximately 50 % higher intensity in the biotic compared to the abiotic experiments, as shown in Figure 3.18 than in Figure 3.19. However, peak intensity variation is likely, but not limited to, the result of imperfect packing and inconsistencies in the grinding process. In addition, because the substrate is a mixture of the mineral aegirine and a basalt, imperfect mixing of the two may have led to variations in composition in the analysed samples. Consequently, variations in peak intensity were not considered reliable indicators of mineralogical change.

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3.7. Discussion

This chapter investigated the processes of colonisation and ecological succession in a Mars analogue environment, and furthermore, tested whether the microbial community could utilise bio-essential elements from a Mars regolith analogue and produce distinguishable biosignatures.

3.7.1. Trends in microbial diversity

3.7.1.1. Microbial succession

In considering microbial succession processes within this experimental system, it is important to clarify whether the colonisation of the system represents a primary or secondary succession. As discussed in Chapter 1, primary successions occur where previously uninhabited environments are colonised, and secondary successions occur where some event reduces biological diversity and frees up niche space.

In this experiment, the environment was artificially constructed using water, a Mars gas analogue and a Mars regolith analogue containing aegirine and basalt. Aside from the Mars gas, the water and regolith analogue would most likely have been inhabited by microbial communities prior to the experiment. Assuming the efficacy of the sterilisation procedure described above (Section 3.3.5) the artificially constructed system can be considered to have been uninhabited prior to inoculation. Thus, the type of succession to be considered in this study is that of primary succession.

Immediately after submersion in the medium, the regolith analogue would have been available for microbial attachment (Jackson *et al.*, 2001). According to Costerton *et al.*

(1987), the majority of bacteria within aquatic environments can be found attached to the surface of sediment or substrate and within hours of inoculation, an organic film forms on the surface. This time frame is based upon the submersion of sterile sediment or rock into an aquatic environment already colonised by communities of microorganisms and may therefore not be directly applied to this experimental system because, in this experiment a live microbial inoculum was added to a sterile environment. However, as discussed above, the only source of several bioessential elements within the experimental system is the Mars regolith analogue and therefore it is likely that microbial growth centred around the substrate surface or the aquatic environment immediately adjacent to its surface where microbially-induced dissolution would have increased the availability of these essential elements.

Little evidence of microbial attachment or the presence of biofilm was found when the regolith analogue was analysed post-experiment and therefore it is possible that the microbial community existed mainly as planktonic cells floating above the substrate. It is prudent to note that mineral dissolution does not require microbial attachment and may occur indirectly as a result of metabolism. Thus, the absence of evidence of the presence of microorganisms on the observed mineral surfaces does not preclude their potential interaction with the regolith analogue.

The presence of the amorphous silicate in the biotic experiment may indicate an inorganic alternative to biofilm, though no evidence of microorganisms was observed on the surface of the deposit. In either case, the colonisation of the experimental system can be considered to be relevant to primary succession because successful growth and survival was achieved by the microbial community within the Mars analogue environment.

The early, mid and late phases in the model of microbial succession shown in Figure 1.3 and discussed in Chapter 1 (Jackson *et al.*, 2001; Jackson, 2003) in the context of microbial growth phases, described by Dykhuizen (1990), may be compared. In this case, the early phase represents the lag phase with the first peak in S shown in Figure 1.3 being attributable to the addition of the inoculum. The mid phase represents the early exponential phase and the late phase the late exponential phase. The stationary phase and death phase are not represented here as the model does not extend to stable populations or potential secondary successions. The death phase is also not evident in the data collected for this study and thus the comparison may still be of use.

Therefore, if this model is applicable to the bacterial community structure data shown in Figure 3.7, the number of species at 0 hr should have been significantly higher than the time periods immediately following it. At 0 hr 19 different bacterial groups were identified and this was followed by a drop to 12 at 117 hr and a further drop to 10 at 170 hr. Cell enumeration showed that the exponential phase began between 0 and 117 hr and continued until 290 hr. According to Jackson (2003) this phase should have been characterised by an increase in the number of bacterial groups, however, this was not observed. The end of the exponential phase was recorded by cell enumeration at 290 hr and by this stage only 12 groups were detected.

The prediction of steadily increasing number of groups is based on the prediction that niche space (R) would increase with time (Figure 1.3). A major factor in this increase is attributed to the shift from a two-dimensional environment (i.e. attachment to a mineral surface) to a three-dimensional one (i.e. a complex biofilm structure). However, the inoculum in this study had already grown within a three-dimensional environment, thus the lack of increase in R is expected.

Interestingly, though R did not apparently increase during the exponential phase as expected, 16 groups were detected at 674 hr. This fell to 10 by 743 hr. This may represent the short-term expansion of niche space, perhaps induced by the dissolution of a rare mineral inclusion within the Mars regolith analogue, thus releasing a small amount of previously absent elements. However, presently there is no other evidence of such a process occurring.

Sn, the addition of new bacterial groups, is not wholly applicable to this study as the model assumes an open system with free immigration into the ostensibly continuous increase in niche space. In this study, only groups lying dormant are capable of occupying niches made available at later stages.

As discussed above, during the exponential phase the Acidobacteria Gp9 became dominant. According to Jackson (2003), broad scale competition should have been dominant during the beginning of this stage. Therefore, given that this is when the Acidobacteria Gp9 became dominant, it is likely that it was capable of out-competing the other bacterial groups through broad scale competition. This type of competition affects all species and thus likely reflects the ability of this organism to utilise one of the main bioessential elements within the experimental system, e.g. the carbon source or the nitrogen source. Figure 3.6 shows that the concentrations of both Fe and Ca in the growth medium increased rapidly during this stage. Fierer *et al.,*(2007) found a negative correlation between the abundance of Acidobacteria and soil carbon availability. It is possible that it is this preference for low carbon that allowed the Acidobacteria Gp9 to out-compete other groups during this stage.

The transition away from broad scale competition to fine scale competition by the late exponential phase does not appear to have affected the abundance of Acidobacteria Gp9 until the late stationary phase, at 734 hr, when it was no longer the dominant

group. If the intensity of fine scale competition continued to increase as per Figure 1.3, it may have resulted in the eventual reduction of the Acidobacteria Gp9 population. Fine scale competition occurs over nutrients that are only required by certain microbial populations, thus at 734 hr the Acidobacteria Gp9 were out-competed for a resource likely to be specific to both them and the group that out-competed them.

The microbial community structure variation over time in this experiment fits the primary succession model developed by Jackson (2003) and can be used to explain the observed trends in the data.

3.7.1.2. Relative proportions of bacterial groups

In Chapter 2, the microbial community was collected and phylogenetically characterised from the anaerobic intertidal zone of the River Dee. The microbial community would likely have been influenced by the environmental conditions in the intertidal sandflat, which were of circumneutral pH, ~12 °C temperature, low carbon availability and oligotrophic nutritional conditions.

In the natural environment, the Class *Hyphomicrobiaceae* was the most prominent bacterial group; however, when the microbial community was grown in a Mars simulated regolith, at 14.8 °C and pH 7 with a simulated Mars gas atmosphere, the *Hyphomicrobiaceae* diminished quickly, falling below detection limits by 321.5 hr, and reappearing as a minor constituent at 743 hr. The microbial community was instead dominated by *Acidobacteria.* Among the Acidobacteria, facultatively anaerobic species have previously been isolated (Pankratov *et al.*, 2012), thus potentially providing an explanation for their success in the anaerobic experimental system.

The shift in community diversity can also be seen clearly by the loss of other major bacterial groups over the first 177 hr (Figure 3.7). For example, *Ectothiorhodospiraceae* accounted for approximately 20 % of the microbial community at the start of the experiment, but were below detection limits for the remainder of the experiment.

After this initial shift in microbial diversity due to incubation within the Mars analogue system, there were further shifts in community diversity based on the relationship to the microbial growth phases. It was expected, for example, that during lag phase, the species that are best able to adapt to new environmental conditions will dominate (D. Dykhuizen, 1990). In this case determining the most successful group during the lag phase is problematic, since the second sampling period (at 117 hr), is instead representative of the very early exponential phase according to Figure 3.4.

During the exponential growth phase, species that are able to replicate at a faster rate than their competitors would thrive (D. Dykhuizen, 1990), e.g. the Acidobacteria Gp9, which dominated at 290 hr, at the end of the exponential phase (Figure 3.4). Finally, during the stationary phase (321 hr – 743 hr in this experiment) those species that are best able to survive in a community of maximum population size and limited resources will be most prevalent (D. Dykhuizen, 1990). In addition to dominating the exponential growth phase, the Acidobacteria Gp9 was the major constituent of the microbial community throughout the stationary phase.

The Acidobacteria is a highly diversified (Jones *et al.*, 2009) and poorly understood phylum of bacteria that has been found to be extremely prevalent in soils, making up between 10 and 50 % of 16S rRNA clone libraries (Barns *et al.*, 2007). However, most are uncultivated (Webster *et al.*, 2006), since they require specific culture conditions (Jones *et al.*, 2009). Less is known about the Acidobacteria subgroup 9 which is known to be among the less abundant within the 26 subgroups set out in Barns *et al.*, (2007),

with subgroups 1, 3, 4 and 6 being the most abundant in worldwide soil surveys (Barns *et al.*, 1999; Janssen, 2006). Acidobacteria have also been shown to be prevalent in marine, freshwater and intertidal sediments, ranking fourth in abundance in a study comparing the diversity in those environments (Wang *et al.*, 2012).

Studies on bacterial weathering have shown the Acidobacteria to be prevalent among several basaltic environments, e.g. sub-glacial basaltic glass in Iceland (Cockell *et al.*, 2009), basaltic lava flows in Hawaii (Gomez-Alvarez *et al.*, 2007) and oligotrophic volcanic rock (obsidian) environments in Iceland (Herrera *et al.*, 2008). Mason *et al.*, (2007) showed that all Acidobacteria from basalts form a monophyletic clade, all members of which are from North Sea and coastal marine sediments (Asami *et al.*, 2005; Musat *et al.*, 2006). Given the presence of the Acidobacteria Gp9 in this study within a coastal marine sediment, and its success in a nutritionally-limiting medium based on a basalt substrate, it is possible to suggest that it may be a member of the clade described by Mason *et al.,* (2007).

Presently, there is little known about the physiology or ecological role played by Acidobacteria in their environment. However, findings from recent studies provide a rationale for the success of Acidobacteria in the experimental system used in this study. Fierer *et al.,* (2007) found that the abundance of Acidobacteria was negatively correlated to soil carbon availability, and studies of microbial succession have found greater abundances in old soils when compared to young soils (Nemergut *et al.*, 2007; Tarlera *et al.*, 2008). This has been taken to suggest that Acidobacteria are oligotrophs that thrive in environments with low nutrient availability (Jones *et al.*, 2009).

Interestingly, the abundance of Acidobacteria has been shown to be regulated by pH, with higher abundances at lower pH values (Fierer *et al.,* 2007; Männistö *et al.,*, 2007; Lauber *et al.*, 2008; Jones *et al.*, 2009). However, the pH of the experimental system

(Figure 3.5) increased over time, reaching its peak after 170 hr. The abundance of Acidobacteria (Figure 3.7) also increased during this period and dominated the community after approximately 290 hr when the pH remained higher than its initial value.

Further work on the relationship of pH and Acidobacteria diversity found that some subgroups had a negative correlation to pH while others had a positive correlation (Jones *et al.*, 2009). Although Jones *et al.*, (2009) did not consider subgroup 9 specifically, it is not inconceivable that it would have a positive relationship with pH, in keeping with the results of this study.

The final stage of the experiment, where the Acidobacteria Gp9 failed to be the dominant group for the first time since its initial exponential growth phase, could represent the secondary succession, whereby other groups outcompete the pioneer organism because of changes it made to the environment. Unfortunately, because of the lack of physiological data regarding Acidobacteria Gp9 it is not possible to say with certainty what processes it was involved in and how that may have facilitated or debilitated other microbial life.

3.7.1.3. pH

pH reached a peak of approximately 7.35 at approximately 290 hr and then stabilised to around 7.25 for the remainder of the experiment. Pilot data suggests that this represents the buffered liquid medium and the acidifying CO_2 rich atmosphere reaching equilibrium, in addition to organic acids produced as a result of microbial metabolism.

Variations in elemental composition of the medium were observed during the stationary phase between both, approximately 290 hr and 321 hr, and 321 hr and 409 hr. During this first period, Al, K and Si all increased in concentration, and in the second, both Fe and Mg also increased. Therefore, it appears that the microbial community enhanced mineral dissolution.

In the approximately 120-hr stationary phase Al concentration increased from 113 to 189 µmol L^{-1}. Al concentration at the substrate surface is potentially higher than measured in the medium because it adsorbs to mineral surfaces and aluminium hydroxides are insoluble (Piña and Cervantes, 1996). However, no such precipitate was detected through FEG-SEM or XRD analysis. Al is toxic to microbial life (Piña and Cervantes, 1996), however the degree of toxicity varies. Therefore, some bacterial groups within the community may have been affected by this toxicity. Further experiments testing the variation in community diversity in response to varying concentrations of Al could be used to investigate the effects of Al toxicity in the system.

Sensitivity of *Escherichia coli* to Al toxicity has been shown to be dependent on pH, with lower sensitivities toward neutral pH (Guida *et al.*, 1991). Al toxicity has been shown to increase when Fe was omitted from growth medium (Piña and Cervantes, 1996), which may negate the toxic effects of the increased Al concentration in this study because of the simultaneous increase in Fe concentration in the medium. With sensitivities to Al reported as being as low as 50 µM for *Bradyrhizobium* (Whelan and Alexander, 1986), a genus potentially present in the experimental community because it belongs to the Alphaproteobacteria , it is likely that some Al toxicity was experienced. This may therefore have given Acidobacteria Gp9 a competitive advantage.

K, Fe and Mg are all bioessential elements that are vital for life to survive (Wackett *et al.*, 2004; Pontefract *et al.*, 2012). Considering the uncertainties regarding the specific

microbial pathways utilised by Acidobacteria Gp9 it is not possible to ascertain whether, or how, the release of these elements into the experimental medium would have impacted diversity.

It is likely that Acidobacteria Gp9 played an important role in modifying the experimental environment while it was the dominant bacterial group. The increase of bioessential elements, and the potential inhibition by Al with parallel increases in Fe concentration may have altered the conditions such that other bacterial groups could become dominant. Further sampling points from the stationary phase would be necessary for confirmation but it is likely that, as a result of this secondary succession, a climax community of relatively stable composition could be formed, potentially lacking Acidobacteria Gp9 as conditions tend away from oligotrophy (Kuznetsov *et al.*, 1979).

Acidobacteria are a highly diverse group (Naether *et al.*, 2012). This diversity, and the ubiquity within soil systems has led to suggestions that the Acidobacteria are extremely metabolically diverse (Quaiser *et al.*, 2003). This may explain their unique capability (among the microbial community in this study) to quickly adapt to and utilise the nutrients within the experimental system. This also supports their status as a pioneer organism. This organism may represent a terrestrial biological analogue of putative martian life in similar environments, and could have made martian environments more habitable, facilitating the development of a stable and diverse microbial ecosystem.

3.7.2. Microbial weathering, dissolution and secondary mineralisation

Rocks can be considered as primary ecosystems where only a select group of adapted organisms can survive and grow. However, the microorganisms that colonise rocks can increase the availability of bioessential elements, through direct and indirect action, and these can be used by other microorganisms (Bennet *et al.,* 2001; Welch *et al.,* 2002; Rogers and Bennett, 2004; Uroz *et al.,* 2009; Gadd, 2010; Uroz *et al.,* 2011; Ahmed and Holmström, 2015a). For example, in soil environments, the microbial diversity at the rock surface is often significantly different to the bulk environment (Certini *et al.,* 2004; Uroz *et al.,* 2009; Uroz *et al.,* 2009; Uroz *et al.,* 2011). It has also been shown that rock composition can alter the composition of microbial communities (Gleeson *et al.,* 2006; Carson *et al.,* 2007). Therefore, in an oligotrophic (low nutrient) system, where the nutrients predominantly come from rocks, e.g. the Mars analogue system used in this study, microbial weathering would therefore be a significant factor in determining the microbial diversity and the formation of a thriving microbial community.

As discussed in Chapter 1, microbial weathering of rock surfaces can be responsible for generating biosignatures. The type of biosignatures potentially formed by microbial action is dependent upon the metabolism of the microorganism, as this will determine how microbial metabolism will impact the chemistry of the system. Although, in Chapter 2, the microbial community within the River Dee martian analogue environment was characterised, the impact of such microorganisms on a Mars analogue system was still unclear.

Furthermore, the impact of colonisation and succession processes upon the microbial community are important in order to understand diversity within potential martian microbial communities. Crucially, this may identify species that thrive within the

analogue environment and out-compete other microbial species, therefore elucidating ideal biological analogues for putative martian life.

Dissolution trends elucidated by ICP-AES analysis largely showed that, as cell density increased the presence of elements in the bulk medium also increased. As the microbial community reached the stationary phase, the increased concentration of bio-essential elements, e.g. Fe, K, Mg, in the medium finally exceeded that of the abiotic experiment. Thus, at this stage it was possible for excess elements to accumulate in the bulk medium.

Microbial activity is thought to increase dissolution rates because of the production of metabolic by-products, e.g. organic acids, and alteration of the pH, as discussed in Chapter 2. In addition, pH-induced elemental release has been hard to distinguish from the elemental release caused by organic acids and chelating ligands (Uroz *et al.,* 2009). However, microbial weathering in an environment with near neutral pH was investigated by Vandevivere *et al.* (1994) and several strains of bacteria were shown to increase Si dissolution by 1.1-1.4-fold over a 5-day incubation. Furthermore, analyses showed that enhanced dissolution occurred independently of microbial attachment.

The inclusion of a buffer in the system may have prevented elemental release as a result of pH changes, therefore explaining the contradiction between microbe-basalt interaction data collected by Wu *et al.*, (2007b) and this study. Wu et al (2007) found between 2 and 9-fold increases in elemental release rates in biotic samples compared to abiotic controls, whereas in this study the differences in elemental release rates was always less than a 1-fold increase or even decrease. The inclusion of a buffer in the system is believed to be more realistic as it models open systems where pH change is tempered by regular cycling of medium conditions.

Secondary mineralisation in similar systems is thought to occur because of the alterations to the rock substrate made by microbial community interaction, often through acidification or the production of chelating molecules (Wanner and Egli, 1990; Barker *et al.*, 1998; Kalinowski *et al.*, 2000; Bennet *et al.*, 2001; Uroz, Calvaruso, Turpault, Sarniguet, *et al.*, 2009; Gadd, 2010). This biomineralisation process (Gadd, 2010) either occurs directly at the microbial binding site or as a result of altered system chemistry after microbe-induced weathering (Banfield *et al.*, 2001; Welch *et al.*, 2002; Konhauser, 2007; Lian *et al.*, 2008; Uroz *et al.,* 2009; Gadd, 2010). Since there was little evidence of microbial attachment in this study it is likely that any secondary mineralisation would have had to occur indirectly, as a result of changes to the system chemistry. However, to ensure that cell enumeration counts were accurate, the experimental system was shaken before aliquots were taken. This facet of the experimental procedure may be responsible for the lack of attachment observed in this study.

Detailed analyses of secondary mineralisation were problematic because of the small grain size used in this experiment (100 µm). FEG-SEM and XRD analyses were both impeded by the overwhelming bulk composition and the rarity of potential secondary minerals at detectable abundances. Future work (see Chapter 5) replicating this experimental system over a significantly larger time period (3-6 months) could allow for the production of larger quantities of secondary minerals and more easily detectable weathering features. Thus, it may be possible to carry out more detailed analyses.

Comparisons of abiotic and biotic samples under the FEG-SEM, however, highlighted an amorphous siliceous structure in the biotic experiments that was not observed in the abiotic experiments. This structure was rare within the sample, with three examples found within a 1 cm diameter SEM stub. Analysing the spectra (Figures 3.16

and 3.19) shows that the underlying grain, and seemingly the siliceous deposit, had approximately a 30 % Fe content, which was consistent with aegirine. The remaining major peaks were for Na and Cl which is likely a result of NaCl deposits beneath or within the siliceous deposit. Therefore, it may represent partially dissolved aegirine, although this does not rule out the possibility that it represents a biofilm or extracellular polymeric substances (EPS) produced by the bacteria. Confirmation of any organic residue that may support this hypothesis was problematic because carbon is omitted from spectra data as the samples were carbon coated prior to analysis.

SEM analysis of bacterial biofilms has shown they may consist of web-like structures with individual cells often visible (Araujo *et al.*, 2003). The amorphous structure in Figures 3.13 and 3.14 were uniform, smooth and contained no evidence of bacterial cells within the deposit (though Figure 3.14 shows a microbial cell in the vicinity of the deposit). EPS, however, is a network of organic compounds including proteins and polysaccharides (McSwain *et al.*, 2005; D'Abzac *et al.*, 2010), and has been described as a "continuous film" (Plee *et al.*, 2008). The smooth and uniform surface of the "siliceous deposit" observed in this study is similar to the surface of the EPS in images captured by Prasanna et al (2012) (Figure 3.20) and they are both of the same approximate size (~10 μm in width). Perhaps the siliceous deposit represents EPS disturbed by the agitation of the bottles before cell enumeration.
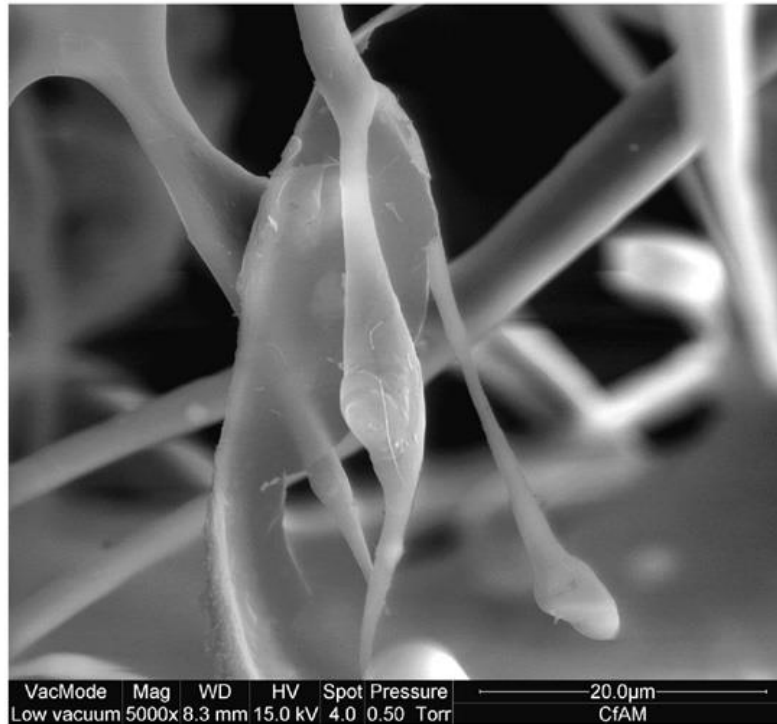
Figure 3.20. SEM image of EPS taken from Prasanna et al., (2012).

EPS have been shown to accelerate silicate dissolution (Bennet *et al.,* 2001) and could therefore have been responsible for the increased elemental release observed in this study at around 300-400 hr. However, at pH7, Barker et al (1998) found that EPS had little effect on plagioclase dissolution. This, in conjunction with the relatively rare occurrence of EPS in the biotic samples suggests that the EPS probably did not have a significant effect on elemental release. Had the experiments proceeded for longer, however, it could have served as a nucleation site for secondary mineralisation (Barker *et al.*, 1998).

With relative confidence, the presence of this siliceous deposit can be suggested to be the result of microbial action, although its rarity within the experimental system likely means that its effects would be difficult to elucidate, especially with regard to the detection of biosignatures on Mars limited to *in situ* analyses. In addition, the MSL Curiosity Rover does not have the capability of imaging such small structures, with the resolution of the Mars Hand Lens Imager (MAHLI) being ~30 μm per pixel (Edgett *et*

al., 2012). Nonetheless, EPS, including its fossilised form (FPS), has been suggested as a biosignature (Westall *et al.*, 2000) to infer the presence of life, both on contemporary Mars and the ancient habitable lacustrine environment described by Grotzinger et al (2014).

3.8. Conclusions

This study sought to investigate the microbial succession processes in an ancient martian lacustrine environment. Chapter 2 demonstrated that an intertidal anaerobic sandflat is a potential analogue for an ancient lacustrine system on Mars. Using the microbial community from this environment as a biological analogue for a martian microbial community, this study observed and measured changes in the community structure and the effect that this had on the geochemistry of a Mars analogue system.

Concentrations of bioessential elements were seen to increase as one bacterial group, the Acidobacteria Gp9, out-competed all other microbial groups, representing a potential ecological succession. Selection pressures during the different microbial growth stages were discussed and the reasons for the Acidobacteria Gp9 dominance were considered. This pioneer organism was suggested to have altered the environmental conditions such that a secondary succession could occur, potentially framing the long-term chemistry and microbial diversity of the system, although further work is needed to confirm this.

Geochemical investigations were carried out to identify any biologically unique phases within the mineralogy of the Mars regolith analogue, and though the bulk composition of the substrate appeared to obscure small-scale changes, EPS were discovered. The

identification of EPS in martian samples would be strong evidence in favour of martian life.

Further work would utilise FEG-SEM, with efforts focused on identifying specific mineral surfaces, mapping their elemental and mineralogical composition, and then re-mapping after exposure to microbial weathering. This would require the use of larger grain size substrate material. Furthermore, this study focused on the interactions of the microbial community and the geochemical environment during microbial colonisation. An extension of this work (both in terms of geochemical and ecological processes) would include investigation into long term processes that occur once the microbial community has reached an equilibrium and there is sufficient time to allow significant secondary mineralisation and to occur.

The next chapter will focus on isolating representatives of the dominant bacterial groups, and investigating their capability to thrive within a range of different environments, for example lower temperature and/or higher salinity/pH.

Chapter 4- Isolation and characterisation of microbial analogues for Mars

4.1. Introduction

4.1.1. Summary of previous chapters

In Chapter 2, a sub-surface microbial community from a Mars analogue environment was characterised, which were then grown within a simulated analogue environment designed to further replicate conditions in potentially habitable ancient martian environments, e.g. Gale Crater (Chapter 3). It was demonstrated that, given sources of both nitrogen and carbon, the microbial community could utilise the martian regolith as a source of bioessential elements. Therefore, the members of the microbial community would likely possess a mineral weathering phenotype so they can enhance mineral dissolution.

The growth experiments using a simulated analogue environment led to the formation of a microbial community, which differed in composition from that of the original community. The new microbial community is considered relevant to putative martian life within ancient potentially-habitable environments because of the similar conditions in the experimental and martian environments. Therefore, this chapter focuses on the isolation and identification of members of the analogue community that may be similar to microorganisms that could have inhabited these environments. This approach, whereby isolates are selected by environmental selection, is uncommon, because research is usually focused on groups of microorganisms pre-selected by researchers, e.g. methanogenic archaea (Reid *et al.*, 2006; Kral *et al.*, 2016) and iron-reducing bacteria (Nixon *et al.*, 2012), that can survive the adverse environmental

conditions associated with Mars, and are based upon common characteristics e.g. UV, cold and salinity resistance or chemolithotrophic metabolism. This approach provides the advantage of being a more realistic representation of microbial communities that naturally exist as mixed cultures, with a variety of metabolisms represented among the constituent microorganisms and often some mutualistic inter-species relationships. However, the impact of individual members of the microbial community is difficult to distinguish, as was found in Chapter 3.

4.1.2. Putative martian life

If life existed on Mars later than the Hesperian, it would most likely be in the sub-surface environment where it would not be exposed to the surface conditions, for example, the harsh radiation environment (Dartnell, Desorgher, Ward and a. J. Coates, 2007; Parnell *et al.*, 2007; de la Torre *et al.*, 2010) exacerbated by the lack of a significant magnetic field (Acuña *et al.*, 1998), and a depleted atmosphere (Chassefière *et al.*, 2007; Hassler *et al.*, 2014). In the sub-surface environment, below the photosynthetically active region (400 - 700 nm) (Cockell and Raven, 2004), photosynthesis is not possible and microorganisms would likely have had to rely on chemolithoautotrophy and/or chemoorganotrophy. At Gale Crater, during this time, surface conditions are considered likely to be warm enough to support a palaeolake (Grotzinger *et al.*, 2014) (see below), and thus, a subsurface environment at Gale Crater is considered to be potentially habitable.

Chemolithotrophs and chemoorganotrophs use inorganic and organic compounds as electron donors, respectively. Coupled with the oxidation of an electron donor, an electron acceptor (e.g. oxygen in aerobic microorganisms, or nitrate in anaerobic microorganisms (Straub *et al.*, 1996b; Nixon *et al.*, 2013)) is reduced. The coupling of

these two reactions represents respiration, for example, nitrate reducing and iron oxidising bacteria. These two types of bacteria are focussed on because of their relevance to the nutritional environment at Gale Crater, and martian environmental conditions.

1. Nitrate can act as an electron acceptor for anaerobic chemotrophic microorganisms (Straub *et al.*, 1996b). Nitrate reducing bacteria are heterotrophs that carry out an important role within the nitrogen cycle because they reduce nitrate to nitrite, which can then be reduced to form gaseous nitrogen compounds by denitrification (López-Gutiérrez *et al.*, 2004). It has also been shown that NR bacteria play an important role in the oxidation of ferrous iron (Benz *et al.*, 1998; Straub and Buchholz-Cleven, 1998), which may be applicable to the iron-rich basalt on Mars (Schmidt *et al.*, 2014). Furthermore, since nitrogen-bearing compounds have recently been discovered at Gale Crater (Leshin *et al.*, 2013, Stern *et al.,* 2015), the isolation of a species of nitrate reducing bacteria has relevance to studies of putative martian biota.

2. Iron oxidation has been suggested as a plausible metabolism for putative martian biota (Nixon *et al.*, 2012, 2013). IO bacteria are chemolithotrophs that use inorganic ferrous iron as an electron donor. They subsequently split into distinct groups based on their terminal electron acceptor, including both oxygen and nitrate depending on their tolerance of oxygen (Straub *et al.*, 1996a; Emerson and Moyer, 1997). Some can also use light to drive iron oxidation (Widdel *et al.*, 1993; Jiao *et al.*, 2005).

Both Chemolithoautotrophic and chemoorganotrophic microorganisms have long been a focus of life detection efforts on Mars, with the suggestion that they could be a biological analogue of past or present martian life, see for example the work by Boston, Horneck, Amils, Grotzinger and their respective co-authors (Boston *et al.*, 1992;

Horneck, 2000; Amils *et al.*, 2007; Grotzinger *et al.*, 2014). However, chemoorganotrophic microorganisms are also important within microbial ecosystems acting as detritivores, oxidising reduced, organic matter for use by the autotrophs (Fisher and Schulze-Makuch, 2013) These too may be used as analogues of potential martian life (Oren *et al.*, 2014).

The possibility of subsurface lithoautotrophic (microorganisms that derive energy utilising reduced compounds within minerals) microbial ecosystems (SLiMEs) has therefore been investigated, e.g. by Nealson *et al.*, 2005. The importance of these ecosystems is their independence from photosynthesis, both directly, in that they do not contain photosynthetic organisms, and indirectly, in that they also do not require or utilise the by-products of photosynthetic ecosystems on the surface (Stevens and Mckinley, 1995; Nealson *et al.*, 2005). These systems are considered likely to be driven by the presence of hydrogen and oxidants provided geologically (Nealson *et al.*, 2005). This supports the suggestion that methanogens could be a model organism for contemporary martian life (Kral *et al.*, 2016).

The presence of basaltic substrates, liquid water and bicarbonate in the martian subsurface could also support these systems, with serpentinisation and other mineral alteration supplying hydrogen and necessary oxidants e.g. CO_2 (Oze and Sharma, 2005; Neubeck *et al.*, 2014; Konn *et al.*, 2015). Therefore, Stevens & Mckinley (1995) suggest that SLiMEs could be a model for martian life.

4.1.3. Potentially habitable martian environments

One example of a potentially habitable extra-terrestrial environments is ancient martian environments, e.g. Gale Crater, where the environmental conditions are more

favourable to life than those of present-day Mars (e.g. Grotzinger *et al.*, 2014). The ancient lake at Yellowknife Bay, Gale Crater was proposed by Grotzinger *et al.*, (2014) to be habitable based on the evidence for above-zero temperatures, water of circumneutral pH and bioessential element availability that lends credence to a martian biosphere. Similar conditions have been reported for several other locations on Mars e.g. Terby and Jezero craters (Bethany L. Ehlmann *et al.*, 2008; Ansan *et al.*, 2011; Schon *et al.*, 2012), with the latter being one of the proposed landing sites for NASA's Mars2020 rover (NASA, 2017b). These examples present environments in which microorganisms analogous to those isolated in this study may have existed. In addition, efforts have already been made to characterise terrestrial analogues of the lacustrine system at Gale Crater (Schwenzer *et al.*, 2015; Rubin *et al.*, 2016).

4.1.4. Limits of life

To study the habitability on early and present day Mars it is important to determine the limits within which life can exist. Though the environmental constraints (e.g. pH, temperature, salinity) on microorganisms has been widely researched, e.g. Rothschild and Mancinelli, (2001), Harrison *et al.,* (2013) noted that physiochemical parameters were often considered individually, rather than as a composite. For example, the habitable temperature range for life had been considered to be 0 °C to 108 °C, however, including corresponding pressure data has increased this range to – 12 °C to 121 °C. It is prudent to note, however, that this is based on the assumption that extra-terrestrial life is fundamentally similar to terrestrial life.

4.1.5. Aims of the chapter

This chapter describes the isolation and identification of individual chemolithotrophic and heterotrophic bacterial species from a Mars analogue environment (i.e. the laboratory analogue environment designed in Chapter 3). The isolates are characterised with respect to the environmental conditions associated with potentially habitable ancient environments on Mars (e.g. Gale Crater) to investigate the feasibility of them living within these environments. This is a new approach since, at present, there has been no research carried out to investigate the growth and survivability of terrestrial microorganisms in the conditions of potentially-habitable ancient martian environments.

As mentioned above, previous studies have focused on the applicability of specific types of bacteria with purportedly relevant metabolisms, e.g. sulfate and iron reducing bacteria (Nixon *et al.*, 2013) or have focused on contemporary Mars where conditions are much more constraining on potential life e.g. (Matsubara *et al.*, 2016). Thus, this research, focusing on the isolation and survivability of microorganisms from a Mars analogue experimental system, constructed around a potentially-habitable ancient martian lakebed, is a novel approach to informing life detection investigations on Mars.

The aims of this chapter are twofold:

1) To isolate microorganisms from an intertidal environment (as described in Chapter 2), that are putative analogues for potential life in the Noachian lacustrine environment at Gale Crater, Mars.

2) To characterise the environmental limits within which the isolated microorganisms can survive and grow and thus, investigate their relevance to past, or perhaps present, martian environments.

The microorganisms in this chapter were isolated from the laboratory analogue environment characterised in Chapter 3. Based on their survival and growth within the analogue environment it was hypothesised that the optimal growth and survival conditions of the microorganisms would be relevant to the ancient potentially habitable environment at Gale Crater (Chapter 3).

4.2. Methods

4.2.1. Media preparation

In order to isolate microorganisms that utilised a variety of different metabolic pathways, a selection of growth media, which are shown in Table 4.1 and described in detail below, were used. The media were inoculated with the microbial community from the enrichment step in Chapter 3.

Each medium was prepared, and inoculated, both aerobically and anaerobically to increase the likelihood of successfully isolating a variety of facultative and obligate anaerobes. Once individual species were isolated it was then possible to investigate growth anaerobically to determine their applicability to potential martian environments. Anaerobic media was prepared using the anaerobic techniques detailed in Chapter 3. In brief, Na-thioglycollate (0.1 g/L) and ascorbic acid (0.1 g/L) were added as reducing agents to the anaerobic media (the compositions were otherwise the same as the aerobic media).

The media were dispensed into 125 mL Wheaton bottles, either sealed with septum stoppers or bunged with cotton wool. During environmental stressor investigation, media were dispensed into 10 mL hungate tubes and inoculated with approximately

1×10^7 cells from the respective media stock. All media were autoclaved at 121 ℃ and 15 psi for approximately 15 min.

4.2.1.1. Inoculation

The media were inoculated aseptically with a 5 % inoculum from the mixed community, the preparation of which was outlined in Chapter 3. Aerobic media were inoculated in a biological safety cabinet to prevent contamination, and the anaerobic media were inoculated using anaerobic techniques as described in Chapter 3. The selective media were then incubated at 14.8 °C and room temperature.

Table 4.1. Relevant metabolisms, electron donors and electron acceptors for the media used in this study. *Only in anaerobic media. – indicates where there is more than one electron donor.

Media type	Electron donor	Electron acceptor	Metabolism
Marine	-	CO_2*	Chemoorganotroph
Iron-Oxidising	Fe(II) compounds	O_2, NO_3, CO_2*	Chemolithotroph
Nitrate-reducing	-	NO_3, CO_2*	Chemoorganotroph
Enrichment	-	CO_2*	Chemoorganotroph
LB	-	CO_2*	Chemoorganotroph

4.2.1.2. Marine bacteria medium

Due to the nature of the sample site a marine growth medium was used, which had a similar salinity to terrestrial marine environments. This medium selected for chemoorganotrophic, mildly halophilic microorganisms. The growth medium contained the following (per litre): 19.45 g of NaCl, 8.8 g of $MgCl_2$; 5 g of Peptone; 3.24

g of NaSO$_3$; 1.8 g of CaCl$_2$; 1 g of yeast extract; 0.55 g of KCl; 0.16 g of NaHCO$_3$; 0.10 g of ferric citrate; 0.02 g of H$_3$BO$_3$ and 0.008 g of K$_2$HPO$_4$. The pH of the media was adjusted to 7.0 using 1M HCl.

4.2.1.3. Lysogeny Broth (LB) medium

LB medium was used to isolate chemoorganotrophs that can utilise the organic sources within typtone and/or yeast. This medium contained the following (per litre): 10.0 g of tryptone; 5.0 g of yeast extract and 10.0 g of NaCl. The pH was adjusted to 7 using 1M HCl.

4.2.1.4. Nitrate reducing (NR) bacteria medium

To select for NR bacteria, NaNO$_3$ was added to the medium as the electron acceptor and an organic substrate within peptone as the electron donor.

The medium contained the following (per litre): 20 g of peptone and 0.4 g of NaNO$_3$. The pH was adjusted to 7 with 1M NaOH.

The medium was dispensed into a 500 mL flask, which was bunged with cotton wool and covered with aluminium foil to reduce the risk of contamination. This medium was autoclaved at 121 ℃ and 15 psi for approximately 15 min.

4.2.1.5. Iron oxidising (IO) bacteria medium

This medium was prepared by combining four solutions (A, B, C and D), each prepared separately. The final concentrations of each substance are shown below (per litre):

2 g of KH$_2$PO$_4$, 2 g of KNO$_3$, 1 g of NH$_4$Cl, 0.8 g of MgSO$_4$ × 7H$_2$O, 0.1 g of ascorbic acid and 0.1 g of Na-thioglycollate, 5 g of Na$_2$S$_2$O$_3$, 1 g of NaHCO$_3$, 0.2 g FeSO$_4$ × 7H$_2$O.

Solution A was prepared as follows: To 979 mL of dH_2O, 2 g of KH_2PO_4, 2 g of KNO_3, 1 g of NH_4Cl, 0.8 g of $MgSO_4 \times 7H_2O$, 0.1 g of ascorbic acid and 0.1 g of Na-thioglycollate were added. The ascorbic acid and Na-thioglycollate were added once the preparation had cooled after boiling (see anaerobic techniques Chapter 3). The pH of the solution was adjusted to 7 and 9.8 mL was dispensed into hungate tubes and sealed using 10 mm septum stoppers.

Solution B was prepared by adding 5 g of $Na_2S_2O_3 \times 5H_2O$ to 10 ml of dH_2O.

Solution C was prepared by adding 1 g of $NaHCO_3$ to 10 mL pre-autoclaved dH_2O.

Solution D was prepared by adding 2 mg of $FeSO_4 \times 7H_2O$ to 0.1 mL of 0.1 N H_2SO_4 (made up with pre-autoclaved distilled water).

Solutions A and B were autoclaved at 121 ˚C and 15 psi for approximately 15 min and C and D were filter sterilised. To Solution A,100 µL of Solutions B and C, and 10 µL of D were added under anaerobic conditions.

4.2.1.6. Enrichment media

In addition to the above media, the enrichment media detailed in Chapter 3 was also used. This medium was designed to support a diverse microbial community and contained nutrients suitable for several types of bacterial metabolism. It was therefore selected to achieve a high-density stock of the bacterial groups that grew and survived in the Mars analogue system.

The medium contained the following (per litre): 2 g of trypticase peptone, 2 g yeast extract, 0.3 g KCl, 1 g NH_4Cl, 3 g Na_2SO_4, 23 g NaCl, 0.5 g Na-Lactate, 2 g $MgCl_2$, 0.35 g K_2HPO_4, 0.1 g Na-thioglycollate and 0.1 g ascorbic acid. The pH was adjusted to 7.75.

The mixture was then autoclaved at 121 ℃ and 15 psi for 15 min. Post autoclave 40 μL of a 10 × diluted vitamin solution and 100 μL of a pre-made SL10 trace element solution were added. This non-specific medium was used to increase the likelihood of isolation with no specific target microorganisms.

4.2.1.7. Agar

Agar plates of the above-mentioned media were made by adding 15 g L^{-1} bacteriological agar. The plates for anaerobic incubation were prepared and stored inside a COY anaerobic glove box (Coy Labs, USA), and contained 0.1 g L^{-1} Na-thioglycollate and ascorbic acid in addition. Agar plates were used to facilitate isolation.

4.2.2. Isolation

The culture enrichments were incubated at 21 ℃. Microbial growth was monitored periodically using the Sybr Green cell enumeration method, as previously described (see Chapter 3). For isolation two techniques were used to obtain pure cultures from each of the selective media above. Firstly, serial dilutions were prepared, which was followed by spread plating and streaking.

4.2.2.1. Serial dilution

This method ensured that the community was dilute enough to form single cells on a plate. Cell enumeration demonstrated that the cell density of the inoculum did not exceed 10^8 cells mL^{-1}, and therefore serial dilutions were carried out from 10^{-1} to 10^{-9}. Growth was monitored using cell enumeration by Sybr Green staining, previously

described in Chapter 3. Microorganisms from the highest dilution factor for each medium that demonstrated successful growth were used in the streak plate method.
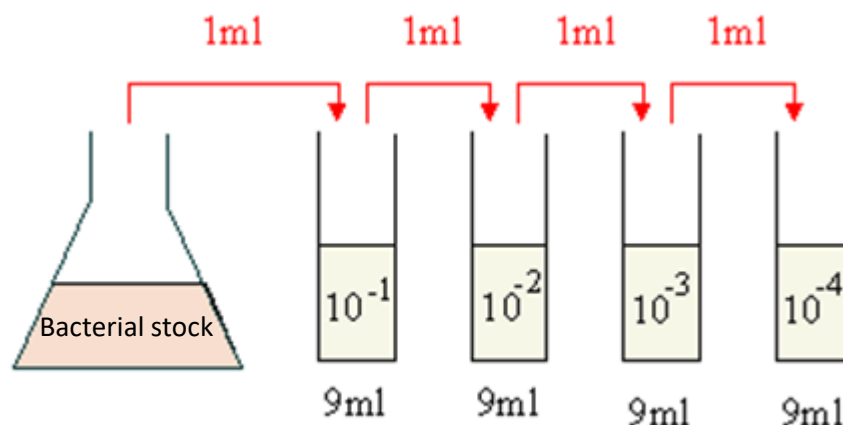
Figure 4.1. The serial dilution method used in this study.

4.2.2.2. Spread plate isolation

To obtain pure colonies with which to carry out plate streaking, 0.1 mL of successfully growing, serially diluted media was pipetted onto agar plates, either their original growth media composition or on LB medium, since LB medium contains a wider range of nutrients. Anaerobic plates were prepared, inoculated and incubated in the anaerobic chamber. Incubation was carried out at room temperature for 7 days. Visible colonies were steaked onto fresh plates as described below.

4.2.2.3. Streak plate isolation

To obtain pure isolates the streak plate method (as described in Zaky *et al.* (2016)) was used. This method involved using the quadrant streak technique to facilitate sequential dilution of the original inoculum over the entire surface of the agar plate.

Using a sterile inoculation loop, a single colony was streaked over a fresh plate. The streaked plates were then incubated at room temperature for 7 days. Visible single colonies were then selected for molecular analyses to determine whether the colony represented a pure culture, and to identify the bacterial species present.

Isolates were stored at -80 °C in glycerol for long term storage.

4.2.3. DNA sequencing and 16S rRNA contig construction

Taxonomic affiliation of the bacterial isolates was achieved through sequencing of the 16S rRNA gene. The bacteria universal primers 27F- COM2 and COM1-1541r were used (Olsson-francis *et al.*, 2015; Olsson-Francis *et al.*, 2016). Sequencing was carried out in both the forward and reverse directions to aid in the construction of a contig, which is a pair of overlapping DNA fragments that in combination represent a consensus region of DNA.

PCR amplification was performed in 50 μL reactions containing the following: 250 nM of each primer, 5 μL of 10 × Taq buffer, 2 mM $MgCl_2$, 0.1 mM of each Deoxynucleotide Triphosphate (dNTP), 5 μg bovine serum albumin (BSA) and 1.75U of Taq polymerase. DNA was not extracted prior to PCR. Instead the PCR was carried out directly on colonies that could grow on plates (all of the aerobic isolates) and with liquid medium (the anaerobic isolates). For liquid medium 1 mL of culture was aseptically centrifuged at 10,000 × g and the pellet was resuspended in sterile phosphate buffered saline (PBS). 1 μL of the suspension was added to the PCR reaction.

PCR products were purified using a Qiagen PCR purification kit and DNA concentration quantified using a ThermoScientific NanoDrop 100 spectrophotometer. These purified PCR products were sequenced (Macrogen: Seoul, South Korea). Subsequent

phylogenetic sequence analysis was carried out using the Bioedit Sequence Alignment Editor (Hall, 1999) and the CAP3 contig assembly program (Huang and Madan, 1999). Manual quality alignment was carried out in Bioedit Sequence Alignment Editor and the contigs were created using the CAP contig assembly program to assemble contigs of approximately 1000 bp.

Taxonomic classification of the sequences was performed using the Naïve Bayesian rRNA Classifier v1.0 analysis tool within the Ribosomal Database Project II website (Michigan State University) (http://rdp.cme.msu.edu/). Based on the taxonomical hierarchy proposed in Bergey's Manual of Systematic Bacteriology (Garrity *et al.*, 2004).

The NCBI Blast database was also used to identify the most closely related species. This information was noted along with the % homology between the two.

4.2.4. Phenotypic characterisation of isolates

4.2.4.1. Gram staining

Gram staining differentiates between bacteria Gram positive and Gram negative microorganisms based on the cell wall structures. Variations in the amount of peptidoglycan in the cell wall determine whether staining is reversible or irreversible, and therefore staining is a useful means of distinguishing between the two groups. Gram positive bacteria have a thicker peptidoglycan layer and retain the stain even after washing. In comparison, gram negative bacteria have a thin peptidoglycan layer, but have an outer membrane and a higher lipid content.

For Gram staining a sterile, disposable inoculation loop was used to transfer liquid cultures to the surface of a clean glass microscope slide and spread over a small area

(approximately 1 cm^2). The cells were air dried by storage for 5 min in a biological safety cabinet and then passing the slide through the flame of a Bunsen burner, ensuring that the cells were not directly exposed to the flame.

After fixation, the slide was flooded with crystal violet solution and left for one minute. The stain was then removed from the slide with ddH$_2$O and then flooded with Gram's iodine solution for one minute. The slide was then washed again with ddH$_2$O and the excess ddH$_2$O removed by blotting. The slide was then flooded with 95 % alcohol for 10 sec and washed with ddH$_2$O. Finally, the slide was flooded with safranin solution. After 30 sec the slide was washed with ddH$_2$O, blotted dry, and examined under light microscopy at ×100 magnification using an oil immersion lens.

4.2.4.2. Morphology

The morphology of the isolates was examined using Sybr Green, as described in Chapter 3. Stained cells were then observed under a Leica DNRP microscope at ×100 magnification.

4.2.4.3. Mineral Phosphate Solubilisation

Following on from Chapter 3, the isolated bacteria were investigated for the presence of a mineral phosphate solubilisation (MPS) phenotype. The MPS phenotype is associated with mineral weathering and demonstrates that mineral-bound phosphate can be solubilised and therefore made accessible for microbial metabolism. MPS plates were prepared as described by (Mailloux *et al.*, 2009) using tri-calcium phosphate. The agar plates contained two layers, one transparent layer below an opaque mineral phosphate-containing layer, which consisted of the following (percentages are w/v %):

1. Bottom layer 1 % agarose

2. Top layer 2 % agarose, 1% glucose, 0.4 % $MgSO_4.7H_2O$, 0.1 % NaCl, 0.5 % NH_4Cl

Each were autoclaved separately at 121 ℃ and 15 psi for approximately 15 min. After autoclave (while still molten), 1 g L^{-1} of tri-calcium phosphate (TCP) was added to the top layer to provide a solid phosphate source.

The two molten agar preparations were poured into sterile agar plates in a biological safety cabinet to prevent contamination, ensuring that the bottom layer was set before the top layer was poured. Once set, the plates were divided into quarters by marking the underside of the plate. Three 50 µL aliquots were then pipetted onto the surface of the agar in each of the four quadrants. The plates were incubated at 30 °C for 72 hr. This was repeated for all aerobic isolates. For obligately anaerobic isolates, agar was prepared using the anaerobic techniques described in Chapter 3 and the plates were prepared and stored at room temperature in an anaerobic chamber.

4.2.4.4. Endospore formation

The isolates were also tested for their ability to form spores. Spores provide protection from environmental stresses and allow the survival of microorganisms through extended periods of unfavourable conditions.

Approximately 1×10^6 cells of each isolate were prepared for staining as above (see Gram staining). A small, approximately 1 cm^2, piece of blotting paper was placed on the fixed cells. Schaeffer and Fulton Spore Stain Solution A was added until the blotting paper was saturated. The slide was then heated gently above a beaker of boiling water for 5 min. When the blotting paper began to dry, additional Spore Stain Solution A was added to keep it moist.

After 5 min the slide was removed from the heat, the blotting paper removed and the slide allowed to cool for two min. The slide was then rinsed with ddH$_2$O and stained with safranin for two min. Both sides of the slides were then washed with ddH$_2$O and the slide was blotted and viewed under oil-immersion light microscopy.

Vegetative bacterial cells were stained pink/red by the safranin whereas endospores were stained green. Slides were examined for the presence of green-stained endospores using a Leica DNRP microscope at ×100 magnification.

4.2.5. Determination of carbon source utilisation by BIOLOG tests

To investigate the range of organic carbon sources that each of the isolates could utilise, Technopath Biolog Ecoplates were used. These 96-well plates contain 31 different organic carbon sources selected for their applicability to soil microbial community analysis (Insam, 1997). These were dried with other essential nutrients in triplicate within the wells, along with control wells, as shown in Figure 4.2. A tetrazolium dye is included within each well and as the organic carbon source is metabolised, the dye is reduced resulting in a colour change.

These organic carbon sources were not selected specifically because of their applicability to martian environments, however five of the carbon sources contained within the Bioplates were amino acids, thought to have been provided to the early Earth and early Mars by meteorite and cometary impacts (Chyba *et al.*, 1990; Jull *et al.*, 2000; Botta and Bada, 2002).

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

Figure 4.2. A schematic layout of the BIOLOG Ecoplates used in this study.

To inoculate the plates, 10 mL aliquots of the isolate were centrifuged at 10,000 × g, washed and resuspended in 1 × PBS. Each well was inoculated with 150 μL of inoculum. Optical density readings at 590 nm were taken every 15 min over 18 hr at 25 °C using a Biotek ELx808 microplate reader.

4.2.6. Survivability during exposure to environmental stressors

The survivability of organisms during exposure to environmental stressors was investigated in terms of pH, temperature and salinity, and, tolerance to freeze-thaw cycling.

4.2.6.1. pH, temperature, NaCl concentration

To determine the growth limits for each of the isolates, they were grown in their selective medium over a range of pH values (3 – 10), NaCl concentrations (0 % – 30 %) and were incubated at varying temperatures (4 °C – 55 °C). Each growth experiment was carried out in triplicate and inoculated with a 5 % inoculum from the above selective media stocks. Abiotic controls were carried out in parallel.

To measure cell growth, optical density measurements were taken every 18 – 24 hr using a Camspec M107 spectrophotometer. Hungate tubes were placed into the instrument and OD readings were taken at 660 nm. The hungate tubes were marked to ensure that the measurement was taken from the same point throughout analysis. This ensured that variations and imperfections in the glass surface did not impact the measurements. OD measurements were continued until the isolate had reached stationary growth phase.

Instrument blanks (ddH2O) were analysed prior to each set of measurements and after every 5 individual analyses to monitor instrumental drift.

4.2.6.2. Freeze-thaw tolerance

Diurnal fluctuations in temperature may have led to freeze-thaw cycles within the aqueous environments on Mars, especially as the climate became colder at the end of the Hesperian (Carr and Head, 2010; Clifford *et al.*, 2010; Fairen, 2010). Formation of ice crystals within the cytoplasm is known to cause damage to bacterial cells and often death. Mechanisms of resistance are varied and could be essential for the survival of putative martian organisms.

To investigate survivability 10 mL of culture was centrifuged at $10,000 \times g$ and the cells were washed with 50 mM Na-phosphate buffer solution (pH 7) and resuspended in the appropriate growth medium. 1 mL aliquots (in triplicate) of the cell suspension were frozen at $-20\,°C$. The culture was allowed to freeze and held at that temperature for 10 min (40 min total). Samples were then thawed for 40 min at $37\,°C$ before being stained and cell counts were carried out (see below). This process was repeated three times for aerobic samples. For anaerobic samples, 1 mL aliquots were transferred into Eppendorf tubes and, under anaerobic conditions, placed within a 100 mL Wheaton bottle. The Wheaton bottle was sealed prior to removal from the anaerobic hood and then placed in the freezer or incubator respectively. The anaerobic Wheaton bottle was transferred back into the anaerobic hood after thawing and opened to carry out the staining procedure.

Short durations of freezing and thawing were used to isolate the effects of the freezing and thawing processes from the likely increased growth rate induced by over-exposure to $37\,°C$ and the likely cell death associated with extended periods at $-20\,°C$.

As a control, an identical sample was stored at room temperature and cells were counted at the start and end of the experiment.

4.2.6.3. Live/Dead staining

The quantity of live, dead and damaged cells remaining after desiccation and freeze-thaw was ascertained by Live/Dead staining. For this, 3 µL of Live/Dead stain was added to the 1 mL of culture in a sterile Eppendorf tube and stored in darkness for 20 min to allow staining to take place. 20 µL was then pipetted onto a clean microscope slide and viewed under oil-immersion microscopy. Live, dead and damaged cells appear green, red and yellow respectively and each were counted in 20 fields of view.

4.3. Results

4.3.1. Identification of isolates

All isolates were sequenced to give taxonomical identification to genus level using the NCBI Blast database, (Table 4.2). Each of the isolates represent five distinct bacterial species and were closely related to species isolated from marine and soil environments, as well as compost and anaerobic sludge (Bouvet and Grimont, 1986a; Parshina *et al.*, 2003; Heyrman *et al.*, 2004; Kwon *et al.*, 2007; Jimenez *et al.*, 2013). In addition, one of the isolates belonged to the *Acinetobacter* genus and was most closely related to *Acinetobacter johnsonii* which had been found in eviscerated chickens.

Table 4.2. The taxonomical identification of the five isolates characterised in this study with associated sequence lengths and identity to the best matched sequence in the NCBI database (see Appendix for sequences).

Isolate	Selective media used	Contig sequence length (bp)	NCBI Blast closest relative	Identity to best matched sequence	Source of closest relative
E_01	Nitrate reducer	1243	*Acinetobacter johnsonii*	99 %	Clinical samples and eviscerated chickens (Bouvet and Grimont, 1986b)
E_02	Iron oxidising	1341	*Clostridium amygdalinum*	98 %	Anaerobic sludge bed
E_03	Enrichment	741	*Bacillus toyonensis*	99 %	Algoya Bay, South Africa (marine environment)(Okaiyeto *et al.*, 2015)
E_04	Marine 1 (red)	958	*Bacillus drentensis*	99 %	Soil. Drente, Netherlands
E_05	Marine 2 (white)	765	*Bacillus niabensis*	99 %	Cotton-waste compost. Suwon, South Korea

All of the sequences except for E_02, were 99 % similar to sequences in the database; however, E_02 was only 98 % homologous with its nearest relative, *Clostridium amygdalinum*. It is therefore possible that this represents a novel *Clostridium* species.

4.3.2. Phenotypic characterisation of isolates

4.3.2.1. Gram staining, morphology, spore formation

All five of the isolates in this study were gram positive. Gram positive bacteria often live in extreme environments, and are protected from harsh environmental conditions by their thick peptidoglycan cell wall (Silhavy *et al.*, 2010). Given the experimental conditions used in Chapter 3 it is therefore not surprising that the isolated species are all gram positive.

Isolates E_02, E_04 and E_05 were rod shaped, whereas E_01 and E_03 were both coccoid.

Phenotypic tests for phosphate solubilisation of the isolates indicated halos around colonies inoculated on MPS plates, which demonstrated the presence of the MPS phenotype (the colonies on the agar solubilised the TCP in the top layer of the agar, making the agar turn transparent where TCP has been removed (Goldstein and Liu, 1987)). This is observed as a halo around the colonies and was used to infer the presence of the MPS phenotype in the isolates.

Two of the five isolates (E_03 and E_04) were able to solubilise phosphate (Table 4.3).

Table 4.3. Phenotypic characteristics of the isolates used in this study.

Isolate	Gram stain	Morphology	MPS	Spores
B. toyonensis (E_03)	+	coccoid	Yes	No
B. drentensis (E_04)	+	bacillus	Yes	Yes
B. niabensis (E_05)	+	bacillus	No	Yes
A. jonsonii (E_01)	+	diplococci	No	No
C. amygdalinum (E_02)	+	bacillus	No	Yes

4.3.2.2. Effect of temperature on growth

For all of the isolates, the optimum specific growth rate was observed between 14.8 °C and 40.0 °C (Figure 4.3). Below, each isolate will be considered in turn.

For *B. drentensis* the general trend showed specific growth rate increased with temperature until 37 °C. Specific growth rate increased from 0 hr^{-1} at 4 °C, to 0.015 hr^{-1} at 14.8 °C and rapidly increased to 0.037 hr^{-1} at 37 °C. The optimum temperature was identified as 37 °C. Biphasic growth was observed in both the 21 °C and 25 °C conditions, and the specific growth rates were calculated for the first step in the log phases to facilitate direct comparison with the other isolates.

For *B.toyonensis* (Figure 4.3B), the highest specific growth rate (0.064 hr^{-1}) was observed at 14.8 °C. Above and below this temperature the specific growth rate was
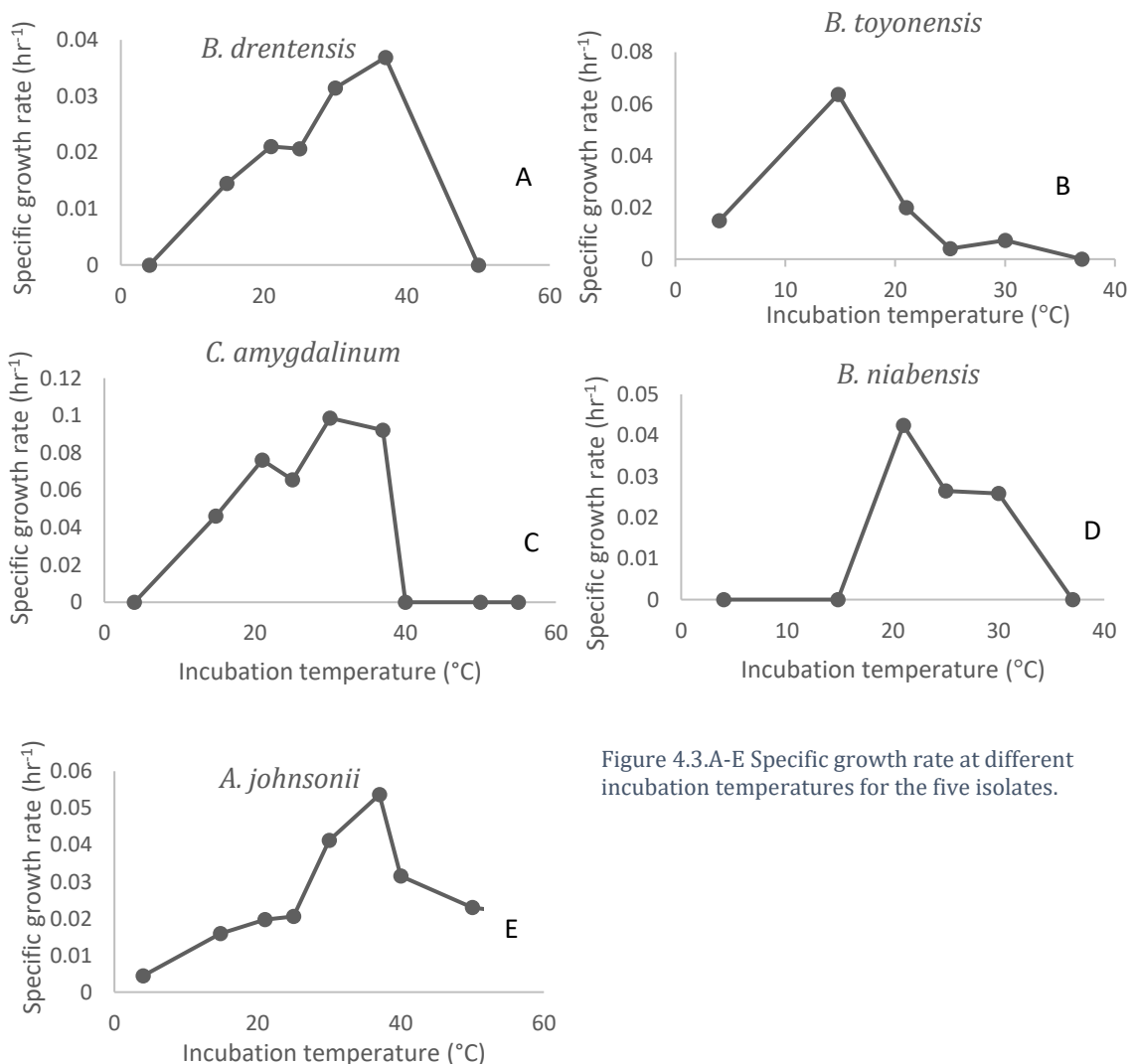
Figure 4.3.A-E Specific growth rate at different incubation temperatures for the five isolates.

considerably lower (0.015 and 0.02 hr^{-1} at 4 °C and 20 °C respectively).

With regard to incubation temperature, the optimum growth condition for *C.amygdalinum* (Figure 4.3C) is between 25 °C and 37 °C. The optimum specific growth rate of 0.099 hr^{-1} was observed at 30 °C. The specific growth rates decreased to 0.046 hr^{-1} at 14.8 °C and no growth was detected at 4 °C.

For *B. niabensis* (Figure 4.3D), optimum growth was observed at 21 °C where a specific growth rate of 0.042 hr^{-1} was calculated. However, the specific growth rate decreased to 0.025 hr^{-1} at 30 °C. There was no growth detected above 30 °C, or below 20 °C.

Figure 4.3E shows that the optimum specific growth rate for *A.johnsonii* occurred at 37 °C, with a specific growth rate of approximately 0.054 hr^{-1}. The specific growth rate decreased at 30 °C to 0.041 hr^{-1} and at 40 °C to 0.032 hr^{-1}. There was no growth detected at 4 °C.

4.3.2.3. Effect of pH on growth

Growth was not observed below pH 5 and above pH 10 for any of the isolates. Specific growth rates rapidly decreased to zero, below pH 5 and above pH 10.

Figure 4.4A shows that, for *B. drentensis*, the optimum specific growth rate occurred at pH 7, with a specific growth rate of approximately 0.028 hr^{-1} was calculated. Growth was not observed at pH 5.

For *B. toyonensis*, specific growth rates calculated at pH 5, 6 and 10 (Figures 4.4B) can be accounted for by extended lag periods (at 75 and 139 hr). In all other conditions, where growth was observed the log growth phase had finished by approximately 139 hrs. The relatively high specific growth rate calculated for growth at pH 5 (0.016 hr^{-1}), the similar value (0.015 hr^{-1}) calculated at pH 8, and the steady increase from pH 6 and

7 (0.009 hr^{-1} and 0.011 hr^{-1}) indicate that the value for pH 5 may have been inflated by the extended lag period, and thus pH 8 is considered to be the optimum pH. The specific growth rate at pH 8 was 0.015 hr^{-1}. No growth was observed at pH 4 or below, and at pH 10.

For *C.amygdalinum* (Figure 4.4C) the optimum specific growth rate occurred at pH 7, with a specific growth rate of 0.100 hr^{-1}. Specific growth rates at pH 6 and pH 8 were relatively high, at 0.078 hr^{-1} and 0.070 hr^{-1} respectively. No growth was observed at pH 5 or pH 10.

The optimum pH for *B. niabensis* (Figure 4.4D) occurred at pH 6.5 with a specific growth rate of 0.051 hr^{-1}. No growth was observed at pH 6 and 7.5.
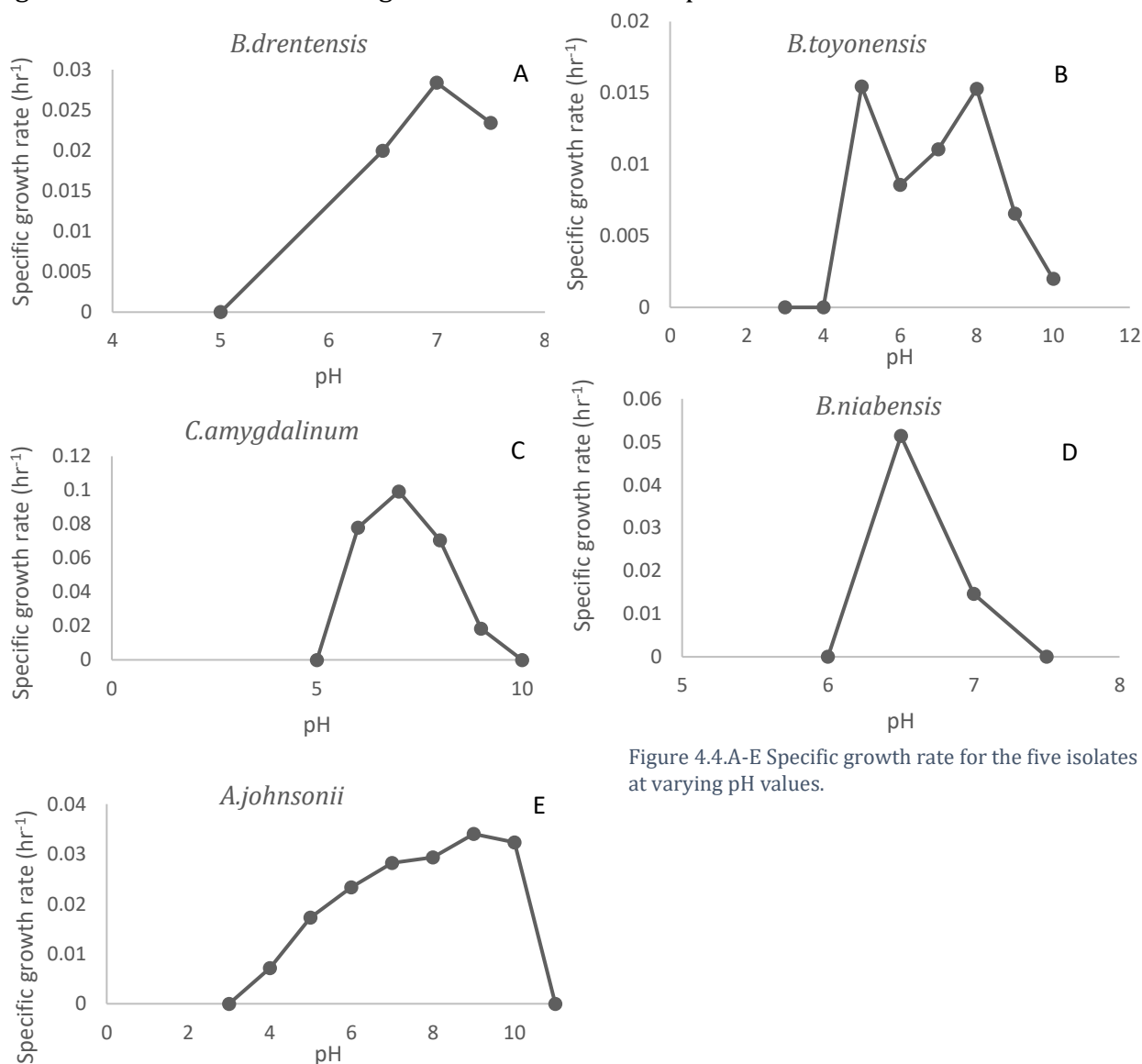
Figure 4.4.A-E Specific growth rate for the five isolates at varying pH values.

The optimum pH for *A.johnsonii* (Figure 4.4E). pH 9 where a specific growth rate of 0.034 hr^{-1} was calculated. No growth was observed at pH 3, 4,5 and 11. Unlike the specific growth rate for all other pH conditions, the *A.johnsonii* samples at pH 10 had a lag phase 21 hr longer than the almost immediate onset of the log phase in the other samples.

4.3.2.4. Effect of salinity on growth

Figure 4.5A show that specific growth rates for *B. drentensis*, with the optimum recorded at 0.077 hr^{-1} for 0.5 %, decreasing to 0.058 hr^{-1} at 3 %. No growth was observed below 0.5 % or above 3 %.

Figure 4.5B shows that the optimum specific growth rate for *B. toyonensis* was calculated at 0 % and was 0.094 hr^{-1}. The specific growth rate decreased with increasing NaCl concentration and no growth was observed at 10 %.

For *C.amygdalinum* (Figure 4.5C) the optimum growth rate was recorded at 2 % (0.122 hr^{-1}). Specific growth rate decreased with increasing and decreasing NaCl concentrations, reaching 0.05 hr^{-1} at 0 % and 0.034 hr^{-1} at 5 %.

For *B. niabensis* (Figures 4.5D) the optimum specific growth rate was calculated at 2% NaCl and was 0.120 hr^{-1}. No growth was observed at 0.5 % or 3 % conditions.

The specific growth rates calculated for *A.johnsonii* (Figure 4.5E) show that 0.5 % NaCl is the optimum for growth (0.112 hr^{-1}). However, at 3 % and 10 % rates of 0.045 hr^{-1} and 0.056 hr^{-1} were calculated, No growth was observed at 15 % and 20 %.
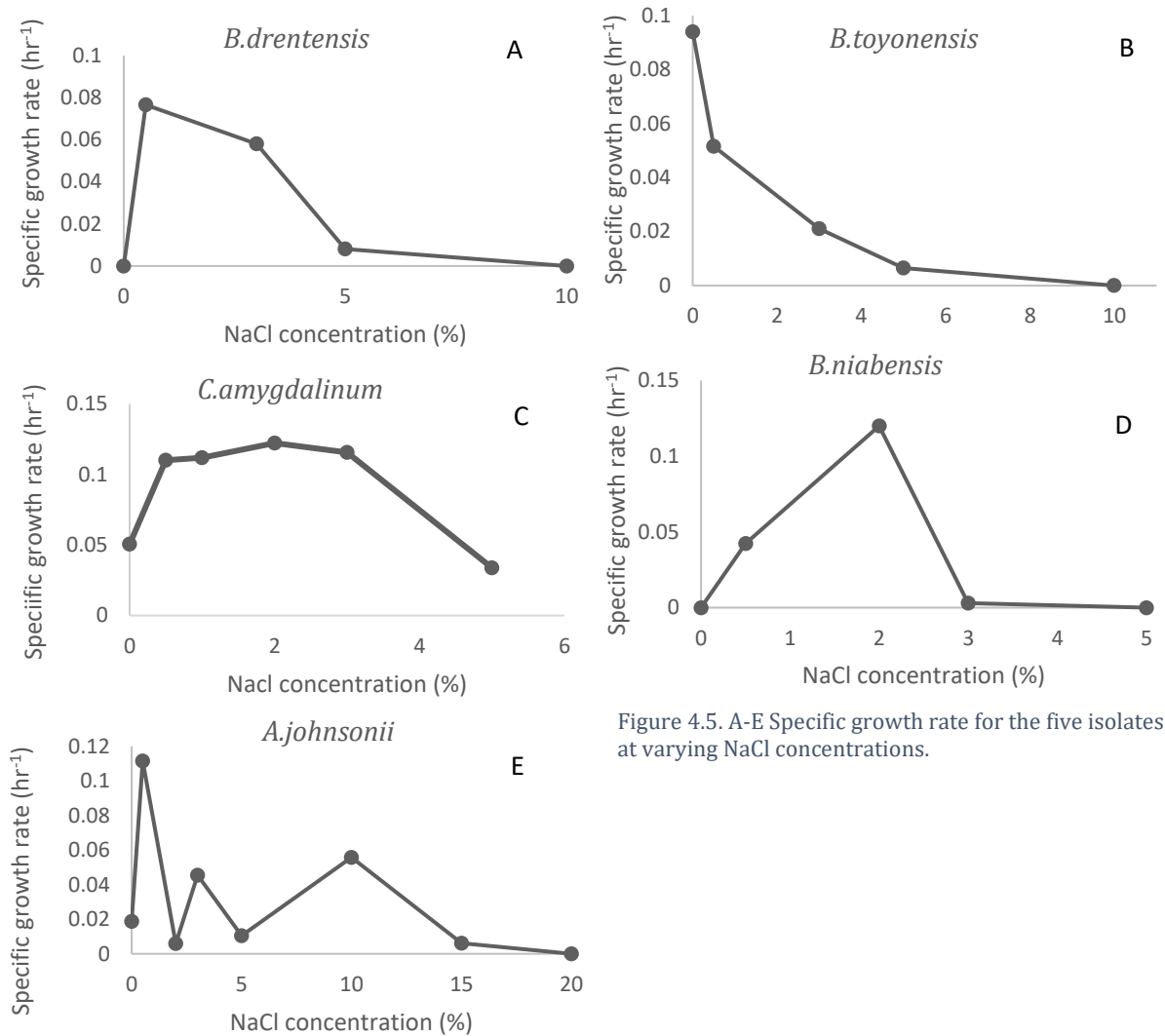
Figure 4.5. A-E Specific growth rate for the five isolates at varying NaCl concentrations.

4.3.2.5. Survival under anaerobic conditions

Mars has an approximately 96 % CO_2 atmosphere. Therefore, in consideration of the suitability of each of the isolates to potential martian environments, it was important to note where growth and/or survival are possible under anaerobic conditions.

Table 4.4 Favoured oxygenation conditions for the five isolates in this study.

Isolate	Anaerobic conditions	Aerobic conditions
B.toyonensis	Yes	Yes
B.drentensis	No	Yes
B.niabensis	No	Yes
A.jonsonii	Yes	Yes
C.amygdalinum	Yes	No

As described above, the microorganisms were incubated under both aerobic and anaerobic (N_2 atmosphere) conditions. Two isolates, *B.toyonensis* and *A.jonsonii,* were facultative anaerobes and were, therefore, able to grow under both conditions. Both *B.drentensis* and *B.niabensis* were found to be obligate aerobes, and *C.amygdalinum* was found to be an obligate anaerobe.

4.3.3. Freeze-thaw survival

Repeated cycling between above-zero and sub-zero temperatures form ice crystals in the cytoplasm of the cell causing stress. Diurnal temperature fluctuation, along with gradual shift toward a colder climate at the end of the Noachian, would have likely caused such freeze-thaw conditions to exist on Mars. The ability of the isolates in this study to survive freeze-thaw cycles was therefore investigated (Figure 4.6)**.**

A *B.toyonensis*

B *B.drentensis*

C *B.niabensis*

D *A.johnsonii*

E *C.amygdalinum*
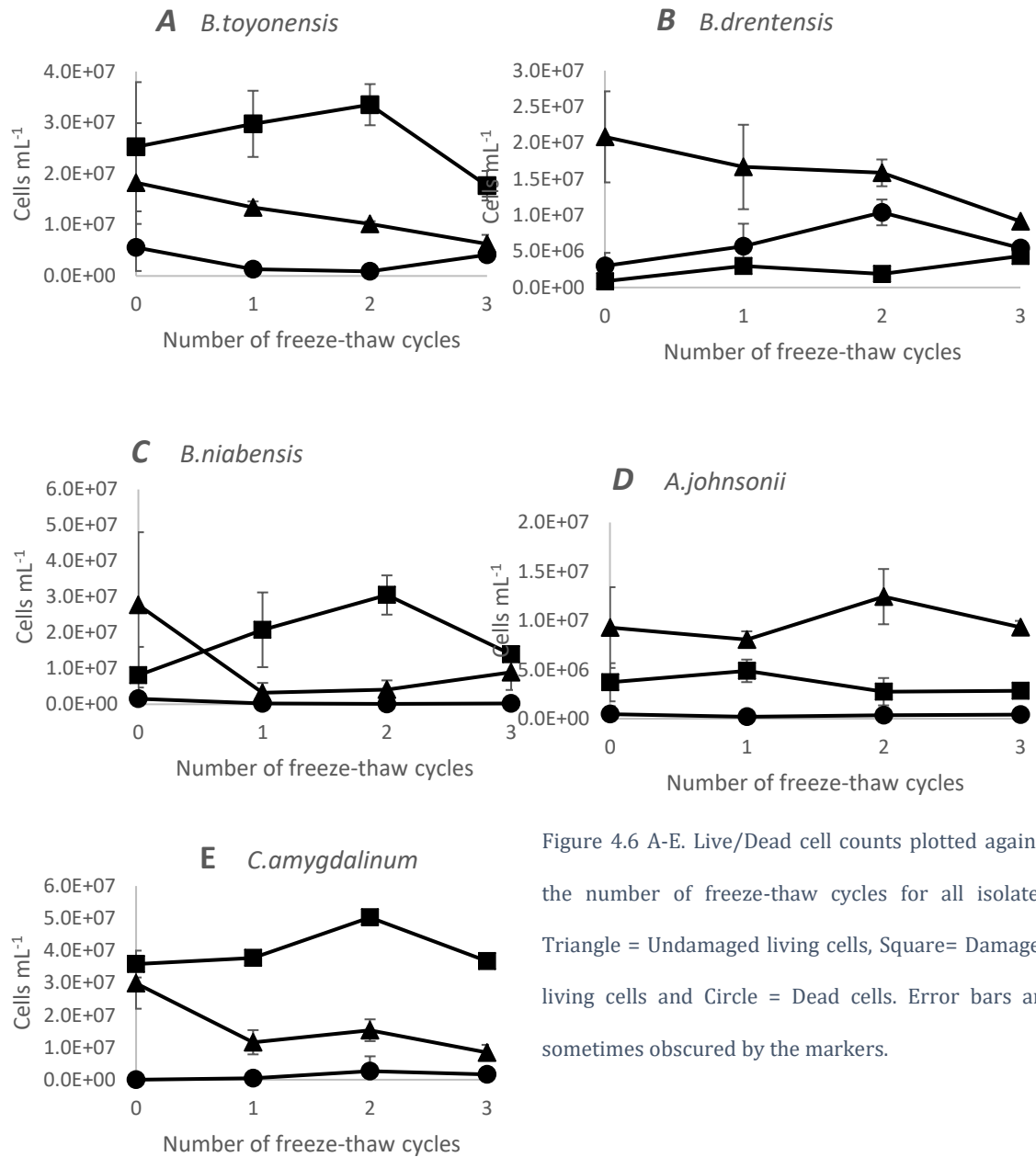
Figure 4.6 A-E. Live/Dead cell counts plotted against the number of freeze-thaw cycles for all isolates. Triangle = Undamaged living cells, Square= Damaged living cells and Circle = Dead cells. Error bars are sometimes obscured by the markers.

For all isolates, the number of undamaged living cells decreased with successive freeze-thaw cycles, except for *A. johnsonii,* where the number of undamaged living cells did not decrease considerably throughout all four freeze-thaw cycles, therefore indicating a resistance to freeze-thaw cycles.

The reduction of undamaged living cells in *B.toyonensis, B.niabensis* and *C.amygdalinum,* from approximately $1.8 \times 10^7 - 6.2 \times 10^6$ cells mL^{-1} , $2.8 \times 10^7 - 8.9 \times 10^6$ cells mL^{-1} and $2.9 \times 10^7 - 8.4 \times 10^6$ cells mL^{-1} respectively, corresponded with an

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increase in the detection of damaged cells during the first and second freeze-thaw cycles, from $2.5 \times 10^7 - 3.4 \times 10^7$, $8.0 \times 10^7 - 3.1 \times 10^7$ and $3.6 \times 10^7 - 5.0 \times 10^7$ cells respectively. However, after the third cycle the number of dead cells increased and the number of damaged living cells corresponded with an increase the number of dead cells detected. In these isolates, successive freeze-thaw cycles seemingly caused cell damage and eventual death.

Freeze-thaw cycles appeared to affected *B.drentensis* more than the other isolates, with an approximately 16 % reduction in undamaged living cells after three freeze-thaw cycles (compared to a reduction of 3 % in *B. toyonensis*, the second highest among the isolates). A drop in undamaged living cells after each successive freeze-thaw cycle appeared to correspond to an increase in the number of dead cells detected. The number of damaged cells did not increase significantly over the three freeze-thaw cycles.

4.3.4. Determination of carbon source utility by BIOLOG tests

31 carbon sources were tested using the Biolog plates but only 13 of them were utilised by the isolates in this study (Table 4.5).

The different carbon sources utilised by the five isolates had several similarities and there were few carbon sources uniquely utilised by a specific isolate. All five isolates were able to utilise Tween 40, and four isolates were able to utilise Tween 80 and L-asparagine (excluding *B.toyonensis* and *C.amygdalinum* respectively). γ-Hydroxybutyric Acid (HBA*) and itaconic acid were only utilised by all three *Bacillus* isolates, and 4- hydroxy benzoic acid (HBA) was utilised by *B. toyonensis, B. niabensis* and *A. johnsonii*. L-Serine and PAME were utilised by *B. toyonensis* and *B. drentensis*.

Carbon sources that were utilised by a single isolate were: phenylalanine by *A.johnsonii*; D-xylose by *C.amygdalinum*; L-threonine and glycly- L- glutamic acid (GGA) by *B.toyonensis;* and D- galacturonic acid (GLA) by *B.drentensis.*

Table 4.5 Specific growth rates for different carbon sources utilised by the five isolates used in this study.

	L-asparagine	Tween 40	Tween 80	L-serine	γ-HBA*	4-HBA	Itaconic acid	Phenyl alanine	D-xylose	PAME	L-threonine	GGA	D-GLA*
B.toyonensis	0.088	0.043	-	0.046	0.076	0.027	0.046	-	-	0.039	0.032	0.041	-
B.drentensis	0.115	0.062	0.051	0.066	0.129	-	0.052	-	-	0.047	-	-	0.107
B.niabensis	0.078	0.064	0.55	-	0.110	0.070	0.058	-	-	-	-	-	-
A.jonsonii	0.048	0.075	0.056	-	-	0.014	-	0.021	-	-	-	-	-
C.amygdalinum	-	0.050	0.018	-	-	-	-	-	0.032	-	-	-	-

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4.4. Discussion

The identification of a biological analogue for putative martian life would allow researchers to constrain life and biomarker detection efforts, as well as better identify target environments for life detection missions. Terrestrial microorganisms that can survive and grow within simulated martian environments give essential insight into potential microbe-environment interactions in the target martian habitat because their interactions with the system may model those of the target habitat. Thus, it was important to follow the microbial weathering experiment in Chapter 3 with an attempt to isolate and comprehensively characterise the bacteria from within the microbial community such that their suitability as a martian analogue organisms could be discussed.

4.4.1. Metabolism

The five microorganisms isolated in this study represent two types of metabolism: chemoorganotrophy and chemolithotrophy. Chemoorganotrophic organisms require organic carbon sources for growth and therefore, alone, are unlikely to be good candidates for pure culture communities on Mars, where the likely organic carbon source (meteorites) is unreliable because of unpredictable and limited infall, and concerns around bioavailability. Despite this unreliability, primary production on early Mars could have developed by utilising amino acids or carboxylic acids, known to be present in some meteorites (Yuen *et al.*, 1984; Cronin *et al.*, 1993; Botta and Bada, 2002; Sephton, 2002; Cooper *et al.*, 2011; Cobb and Pudritz, 2014), providing the necessary initial carbon source to begin primary production. Therefore, as part of a mixed microbial community, chemoorganotrophs are good candidates for putative martian life.

Four amino acids are among the 13 organic carbon sources utilised by the five isolates in this study. Of these, serine and threonine are thought to have been present on the early Earth, and phenylalanine and asparagine are essential to life on Earth (Cobb and Pudritz, 2014). If conditions on Noachian Mars are to be replicated with regard to modelling the action of potential microbial life, bacterial species capable of utilising carbon sources that were likely present would lend credence to any analogue system. The ability of *B.toyonensis* and *B.drentensis* to utilise amino acids likely present on early Earth (Cobb and Pudritz, 2014), makes them especially relevant to metabolism on early Mars.

Usually heterotrophic organisms exist within an ecosystem where some form of autotrophy exists, e.g. chemolithoautotrophy. However, in the presence of sufficient external organic carbon sources, heterotrophs could conceivably establish an ecosystem without autotrophy, though its longevity would be uncertain because of the reliance upon external carbon flux. Therefore, the heterotrophic microorganisms isolated in this study are relevant, both as pioneer species, and as part of a microbial community.

Another form of metabolism on Mars could be chemolithotrophy. *C.amygdalinum*, isolated in this study, has been shown to be chemolithotrophic (Parshina *et al.*, 2003) and, since most chemolithotrophs are autotrophs (Amils, 2011), it may be that *C.amygdalinum* is also an autotroph. Fine-grained sedimentary rocks discovered by the MSL Curiosity Rover at Gale Crater indicated the presence of an ancient circumneutral, lacustrine environment and is thought to have been suitable for a martian biosphere founded on chemolithoautotrophy (Grotzinger *et al.*, 2014). Thus, the applicability *C.amygdalinum* to such ancient martian environments is heightened with the isolation of a potentially chemolithoautotrophic bacterium with growth optima that are well

aligned with environmental conditions at Gale Crater (i.e. obligate anaerobe, 14.8 °C, pH 7 and 1 - 2 % NaCl growth optima).

As discussed in Chapter 2, there may have been a variety of carbon sources available for potential microbial life in the ancient lacustrine environment at Gale Crater, e.g. meteorite organic matter (Flynn, 1996; Sephton, 2012) and aromatic hydrocarbons (e.g. 150 - 300 ppbw chlorobenzene) have been tentatively identified in analyses of the Sheepbed mudstone (Freissinet *et al.*, 2015, 2016). In addition, several carbon sources have been detected in martian fines sampled at the Rocknest location, Gale Crater (Leshin, Mahaffy, Webster, Cabane, Coll, Conrad, Archer, Atreya, Brunner, a Buch, *et al.*, 2013). However, the ability to utilise aromatic hydrocarbons was not specifically investigated in this study, though phenylalanine, an aromatic amino acid, was included in the Biolog Ecoplate and was utilised by *A.johnsonii* only.

Microbes that are only able to utilise a small number of organic carbon sources are likely to have been selected against in oligotrophic early martian environments. Therefore, among putative martian microbial communities, high versatility with regard to carbon source utilisation would likely be prevalent. Therefore, since the bacteria isolated in this study are able to utilise a range of carbon sources, their status as potential biological analogues is further supported.

4.4.2. Optimum growth conditions

Characterisation of the optimum growth conditions and the associated limits of growth provided useful insight into the suitability of each isolate to martian environments, since there may be an overlap. As with Chapters 2 and 3, the focus of these investigations was the ancient lacustrine environment at Gale Crater. Comparisons

made between the proposed conditions in the ancient lacustrine system at Gale Crater

and growth limits of the five isolates yielded the results shown in Table 4.6.

Table 4.6. A comparison of growth optima and limits for survival of the five isolates with environmental conditions thought to have been present in the ancient lake at Gale Crater. † = (Heyrman *et al.*, 2004).

Mars ancient lake conditions	11-15 °C	Circumneutral-slightly basic pH	Anaerobic CO_2 atmosphere	Low salinity (NaCl) (1-2 %)
Clostridium amygdalinum	High specific growth rate at 14.8 °C	Optimum pH 7	Obligate anaerobe	Optimum growth rate at 1 - 2 %
Acinetobacter johnsonii	Slow growth rate	Good growth between pH 7 and 9	Obligate aerobe	10 % optimum, low growth below 5 %
Bacillus drentensis	Slow growth rate	Optimum 7 - 7.5	Facultative anaerobe †	Optimum at or below 3 %
Bacillus toyonensis	Optimum 14.8 - 21 °C	Optimum at pH 10. Slower growth at 7	Facultative anaerobe	No growth above 5 %. 0.5 – 2 % optimum
Bacillus niabensis	Optimum 21 °C. No growth at 14.8 °C	Optimum 7 - 7.5	Facultative anaerobe	Optimum 2 %

Extended lag phases under certain conditions were observed, unsurprisingly where successful growth of the bacteria was close to its limit. Langenheder et al. (2006) described two explanations for an extended lag phase:

1. Environmental conditions are such that there is a very small effective inoculum, with many cells no longer viable after inoculation.

2. A very long time is required for the bacteria to adapt to the new environmental conditions.

In both explanations, the extended lag phase is caused by a shock response, the of which length is usually dependent on the extent to which environmental factors vary between the inoculum environment and the experimental one (Ghaly *et al.*, 1989). Also, Tango & Ghaly (1999) found that increasing the temperature towards the optimum for the species in question decreased the duration of the lag phase. With regard to Mars, this phenomenon could represent the exposure of potential microbial communities to rapid variations in environmental conditions associated with geological phenomena, e.g. bolide impacts.

Abramov and Mojzsis (2009) investigated microbial habitability during the LHB and showed that even with a 100-Myr-long LHB, global habitable volumes were significant even for mesophiles. Furthermore, even when the geothermal temperature gradient was doubled, microbial life was not extinguished. Therefore, despite the extreme temperatures produced during bolide impacts, on Mars putative martian communities could potentially be aided by the temperature increase at distance from the site of impact.

For this study, optical density measurements were used to determine cell density, therefore distinguishing between viable and non-viable cells was not possible. A drop in viable cells shortly after inoculation would be required to determine which of Langenheder *et al.*, (2006)'s explanations for the extended lag period is most likely. It is assumed that, within species variation would be insufficient to support option one within these pure cultures, and therefore where species were able to adapt to the extreme environmental conditions they were considered to have been able to inhabit such an environment.

4.4.3. Other phenotypes

The presence of the MPS phenotype in the isolates was expected to be prevalent since the laboratory analogue system in Chapter 3 had only mineralogical sources of phosphate stored within phosphate-containing minerals in the regolith analogue. Therefore, access to the bioessential mineral was limited by the dissolution of those minerals. Microorganisms that could solubilise phosphate from the substrate would have been able to increase phosphate concentrations in the medium and provide a source of phosphorus for itself and other microbial species.

Previous studies have used the MPS plate technique described above (Section 4.2.4) to identify the weathering capacity of individual bacterial isolates, thereby elucidating two pieces of information. Firstly, a qualitative assessment of whether the isolate possesses the MPS phenotype; and secondly, if the size of the "halo" can be measured accurately, it provides a quantitative means of comparing the weathering efficacy of isolates. In this study the latter was not possible, since an accurate means of measuring the halos was not readily available.

The MPS phenotype would also be likely to be present in putative martian microbial life since only mineralogical sources of phosphorus have presently been identified on Mars. Thus, putative martian microorganisms would be forced to rely on the dissolution of phosphate-bearing minerals for nutrition. For example, phosphorus has been identified in the samples taken from the Rocknest sand shadow, and likely represents phosphorus bound in crystalline phases (Blake *et al.*, 2013). This would likely be a limited source of nutrients for microorganisms that could not actively solubilise it such minerals, or were not closely associated with microorganisms that could.

Phosphorus dissolution from basalt has been shown to be increased significantly by microbial weathering (Wu *et al.*, 2007b). The presence of this phenotype within a microorganism indicates its ability to weather rock substrates, removing phosphate through solubilisation. Therefore, the presence of this phenotype in a putative martian microbial community would be advantageous. Mineral phosphate weathering on Mars could be a key factor in the bioavailability of phosphorus, a key bioessential mineral. Microorganisms capable of solubilising phosphate from the regolith may be able to provide nutrition for other microorganisms, thereby potentially setting up a community.

Endospores are dormant forms of bacteria with distinct structural and biochemical characteristics (Piggot and Coote, 1976) that are formed when bacteria are subjected to environmental stressors (Voelker *et al.*, 1995). Environmental conditions on Mars have changed dramatically throughout its geological history (see Carr & Head (2010) for a review). Conditions are believed to have become drier in the late Noachian (Andrews-Hanna and Lewis, 2011), although evidence of clay mineral formation suggests sustained pH neutral surface water into the Early Hesperian (Grotzinger *et al.*, 2014). Environmental changes on Mars would likely mean that microorganisms capable of endospore formation would have greater survivability since spores have been shown to have dramatically increased resistance to environmental stressors when compared to living bacterial cells (Nicholson *et al.*, 2000; Liebert, 2005; Fajardo-Cavazos *et al.*, 2009).

Temperature decreases, both diurnal (Martín-Torres *et al.*, 2015) and long-term (Carr and Head, 2010), would likely have constrained early subsurface life on Mars, limiting it to those that can survive and/or grow in temperatures that may have fluctuated to below zero. Therefore, the ability to survive through freeze-thaw cycles would likely be beneficial in ancient habitable environments on Mars.

Among the five isolates, discussed further below, microbial metabolisms, growth and survival conditions, tolerance to environmental stressors and other likely useful phenotypes relevant to the palaeolake at Gale Crater, have been identified. Thus, the application of these isolates to studies of putative martian life may be appropriate.

4.4.4. Isolates

To discuss the suitability to the isolates from this study as biological analogues for putative martian life it is necessary to evaluate them individually. The case for each isolate is discussed hence.

4.4.4.1. *Acinetobacter jonsonii*

Acinetobacter jonsonii type strain CIP 64.6 was previously isolated by Bouvet & Grimont (1986) from clinical specimens and eviscerated chickens and from frost-plant-samples collected in China (Li *et al.*, 2012; Tian *et al.*, 2016). It is a Gram-positive species with diplococci cell morphology.

Experimental characterisation of this isolate indicated optimum growth rate at pH 9 and 37 °C, preferring relatively high salt concentrations of 10 % with little tolerance for change. Interestingly, Bouvet & Grimont (1986) found that growth could not be sustained at 37 °C, and selection pressures within the microbial weathering experiment carried out in Chapter 3 would not have helped develop a tolerance for higher than usual temperatures. Therefore, these findings suggest a deviation from the expected growth conditions of *A.johnsonii* and may indicate that the strain isolated in this study is different to the strains used by Bouvet and Grimont (1986).

This species was isolated aerobically in media designed to isolate nitrate reducing bacteria, and subsequently plated out onto agar of the same composition. Efforts to grow this isolate anaerobically were unsuccessful, thus categorising it as an obligate aerobe. Although the *Acinetobacter* genus belongs to the *Gammaproteobacteria* class, and TRFLP data presented in Chapter 3 show several unresolved *Gammaproteobacteria* groups, it is unlikely that this species ever accounted for a considerable portion of the experimental microbial community. It is likely that it merely survived in a dormant state in the media until it was inoculated into more favourable conditions, though it does not form spores (Bouvet and Grimont, 1986a).

The optimal and growth conditions set out in Table 4.6, indicate that *A.jonsonii* would be unlikely to survive within the ancient martian lake system at Gale Crater. Firstly, and perhaps most significantly, it is obligately aerobic and would die quickly without an oxygenated atmosphere. It also requires higher temperatures and salt concentrations than evidenced on ancient Mars. However, resistance of *A.jonsonii* to both desiccation and freeze-thaw cycles is suggestive of its suitability as an analogue when investigating processes involving the diurnal freezing and desiccation in fluctuating martian environments.

A.jonsonii was also found to be unable to solubilise mineral phosphate from its environment and it does not form spores. Of the five isolates it was able to utilise the second lowest number of carbon sources. The main constituent of Tween 40 is palmitic acid, a common fatty acid found in microorganisms, animals and plants, and this was the most effective carbon source for *A.jonsonii*. The ubiquity of palmitic acid in nature may explain its prevalence as a key carbon source in all five isolates. The amino acid, phenylalanine, was uniquely metabolised by *A.jonsonii*. Amino acids are often the first compound class associated with life (Sephton and Botta, 2006) and the ability to utilise

amino acids may inform hypotheses that constrain metabolic activity for early putative martian life.

4.4.4.2. *Clostridium amygdalinum*

Among the five isolates, the *Clostridium amygdalinum* is the most promising candidate for a biological analogue for putative martian life. Characterised by Parshina et al. (2003) this species reportedly grows optimally at 45 °C and at pH 7 - 7.5. However, the experimental characterisation of this isolate determined optimum growth conditions at 30 °C (with no growth recorded above 37 °C), and pH of 7. Since the microbial weathering experiment in Chapter 3 was carried out at 14.8 °C and previous stocks of the microbial community had been stored and grown at that temperature for approximately 1 year, it is likely that the low temperature drove selection toward individuals with lower growth optima. Its requirement to avoid temperatures above 37 °C is likely a side effect of this.

Interestingly Parshina et al. (2003) reported that this species is aerotolerant. This is incongruous with the lack of growth observed in aerobic conditions during plating and media based attempts in this study. The NaCl concentration growth optimum at 2 %, and significant growth at 0.5 % and 3 % is relevant to marine environments and low salinity lacustrine environments like that of Yellowknife Bay, Gale Crater (Grotzinger *et al.*, 2014; S. McLennan *et al.*, 2014).

C.amygdalinum was isolated within a modified LB medium with tryptone and yeast extract providing sources of organic carbon. Parshina *et al.*, (2003) found that *C.amygdalinum* required yeast extract for growth, with benzaldehyde, sulfite, H_2 and thiosulfate being used as electron donors. The main products of metabolism were found to be H_2, CO_2 and acetate.

This species is also known to form spores in the absence of a nitrogen source in the medium/environment (Parshina *et al.*, 2003) and this process could have helped preserve it in the subsurface of ancient martian lakebeds when environmental and nutritional conditions fluctuated.

4.4.4.3. *Bacillus toyonensis*

This species has previously been isolated from a community of soil-dwelling saprophytes for use in medical research by Jimenez et al. (2013). It was characterised to distinguish it from *Bacillus cereus* of which it had originally been assumed to be. It was shown to be facultatively anaerobic which is in keeping with this research since growth, though reduced, was observed when this isolate was inoculated into an anaerobically prepared medium. Growth optima were found to be at 35 °C, pH 6.5 and a 5 % NaCl concentration. The above data are roughly congruous with Jimenez et al., (2013) However, a lower optimum temperature, 30 °C, was found to coincide with significant reduction in specific growth rate when temperature was increased to 35 °C. Cold storage conditions in the previous experiments likely facilitated this selective process.

The growth limits of this species are congruous with that of the ancient lake system at Gale Crater, and though it is only facultatively anaerobic it would be able to survive and perhaps adapt to purely anaerobic conditions.

4.4.4.4. *Bacillus drentensis*

Data collected by Heyrman et al. (2004) support the identification of this isolate, reporting growth optima at 30 °C and pH 7 - 8. Minimum growth was observed both in

this study and in their work, at around pH 5 - 6. This study did not determine the maximum temperature for growth, but the decreasing trend above 30 °C is suggestive of a growth limit at around 50 °C. In addition, the production of a reddish-brown pigment matches the characterisation made by Heyrman et al. (2004) to confirm the presence of *B.drentensis*.

As a facultative anaerobe favouring circumneutral pH conditions, and low NaCl concentrations, only the slow growth at 14.8 °C is problematic with regard to its suitability as a biological analogue.

4.4.4.5. *Bacillus niabensis*

The above characterisation of *Bacillus niabensis* matches closely that of Kwon et al. (2007) who demonstrated optimum growth at 30 °C, pH 6 - 8 and NaCl growth range of 0-5 % (w/v). These growth optima are congruent with the ancient martian lake system in question in all but two factors. Firstly, growth at low temperatures (15 °C or less) was not observed and though evidence supports facultatively anaerobic growth, the growth rate was considerably reduced under anaerobic conditions.

4.4.5. Sources of life on Mars

The applicability of the above isolates as biological analogues for putative martian life depends primarily on the origins of such life, of which there are two main options:

1. Lithopanspermia based colonisation
2. Independent origin of life on Mars

These two theories were discussed in detail in Chapter 1, however they are relevant to this discussion.

Lithopanspermia-based origin, where life originated on Earth and then colonised Mars *via* impact ejecta is considered possible (Mileikowsky, 2000), with evidence suggesting the survival of spores in hypervelocity launch scenarios (Fajardo-Cavazos *et al.*, 2009). In this case, terrestrial facultative anaerobes, e.g. *B.drentensis*, *B.niabensis* and *B.toyonensis*, may have been able to successfully colonise aqueous habitats on ancient Mars. Given that the martian atmosphere is considered to always have been primarily CO_2 (Kahre *et al.*, 2013; Mahaffy *et al.*, 2013), it is unlikely that facultative anaerobes would have evolved independently, with the environment favouring anaerobic microorganisms. It is plausible that an active hydrological cycle may have produced pockets of oxygen in the martian subsurface (Boston *et al.*, 1992), potentially reanimating aerobic, spore-forming bacteria, or provided a location for aerobic and facultative anaerobic microorganisms to evolve. These facultative anaerobic isolates could therefore be used as a means of understanding the potential trajectory of the evolution of life on Mars and the potential biomarkers that such life may have left behind.

Though this situation is possible, it is more plausible that anaerobic microorganisms would have originated on Mars. Thus, *C.amydalinum*, with its high level of congruence with ancient martian environmental data, and an obligately anaerobic nature, could be used as an informative tool to investigate processes associated with ancient martian life. Furthermore, data presented in Chapter 3 demonstrate that this *Clostridium* dominated in the martian analogue environment used in that experiment. Evidence clearly suggests that *Clostridium* would be capable of growing and potentially dominating microbial communities in ancient martian lake systems.

4.5. Conclusions

This study aimed to demonstrate the applicability of terrestrial microbes as a biological analogue for putative martian life in the ancient lake environment at Gale crater, and potentially other similar environments on Mars.

Terrestrial species were isolated from a system designed to replicate martian conditions and thus these isolates represent species selected for by such environmental conditions. Characterisation suggested that their growth optima were altered under the selective pressures of the martian analogue environment, further supporting their applicability to studies of past martian life.

Five species were isolated from this system, and, though they all have some congruency with potential habitats on Mars, *C.amygdalinum* stands out as a model organism for such systems. Future microbial weathering experiments utilising this isolate alone could elucidate potential biomarker production; the mixed community used in Chapter 3 prevented such findings because of likely metacommunity interactions. These findings could therefore be used to support efforts in life detection on Mars, both in ancient and, with consideration of evolutionary trajectories, contemporary environments.

Chapter 5- Summary and conclusions

5.1. Discussion of findings and limitations

This thesis characterises a new environmental analogue for use in studies of potential life on Mars. This site, a subsurface estuarine environment, is analogous to the ancient habitable lacustrine environment at Yellowknife Bay, Gale Crater. Microorganisms isolated from the subsurface of the Dee estuary were used as biological analogues to investigate the limits of potential life on early Mars and potential biosignatures that could be used as evidence of past life.

5.1.1. Environmental analogue

Chapter 2 of this thesis focused on establishing the subsurface (below the RPD) of the River Dee estuary, UK as a new terrestrial analogue of the ancient lacustrine environment at Gale Crater, Mars, and thus was subject to the assumption that aqueous terrestrial environments are similar to their martian counterparts. Therefore, making the simulation and analysis of these environments plausible and meaningful. Gale Crater was chosen as an important site to investigate and mimic because recent studies using data collected by the MSL Curiosity Rover have deemed the environment potentially habitable (e.g. Grotzinger et al. 2014).

The hypothesis posed was that measured environmental parameters at the River Dee estuary would be similar to those of the palaeolake at Yellowknife Bay. Environmental data were collated and compared to that collected *in situ* on Mars. The River Dee sample site was found to be (approximately) pH 8, at 12 °C and with 1 % TOC (see Chapter 2 Table 2.3 for details). This was found to be similar to conditions measured at Gale Crater by the MSL Curiosity Rover, where the detection of clays indicated a

circumneutral pH (Vaniman *et al.*, 2014), evidence of long lasting aqueous conditions indicated above zero temperatures (e.g. Williams et al. 2013; Grotzinger et al. 2014; McLennan et al. 2014; Martín-Torres et al. 2015) and the TOC was suggested to be 2400 ppm (0.24 %) (B Sutter *et al.*, 2016). Further, mineralogical comparison made discussion of the provision of bioessential element availability at the two sites possible (see Section 5.1.2 below).

The data in Chapter 2 showed that there is an undeniable similarity between the River Dee Estuary and the palaeolake at Yellowknife Bay, Gale Crater. This comparison was made with regard to the habitability of both environments, and thus the habitability of Gale Crater for terrestrial microorganisms could be investigated. Results from the experiments in Chapter 3 indicated that the microbial community from the sub-surface of the Dee estuary can survive under simulated conditions associated with the ancient lacustrine environment at Gale Crater, and are therefore applicable as biological analogues for past life on the planet (see Section 5.1.3 below). However, pore size distribution, permeability, subsurface pressure and seasonal/diurnal temperature or pH variations were not tested in this study and, the specific sources of organic carbon were not identified. These parameters are important for microbial metabolism (see Chapter 4) and attachment (Fredrickson *et al.*, 1997), and impact the types of microorganism that can survive within an environment (e.g. Harrison et al. 2013). Therefore, there are further parameters yet to be ascertained and compared when additional data are obtained *in situ* at Gale Crater.

5.1.2. Elemental bioavailability

During this study, the results from mineralogical characterisation of the River Dee analogue site were compared with the results from Gale Crater. The sediment

contained approximately 23 % clays, 9 % sanidine, 5 % haematite (and other minor fractions) and 63 % quartz (See Chapter 2 Section 2.3.1). Aside from quartz, which even if dissolved does not liberate bioessential elements, the samples analysed at Gale Crater by the MSL Curiosity rover (e.g. the John Klein and Cumberland drill holes) had 22 % and 18 % clay respectively, with approximately 40 % of plagioclase feldspar and minor fractions accounting for the remainder. These minerals contain bioessential elements, and the concentrations of which are similar in the River Dee Estuary and Gale Crater.

Although bulk elemental compositions were measured for the River Dee sediments to compare with Gale Crater measurements, the bioavailability of these elements was not determined. Thus, even though Gale Crater ostensibly has a large source of Na and Ca in plagioclase feldspar, Fe in haematite, Fe-forsterite and magnetite and other bioessential elements in the amorphous component (e.g. P, Mg, S (Pontefract *et al.*, 2012)), these elements are not necessarily available to potential microorganisms. Future work should focus on distinguishing bioavailable nutrients from those unavailable for biological action.

5.1.3. Biological analogue

When the microbial community was grown in the Mars analogue experimental system, Acidobacteria Gp9 dominated, with Rhodobiaceae, Gammaproteobacteria, Gemmatimonadaceae and Caldilineaceae making up significant portions of the community at varying times during the experiment (Chapter 3). In comparison, of the five species isolated from this community, only *A.johsonii*, a Gammaproteobacteria, represented one of these dominant groups. Future work would focus on isolating the

Acidobacteria Gp9, in addition to identifying and isolating the species representing the Caldilineaceae Rhodobiaceae and Gemmatimonadaceae families.

Mars simulation experiments have been carried out utilising similar bacteria as those isolates in this study, e.g. the gammaproteobacteria *E.coli* (Berry *et al.*, 2010), *B.subtilis* and *Acinetobacteria radioresistens* (Fajardo-Cavazos *et al.*, 2008), but, to date, there has been no effort to isolate microorganisms from a mixed community that survived and grew in simulated martian conditions. In this study (Chapters 3 and 4), a Mars analogue system was designed and bacterial isolates were obtained from the microbial community at the end of the experiment. In addition to the diverse microbial community evidenced by TRFLP data, investigation of the optimal growth/survival conditions of the isolates showed that the Mars system supported bacteria with diverse metabolic and environmental requirements.

A true biological analogue would be able to survive (ideally thrive) in the exact conditions determined to be present at the target martian environment, in this case the subsurface of the palaeolake at Yellowknife Bay. Therefore, an accurate model system could be constructed, utilising the Mars simulation chambers to provide accurate environmental conditions. A model of this kind would facilitate detailed insight into potential microbial processes in that environment and potentially leading to the formation of biosignatures.

The identification of biosignatures in model environments is better suited to a pure culture community as it would be easier to discern the impact of the specific microorganism on the system. This could be at the cost of reducing the accuracy of the model, depending on the means through which life arose on Mars, and the time from such an origin to the incidence of habitable conditions at Gale Crater.

Since approximately 99 % of bacteria are considered uncultivable (e.g. Kaeberlein et al. 2002; Rappé & Giovannoni 2003), it is assumed that pure microbial cultures do not exist environmentally. Thus, if life arose on Mars through abiogenesis and there was sufficient time for evolution and divergence to occur before or during the period of habitable conditions at Gale Crater, then it is safe to assume that a putative microbial community there would be a mixed community such as was identified in Chapter 3. However, if life on Mars arose as a result of a lithopanspermia event, then the inoculation of martian environments with a pure culture may be possible, owing to the likely highly selective nature of interplanetary travel (e.g. as a result of the radiation environment (Olsson-Francis *et al.*, 2009; De la Torre *et al.*, 2010)).

The model system designed in Chapter 3 was constructed to be as accurate a representation of potentially habitable subsurface martian environments as experimental constraints would allow. This included the use of a regolith analogue compositionally similar to the palaeolake at Yellowknife Bay. However, one significant constraint was the closed experimental system; an open system would have been more representative of the target martian environment. The microbial community utilised in Chapter 3 were forced to live within a system where the products of all biotic and abiotic processes (e.g. bacterial metabolism and weathering/dissolution) remained in their immediate vicinity, impacting the chemistry of the system in a way that would likely not be the case in an open environmental system. This will have placed constraints upon the microbial community so that it was not entirely representative of the martian environment.

However, any open experimental system has limited applicability until the nutrient cycling processes in the ancient habitable environment at Gale Crater is known. Nevertheless, this experiment provided a plausible means of investigating microbial community dynamics and succession, while also providing insight into processes of

microbially-mediated dissolution. An understanding and application of the results of this study could be used to inform further experimentation to identify specific biomarkers and aid instrument design for future life detection missions.

From this community, five distinct bacterial species were isolated (Chapter 4) and their growth limits and optima were studied with regards to their likely survival in the palaeolake at Yellowknife Bay. For this, the community was exposed to freeze-thaw cycles and variations in pH, temperature and salinity. Of the five species, one, *Clostridium amygdalinum*, stood out as a potential biological analogue because it is obligately anaerobic, in accord with Mars' CO_2 atmosphere, and its pH, temperature and salinity optima are compatible with the ancient environment at Gale Crater.

5.1.4. Limitations

It is prudent to mention a limitation of the molecular techniques used to identify the above isolates, and track the microbial community in Chapter 3. Investigations of microbial diversity rely heavily upon extracting the 16S rRNA gene from the microbial community (e.g. Santelli et al. 2009; Janssen 2006; Whiteley et al. 2012; Olsson-Francis et al. 2016), which causes a bias since the diversity of 16S rRNA genes provides a measure of the potential diversity rather than the diversity of functioning microorganisms. This is because DNA fragments containing the 16S rRNA gene can survive after cell death and lysis, even persisting for thousands of years in their environment (Nielsen *et al.*, 2007). These fragments may subsequently be amplified during PCR alongside DNA extracted from living microorganisms, therefore giving an inaccurate picture of microbial diversity.

Therefore, since microbial diversity was measured at several stages throughout the microbial weathering experiment using TRFLP, it is possible that DNA fragments from bacterial groups that had already declined in population size, skewed diversity data for subsequent sampling periods.

Furthermore, since it is estimated that only 0.1 % of bacteria have been cultured (Aslam *et al.*, 2010), it is possible that selection biases were input into the cultivation and isolation techniques used.

The taxonomic resolution obtained by DNA sequencing (used in Chapter 3) is generally low, with few taxa being identified beyond Class level, and rarely reaching Genus or Species level. This is also true for other community profiling analyses and this limitation is common in microbial diversity studies (Nocker *et al.*, 2007). To address this, next-generation sequencing (MiSeq) was used which provided a larger sequence database by which taxonomic identities could be inferred. These large databases provide greater confidence in attributing taxa to TRFLP fragments.

In this thesis, the use of MiSeq sequencing had inconsistent success. For some taxa, it was possible to resolve to Family level, and in some cases Genus and Species. However, for some taxa that are less well described, e.g. Acidobacteria, and for some extremely abundant taxa, e.g. Proteobacteria, identification was limited to Class or even Phylum level resolution. Nevertheless, it was still possible to carry out a broad examination of the bacterial communities and, where resolution was to Genus or Species level, a detailed examination was possible. With future developments in the size of gene libraries, this type of analysis could provide higher resolution with much greater consistency.

5.1.5. Biosignatures

If evidence of ancient life on Mars is to be detected it is likely to be based on the discovery of biosignatures, indicators of uniquely biological activity (Chapter 1 Section 1.6). As discussed in Chapters 1 and 3, there are several types of biosignature that range from secondary alteration minerals and metabolic products to microfossils and microbial cell structures. The formation of these biosignatures is dependent upon the system in question, and is therefore determined by the types of microorganism inhabiting the system and its environmental and chemical characteristics.

To model biosignature production in subsurface environments at Gale Crater, a microbial weathering experiment was carried out within an environmentally analogous system. The production and characterisation of biosignatures in this experimental system would then be used to suggest potential biological processes on Mars and inform life detection strategies.

The microbial weathering experiment in Chapter 3 was designed to promote the interaction of the microbial community with the Mars analogue substrate. Therefore, it was hypothesised that, with adequate abiotic controls to allow for abiotic background corrections, it would be possible to identify unique chemical or mineralogical signatures of the microbial community during analysis. Such signatures may be small scale, but, owing to the presumed dominance of microbial processes in biological conditions, there could also be a bulk change in Mars analogue substrate composition. XRD and FEG-SEM analyses were used to test these hypotheses.

With the exception of potential biofilm, the results of this experiment did not reveal any uniquely biological phenomena, either because the analyses were not of sufficient resolution (e.g. XRD analysis, see Chapter 3) or because of the complexity of the system, i.e. where other microbial weathering experiments focused on single species (e.g. (Wu

et al., 2007b; L. Wu *et al.*, 2008; Olsson-Francis *et al.*, 2012), this study used a mixed community consisting of tens of different microbial groups. This meant that changes in system chemistry were not directly attributable to specific microorganisms, and, since the metabolic pathways utilised by every microorganism present was not known, it was not possible to predict and accurately discern biotic and abiotic processes. Furthermore, syntrophy-like interactions, whereby metabolic products for one species act as an energy source for another, may have obscured or removed any unique biological products as they were forming.

However, ICP-AES data did reveal a pattern of elemental release within the biological samples that coincided with the peak cell density, but these large-scale changes are not reliable signs of purely biological activity and so could not be used as biosignatures. Further, biofilms have limited longevity, so they are not ideal targets as biosignatures on future life detection missions.

A *prima facie* evaluation of the experiment would suggest that the method used should have properly tested the hypothesis; however, retrospectively, there are changes that would have facilitated a more comprehensive investigation and determined with more certainty whether geochemical biosignatures in this system could be detected against the abiotic background (see Section 5.2.2).

The mixed microbial community was an ideal analogue community for martian life; however, the system chemistry was too complex and the microbial community too diverse to allow high resolution investigation of the chemical system and the associated microbial processes. Further experiments are needed using the isolates, to enable an in-depth understanding of the likely metabolic pathways and formation of secondary alteration minerals in the Mars analogue regolith.

Although the complexity of the system made chemical analyses problematic, molecular techniques did indicate patterns in microbial diversity and community structure that provided information regarding potential ecological succession processes on Mars, especially with regards to the colonisation of a habitable martian environment by a diverse community of microorganisms.

5.2. Future work to address research aims

5.2.1. Further characterisation of the River Dee analogue environment

In Chapter 2 the subsurface environment of the River Dee estuary was comprehensively characterised such that it was possible to make a clear comparison to the ancient lacustrine environment at Gale Crater. Among several other parameters, Total Organic Carbon was measured; however, as demonstrated in Chapter 4, microorganisms are only able to utilise certain organic carbon sources (see BIOLOG data, Chapter 4 Section 4.3.3). Therefore, an understanding of the particular organic carbon sources available at the River Dee site could provide a greater understanding of microbial metabolism there. This would allow further and more accurate discussion of its applicability to ancient martian environments.

5.2.2. Clarification of microbial weathering processes and biosignature production

The experimental procedure carried out in Chapter 3 was designed such that it would be most relevant to putative martian microbial communities. However, in ensuring that

the experimental procedure was an accurate reflection of potential environmental systems, this impacted the ability to resolve and identify purely biotic processes. In particular, it was not possible to attribute any observed phenomena to a specific species.

It is possible that the actions of individual microbial species were obscured by the actions of others. Therefore, future experiments could be set up identically but with individual species isolated from the mixed community (including those isolated in Chapter 4), inoculated into the system. This could make biosignatures easier to identify.

Furthermore, to isolate microbial action more effectively, larger pieces of analogue substrate could be added to the system. These could be scored, producing a trench on the mineral surface, to provide sites for microbial attachment and analysed using the FEG/FIB-SEM before being added to the system. Element maps and Point & ID analysis would be carried out, as well as morphological analysis, to comprehensively characterise the substrate surface. These analyses could be carried out again after the experiment and detailed comparisons would therefore be possible. A replicate of this experiment would also be carried out over a larger time scale, such that any weathering processes are given appropriate time to have noticeable impact on the experimental system.

To inform any inferences of microbial metabolism within the experimental system, volatile fatty acid (VFA) analysis could be carried out throughout, with samples taken such that any changes related to microbial growth phases are apparent. In addition, Reverse transcriptase- PCR could be carried out to monitor gene expression. This would provide information on the processes being carried out by the microorganisms at the time of sampling.

5.2.3. Further characterisation of isolates

In Chapter 4, five isolates were characterised and discussed with regards to their applicability to further astrobiological investigations, for example (as discussed above), their use in studies of microbial weathering and potential biosignatures for life detection on Mars. To further constrain the specific martian environments that the isolates may be suitable for, it would be necessary to carry out full phylogenetic characterisation, potentially identifying possible metabolisms and informing hypotheses regarding potential biosignature formation.

Furthermore, their accurate application to martian environments may require further environmental characterisation and testing of their response to additional stressors, for example, to low pressure environments or to the presence of perchlorates. In addition, the present characterisation of tolerance to desiccation and freeze-thaw cycles should be extended to cover much longer periods of desiccation (6 - 12 months), to include cycles of desiccation and rehydration on small (diurnal) and large (monthly) scales and to similarly include investigations of resistance to long term freezing periods and resistance to more freeze thaw cycles. Quantification of the ability to solubilise mineral phosphate could also be carried out during further investigations.

To improve the significance of the conclusions of the martian analogue experiments the level of congruence to the target environment must be high. Thus, the more comprehensive the characterisation of the environment, the stronger the potential analogy to putative martian life, and the more reliable the conclusions. These experiments may purport to inform future life detection efforts, therefore their credibility as analogues is extremely significant.

5.3. Conclusion

This thesis has demonstrated that the subsurface environment of the River Dee Estuary is a meaningful environmental analogue of the ancient fluvio-lacustrine environment at Gale Crater, Mars. By extension the microbial community that lives there has been suggested to be relevant to comparisons of putative martian life, and as such, the community was grown under simulated martian conditions, with findings supporting this claim. Five bacterial species were isolated from this experimental martian environment and have been characterised to demonstrate their applicability to future studies of potential microbial processes on Mars. Of these five isolates, *C.amygdalinum*, has been demonstrated to have significant relevance to models of putative martian life. Therefore, future investigations into potential microbial processes on Mars are informed and facilitated by the improved understanding of microbial succession processes in ancient martian environments, and the environmental and biological analogues presented in this thesis.

Appendix A- Pilot experiments

The below graphs display data collected during pilot experiments carried out prior to undertaking the experiment in Chapter 3. Data from these pilot studies was used to refine the characteristics of the experimental setup.
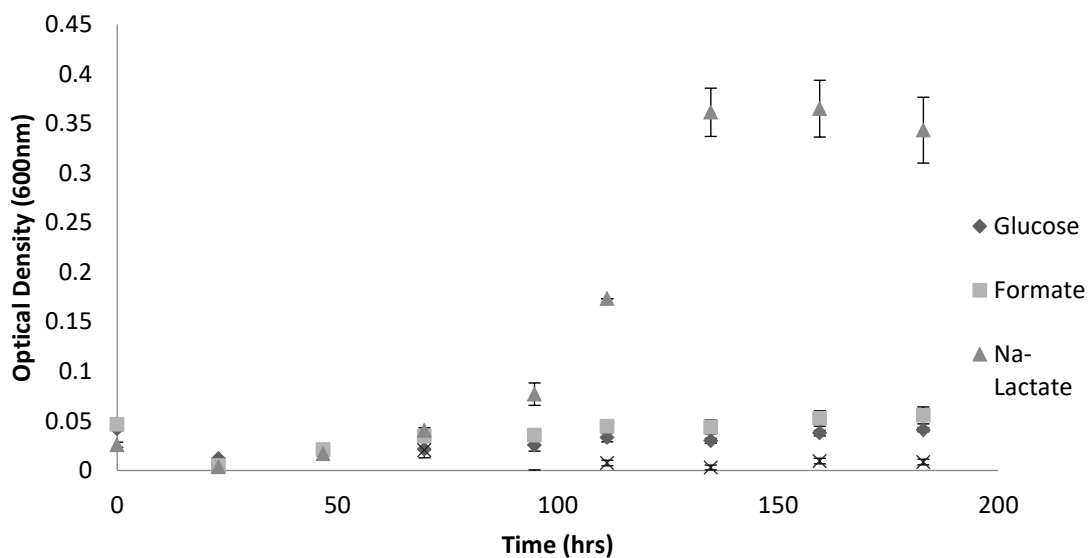
Figure A-1. Optical density at 600 nm measured over time for enrichment media containing different sources of organic carbon. These were compared to an abiotic control to ensure that contamination had not occurred. Error bars denote the standard error of measurement from 3 replicates.
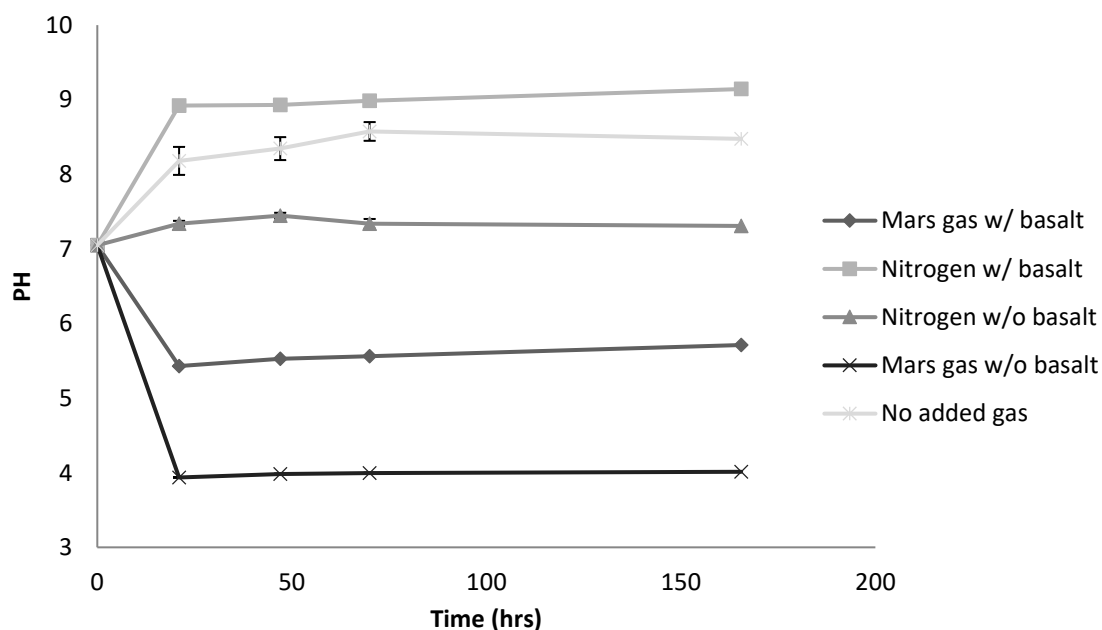
Figure A-2. pH variations over time demonstrated for five different compositions of headspace gas/ basalt substrate. Error bars denote the standard error of measurement from 3 replicates.
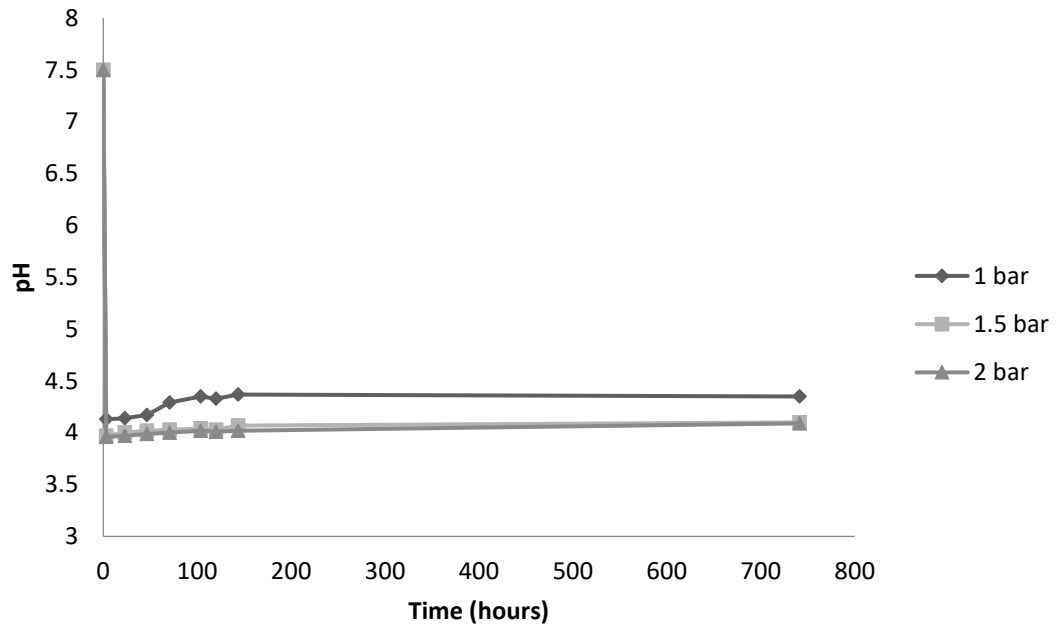
Figure A-3. pH variation in minimal media over time demonstrated for three different pressures of Mars gas headspace. Error bars are obscured by markers.
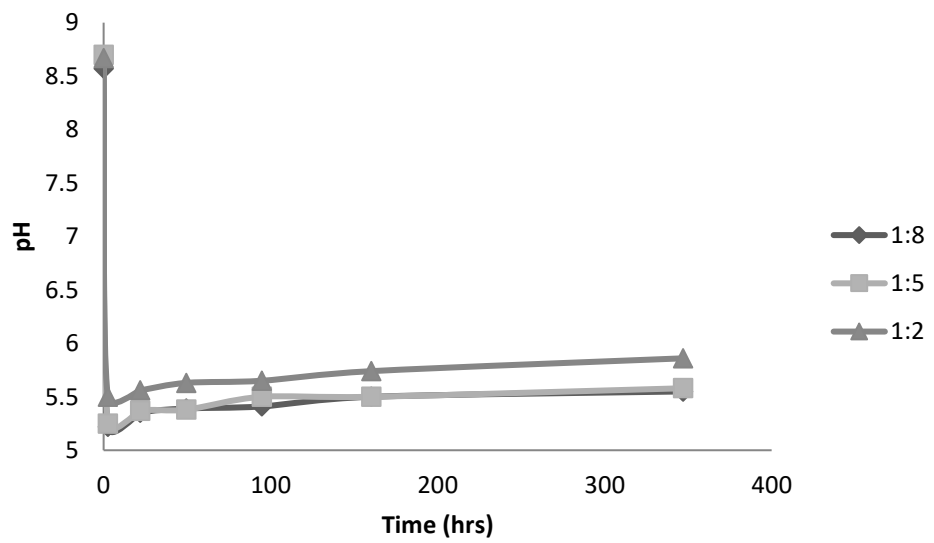
Figure A-4. pH variation in experimental media over time using three different medium : rock ratios. Error bars are obscured by markers

Appendix B- EMPA data

Aegirine- Mt Malosa, Malawi

Table A-1. EMPA data for aegirine showing wt% oxide.

Wt% Oxide

Formula	SO3	P2O5	SiO2	TiO2	Al2O3	Cr2O3	MgO	CaO	MnO	FeO	NiO	Na2O	K2O	Total
1 / 1 .	0.02	0.01	50.78	0.85	0.27	0.04	0.13	1.14	0.53	29.28	0.02	12.26	0.00	95.31
2 / 1 .	0.02	0.02	51.82	0.80	0.72	0.00	0.09	1.21	0.59	29.45	0.00	12.10	0.00	96.81
3 / 1 .	0.04	0.01	51.47	0.78	0.26	0.01	0.10	1.04	0.52	29.29	0.02	12.09	0.00	95.63
4 / 1 .	0.02	0.01	51.22	0.79	0.19	0.00	0.09	1.09	0.55	29.61	0.06	12.22	0.00	95.84
5 / 1 .	0.02	0.00	51.42	0.73	0.28	0.01	0.10	0.81	0.46	29.35	0.01	12.78	0.00	95.97
6 / 1 .	0.02	0.00	51.96	0.72	0.26	0.00	0.08	0.87	0.51	29.72	0.00	12.76	0.00	96.89
7 / 1 .	0.02	0.01	52.14	0.81	0.27	0.01	0.08	0.85	0.51	29.59	0.00	12.45	0.00	96.72
8 / 1 .	0.04	0.03	51.92	1.03	0.25	0.00	0.10	1.16	0.61	29.15	0.00	12.27	0.00	96.55
9 / 1 .	0.03	0.03	52.17	0.89	0.24	0.00	0.08	1.23	0.64	29.22	0.00	12.77	0.00	97.29
10 / 1 .	0.04	0.00	52.12	0.84	0.22	0.01	0.07	1.27	0.58	29.34	0.00	12.41	0.00	96.90
11 / 1 .	0.02	0.00	51.77	0.80	0.23	0.05	0.07	1.24	0.54	29.59	0.00	12.17	0.00	96.45
12 / 1 .	0.06	0.00	52.19	0.91	0.27	0.00	0.06	1.04	0.53	29.17	0.00	12.41	0.00	96.63
13 / 1 .	0.01	0.01	51.68	1.11	0.24	0.00	0.02	1.14	0.56	29.36	0.00	12.50	0.00	96.62
14 / 1 .	0.03	0.05	51.54	1.07	0.21	0.00	0.02	1.13	0.60	29.08	0.01	12.28	0.00	96.02
15 / 1 .	0.04	0.00	52.13	1.11	0.22	0.01	0.05	1.12	0.59	29.58	0.02	12.24	0.00	97.10
16 / 1 .	0.02	0.02	51.52	1.18	0.22	0.02	0.03	1.13	0.56	29.23	0.01	12.20	0.00	96.13
17 / 1 .	0.01	0.00	51.52	1.16	0.21	0.02	0.04	1.14	0.58	29.07	0.00	12.21	0.00	95.97
18 / 1 .	0.04	0.00	51.25	1.18	0.21	0.02	0.03	1.11	0.56	29.11	0.00	12.48	0.00	96.00
19 / 1 .	0.02	0.00	50.76	1.23	0.23	0.04	0.05	1.14	0.55	29.51	0.00	12.20	0.00	95.73
20 / 1 .	0.03	0.06	50.89	1.33	0.21	0.00	0.04	1.34	0.59	28.89	0.02	12.06	0.00	95.45
21 / 1 .	0.01	0.00	49.96	1.14	0.22	0.00	0.05	1.30	0.59	29.23	0.00	12.20	0.00	94.69

22 / 1 .	0.03	0.00	51.06	1.04	0.18	0.01	0.04	1.53	0.54	29.31	0.03	12.17	0.00	95.94
23 / 1 .	0.04	0.01	50.09	1.00	0.17	0.00	0.04	1.47	0.49	29.09	0.00	11.97	0.00	94.36
24 / 1 .	0.04	0.02	49.78	0.96	0.18	0.00	0.03	1.46	0.50	29.49	0.00	11.98	0.00	94.44
25 / 1 .	0.05	0.00	50.63	1.08	0.20	0.01	0.02	1.39	0.57	29.45	0.01	12.46	0.00	95.87
26 / 1 .	0.06	0.00	51.26	1.06	0.16	0.00	0.04	1.59	0.53	29.28	0.01	11.98	0.00	95.97
27 / 1 .	0.03	0.00	52.85	1.01	0.20	0.03	0.04	1.37	0.46	29.26	0.01	12.04	0.00	97.29
28 / 1 .	0.03	0.01	51.79	0.91	0.22	0.00	0.05	1.19	0.52	29.42	0.00	12.63	0.00	96.76
29 / 1 .	0.03	0.00	51.53	0.78	0.22	0.00	0.07	1.34	0.57	29.49	0.03	12.70	0.00	96.74
30 / 1 .	0.05	0.00	51.95	0.98	0.20	0.03	0.05	1.31	0.59	29.05	0.02	12.36	0.00	96.59
31 / 1 .	0.02	0.00	52.14	0.96	0.25	0.00	0.06	1.27	0.57	29.67	0.01	12.68	0.00	97.61
32 / 1 .	0.04	0.00	52.70	0.93	0.23	0.01	0.03	1.32	0.59	29.30	0.00	12.43	0.00	97.57
33 / 1 .	0.05	0.05	53.01	0.94	0.24	0.00	0.03	1.17	0.58	29.19	0.02	12.35	0.00	97.63
34 / 1 .	0.03	0.02	51.19	1.06	0.22	0.00	0.03	1.40	0.58	29.00	0.00	12.19	0.00	95.71
35 / 1 .	0.03	0.03	51.30	0.87	0.22	0.00	0.04	1.55	0.60	29.03	0.00	11.94	0.00	95.60
36 / 1 .	0.05	0.00	52.51	1.00	0.19	0.02	0.05	1.68	0.68	29.17	0.00	12.25	0.00	97.59
37 / 1 .	0.01	0.00	52.80	0.38	0.18	0.00	0.03	0.17	0.04	30.72	0.01	12.88	0.00	97.22
38 / 1 .	0.01	0.00	52.96	1.02	0.13	0.01	0.05	2.36	0.83	28.78	0.00	11.46	0.00	97.61
39 / 1 .	0.04	0.01	52.75	1.11	0.13	0.07	0.03	2.32	0.81	28.88	0.00	11.94	0.00	98.08
40 / 1 .	0.04	0.00	53.06	0.97	0.13	0.00	0.05	2.46	0.77	28.75	0.00	11.80	0.00	98.02
41 / 1 .	0.02	0.00	53.14	1.03	0.12	0.00	0.08	2.38	0.87	29.04	0.00	11.77	0.00	98.45
42 / 1 .	0.03	0.02	53.29	0.98	0.12	0.04	0.06	2.31	0.89	28.88	0.00	12.10	0.00	98.70
43 / 1 .	0.04	0.02	53.39	1.00	0.14	0.01	0.07	2.32	0.80	28.96	0.00	11.82	0.00	98.58
44 / 1 .	0.03	0.00	53.41	0.93	0.13	0.00	0.07	2.33	0.83	28.71	0.02	11.85	0.00	98.29
45 / 1 .	0.02	0.00	51.62	0.95	0.13	0.00	0.08	2.36	0.77	28.91	0.03	11.37	0.00	96.23
46 / 1 .	0.02	0.05	51.66	1.07	0.12	0.00	0.06	2.30	0.87	28.83	0.00	11.84	0.00	96.81
47 / 1 .	0.04	0.00	51.91	0.87	0.12	0.00	0.06	1.95	0.82	28.83	0.04	12.17	0.00	96.81
48 / 1 .	0.03	0.00	52.18	0.99	0.13	0.02	0.05	2.15	0.81	29.12	0.06	11.63	0.00	97.18

49 / 1 .	0.05	0.06	51.95	1.02	0.13	0.00	0.07	2.11	0.80	29.01	0.00	12.16	0.00	97.37
50 / 1 .	0.03	0.00	52.44	1.10	0.12	0.00	0.05	2.10	0.86	28.55	0.00	12.12	0.00	97.40
51 / 1 .	0.02	0.00	52.33	1.02	0.12	0.02	0.06	2.10	0.88	28.77	0.01	12.10	0.00	97.42
52 / 1 .	0.04	0.03	52.24	1.04	0.14	0.02	0.03	2.06	0.85	28.60	0.01	11.75	0.00	96.81
53 / 1 .	0.04	0.00	52.31	1.02	0.13	0.00	0.08	1.99	0.83	28.96	0.02	11.98	0.00	97.35
54 / 1 .	0.04	0.00	52.47	0.96	0.12	0.00	0.06	2.03	0.84	28.79	0.00	11.77	0.00	97.09
55 / 1 .	0.02	0.01	52.78	0.89	0.26	0.01	0.03	0.93	0.54	29.76	0.00	12.98	0.00	98.21
56 / 1 .	0.02	0.00	53.24	0.96	0.27	0.02	0.05	1.05	0.55	29.55	0.00	12.77	0.00	98.45
57 / 1 .	0.05	0.00	52.73	0.94	0.25	0.00	0.06	1.09	0.55	29.16	0.00	12.58	0.00	97.40
58 / 1 .	0.04	0.03	52.89	0.88	0.25	0.01	0.06	0.97	0.47	29.27	0.00	12.44	0.00	97.31
59 / 1 .	0.02	0.00	53.10	0.82	0.27	0.01	0.05	1.04	0.48	29.22	0.00	12.51	0.00	97.51
60 / 1 .	0.02	0.01	53.00	0.89	0.28	0.00	0.07	1.00	0.50	29.42	0.00	12.60	0.00	97.79
61 / 1 .	0.01	0.00	53.18	0.78	0.26	0.00	0.06	0.86	0.52	29.57	0.00	12.57	0.00	97.81
62 / 1 .	0.05	0.04	53.02	0.87	0.25	0.00	0.06	0.79	0.49	29.43	0.00	12.82	0.00	97.83
63 / 1 .	0.02	0.00	52.76	0.88	0.27	0.02	0.10	0.95	0.50	29.19	0.00	12.60	0.00	97.29
64 / 1 .	0.04	0.00	53.34	0.61	0.20	0.00	0.09	1.18	0.57	29.97	0.00	12.05	0.00	98.05
65 / 1 .	0.03	0.00	53.08	0.72	0.13	0.00	0.11	2.12	0.84	28.84	0.02	11.91	0.00	97.80
66 / 1 .	0.03	0.00	53.40	0.68	0.13	0.00	0.09	2.04	0.86	28.91	0.00	11.94	0.00	98.08
67 / 1 .	0.04	0.01	53.38	0.80	0.12	0.01	0.10	2.04	0.88	29.21	0.01	11.86	0.00	98.45
68 / 1 .	0.04	0.00	51.96	0.76	0.13	0.00	0.11	1.95	0.84	29.10	0.01	11.99	0.00	96.89
69 / 1 .	0.04	0.01	52.40	0.73	0.13	0.03	0.10	1.89	0.77	28.83	0.00	11.79	0.00	96.72
70 / 1 .	0.03	0.00	51.87	0.78	0.13	0.00	0.10	1.59	0.77	29.08	0.01	12.05	0.00	96.41
71 / 1 .	0.02	0.00	52.38	0.84	0.12	0.01	0.09	1.56	0.75	29.25	0.00	12.16	0.00	97.16
72 / 1 .	0.03	0.06	52.34	0.77	0.12	0.00	0.11	1.57	0.76	29.09	0.00	12.22	0.00	97.07
73 / 1 .	0.03	0.00	52.08	0.76	0.13	0.03	0.10	1.70	0.69	29.34	0.00	12.24	0.00	97.09
74 / 1 .	0.02	0.01	52.28	0.78	0.12	0.00	0.10	1.67	0.79	28.74	0.02	12.17	0.00	96.69
75 / 1 .	0.02	0.03	52.27	0.73	0.13	0.02	0.10	1.56	0.76	29.27	0.02	11.89	0.00	96.79

76 / 1 .	0.02	0.03	52.23	0.86	0.13	0.00	0.11	1.65	0.71	29.43	0.01	12.29	0.00	97.47
77 / 1 .	0.02	0.00	53.66	0.88	0.14	0.00	0.11	1.73	0.78	29.51	0.00	12.36	0.00	99.19
78 / 1 .	0.03	0.00	53.58	0.87	0.13	0.00	0.10	1.78	0.84	29.45	0.02	12.24	0.00	99.04
79 / 1 .	0.01	0.00	53.18	0.83	0.12	0.02	0.08	1.73	0.84	29.06	0.00	11.98	0.00	97.83
80 / 1 .	0.02	0.01	52.93	0.91	0.11	0.00	0.11	1.81	0.81	29.07	0.03	12.07	0.00	97.87
81 / 1 .	0.04	0.00	53.11	0.76	0.14	0.00	0.09	1.86	0.76	29.16	0.00	12.09	0.00	98.01
82 / 1 .	0.03	0.00	52.88	0.82	0.11	0.01	0.09	1.84	0.82	29.00	0.00	12.23	0.00	97.83
83 / 1 .	0.03	0.01	53.09	0.79	0.13	0.01	0.08	1.87	0.83	29.00	0.03	11.91	0.00	97.77
84 / 1 .	0.04	0.00	53.17	0.81	0.12	0.00	0.08	1.84	0.80	29.40	0.00	12.23	0.00	98.52
85 / 1 .	0.01	0.00	53.07	0.80	0.13	0.00	0.08	1.84	0.85	29.30	0.04	12.27	0.00	98.38
86 / 1 .	0.03	0.01	53.55	0.87	0.13	0.02	0.08	1.87	0.79	29.03	0.02	12.15	0.00	98.55
87 / 1 .	0.03	0.00	53.68	0.91	0.13	0.00	0.09	1.89	0.88	29.25	0.04	12.11	0.00	98.99
88 / 1 .	0.03	0.00	52.48	0.74	0.13	0.00	0.09	1.92	0.82	29.09	0.00	11.73	0.00	97.02
89 / 1 .	0.03	0.00	52.45	0.74	0.14	0.00	0.09	1.90	0.79	29.25	0.02	11.96	0.00	97.37
90 / 1 .	0.04	0.00	52.21	0.85	0.13	0.01	0.07	1.90	0.82	28.84	0.00	11.99	0.00	96.86
91 / 1 .	0.04	0.00	52.90	0.68	0.13	0.06	0.10	1.81	0.79	29.02	0.00	11.95	0.00	97.48
92 / 1 .	0.02	0.00	51.49	0.76	0.12	0.00	0.07	1.99	0.87	29.07	0.01	11.75	0.00	96.16
93 / 1 .	0.02	0.03	53.24	0.76	0.20	0.00	0.06	1.41	0.70	29.44	0.00	12.30	0.00	98.16
94 / 1 .	0.01	0.00	52.96	0.84	0.23	0.05	0.07	1.19	0.65	29.02	0.00	12.58	0.00	97.59
95 / 1 .	0.01	0.00	53.86	0.88	0.23	0.00	0.06	0.99	0.54	29.56	0.00	12.53	0.00	98.65
96 / 1 .	0.02	0.00	53.71	0.83	0.23	0.01	0.06	0.92	0.46	29.80	0.00	12.89	0.00	98.93
97 / 1 .	0.01	0.00	53.74	0.81	0.23	0.00	0.06	1.04	0.49	29.47	0.01	12.66	0.00	98.52
98 / 1 .	0.02	0.00	53.60	0.79	0.21	0.03	0.08	1.18	0.48	29.16	0.00	12.50	0.00	98.05
99 / 1 .	0.02	0.03	52.51	0.56	0.18	0.00	0.09	1.11	0.50	29.30	0.00	12.48	0.00	96.78

Basalt- Le Cheix, Puy de dome, France

Table A-2. EMPA data for basalt showing wt% oxide

Wt% Oxide

Formula	P2O5	SiO2	SO2	TiO2	Al2O3	Cr2O3	MgO	CaO	MnO	FeO	NiO	Na2O	K2O	Total
1 / 1 .	0.33	37.25	0.00	0.13	0.20	0.00	33.77	0.83	0.81	26.50	0.00	0.03	0.00	99.85
2 / 1 .	0.38	37.24	0.03	0.35	0.53	0.00	33.44	2.41	0.72	23.74	0.00	0.10	0.00	98.94
3 / 1 .	0.26	36.89	0.00	0.05	0.05	0.01	34.99	0.66	0.80	25.36	0.00	0.05	0.00	99.12
4 / 1 .	4.46	32.30	0.00	0.07	0.14	0.01	28.87	5.90	0.86	26.81	0.00	0.07	0.05	99.53
5 / 1 .	0.23	36.55	0.00	0.11	0.17	0.01	33.94	0.59	0.80	26.80	0.00	0.05	0.00	99.26
6 / 1 .	0.20	36.59	0.01	0.22	0.24	0.00	33.97	1.31	0.79	25.82	0.01	0.06	0.00	99.22
7 / 1 .	2.37	34.66	0.00	0.23	0.08	0.01	31.65	3.23	0.84	26.68	0.02	0.01	0.00	99.78
8 / 1 .	0.05	43.32	0.00	3.10	8.81	0.00	11.19	23.02	0.10	8.23	0.00	0.53	0.00	98.36
9 / 1 .	0.00	43.22	0.01	3.04	8.78	0.01	11.24	23.01	0.13	8.21	0.00	0.49	0.00	98.13
10 / 1 .	0.05	45.93	0.00	2.84	7.07	0.00	11.97	21.79	0.21	7.91	0.00	0.72	0.25	98.74
11 / 1 .	0.17	43.32	0.03	4.25	8.74	0.00	11.18	22.45	0.16	8.13	0.00	0.53	0.02	98.98
12 / 1 .	0.38	41.76	0.00	4.18	9.91	0.00	10.92	22.07	0.17	9.15	0.00	0.57	0.01	99.12
13 / 1 .	0.11	41.52	0.00	4.64	10.06	0.00	10.40	22.40	0.15	8.79	0.00	0.76	0.10	98.93
14 / 1 .	0.09	45.95	0.00	3.16	7.54	0.00	12.02	22.34	0.20	7.81	0.00	0.61	0.21	99.94
15 / 1 .	0.06	44.50	0.02	3.00	8.35	0.01	11.77	23.25	0.11	7.89	0.02	0.46	0.01	99.46
16 / 1 .	0.14	44.94	0.00	3.21	7.33	0.01	12.29	22.49	0.17	8.71	0.01	0.39	0.02	99.72
17 / 1 .	0.03	46.54	0.00	2.07	6.17	0.01	13.00	23.19	0.14	7.31	0.01	0.43	0.00	98.88
18 / 1 .	0.01	45.77	0.01	2.22	6.34	0.02	12.18	22.70	0.18	8.27	0.00	0.50	0.00	98.21
19 / 1 .	0.04	46.80	0.00	1.96	5.56	0.04	12.63	22.94	0.16	8.00	0.00	0.42	0.00	98.54
20 / 1 .	0.16	44.87	0.01	3.02	6.98	0.00	12.25	22.32	0.20	8.41	0.00	0.42	0.01	98.64
21 / 1 .	0.15	42.95	0.00	4.03	9.13	0.00	11.22	22.43	0.16	8.55	0.01	0.58	0.07	99.28

22 / 1 .	2.33	46.93	0.00	2.50	10.53	0.00	8.48	18.79	0.17	6.70	0.01	2.22	1.79	100.46
23 / 1 .	1.40	35.83	0.00	0.05	0.01	0.00	32.66	1.56	0.88	27.45	0.00	0.04	0.00	99.85
24 / 1 .	0.08	46.00	0.00	3.20	7.02	0.00	12.29	22.83	0.20	8.07	0.00	0.56	0.01	100.26
25 / 1 .	4.77	40.08	0.00	2.80	6.57	0.01	10.60	25.84	0.17	7.05	0.00	0.54	0.03	98.46
26 / 1 .	0.03	51.14	0.00	0.14	29.30	0.01	0.05	13.09	0.00	0.66	0.00	4.20	0.26	98.89
27 / 1 .	0.06	51.38	0.00	0.18	29.31	0.01	0.09	12.79	0.01	0.88	0.01	4.30	0.30	99.33
28 / 1 .	0.08	51.41	0.00	0.20	28.53	0.01	0.07	12.09	0.01	0.78	0.00	4.26	0.37	97.81
29 / 1 .	0.09	51.63	0.01	0.18	28.63	0.00	0.13	11.80	0.02	0.91	0.00	4.50	0.67	98.56
30 / 1 .	0.04	50.96	0.02	0.16	28.73	0.00	0.09	12.54	0.00	0.82	0.01	4.17	0.28	97.82
31 / 1 .	1.04	44.02	0.01	3.79	7.68	0.00	11.38	23.06	0.17	7.86	0.01	0.57	0.03	99.62
32 / 1 .	0.07	44.70	0.00	3.52	7.76	0.02	11.87	22.63	0.16	7.82	0.00	0.70	0.02	99.26
33 / 1 .	0.03	50.91	0.00	0.29	28.81	0.00	0.15	12.76	0.00	1.13	0.02	4.20	0.51	98.81
34 / 1 .	0.07	51.08	0.00	0.14	29.05	0.00	0.08	12.80	0.01	1.04	0.01	4.02	0.26	98.56
35 / 1 .	0.00	0.07	0.00	20.23	4.07	0.05	3.25	0.08	0.97	66.77	0.00	0.04	0.00	95.52
36 / 1 .	0.33	0.11	0.00	18.97	3.64	0.03	2.67	0.95	1.01	66.28	0.00	0.03	0.00	94.00
37 / 1 .	0.00	0.08	0.00	18.65	5.15	0.05	4.14	0.13	0.86	66.20	0.00	0.04	0.00	95.28
38 / 1 .	0.03	0.08	0.00	19.13	4.92	0.03	4.07	0.04	0.87	66.05	0.00	0.02	0.00	95.24
39 / 1 .	0.00	0.07	0.00	19.09	4.92	0.03	4.07	0.05	0.91	66.77	0.02	0.00	0.00	95.93
40 / 1 .	0.00	0.06	0.00	19.24	4.62	0.05	3.52	0.04	0.92	66.85	0.00	0.00	0.00	95.31
41 / 1 .	0.00	0.07	0.00	17.72	5.84	0.07	4.85	0.02	0.73	66.05	0.00	0.06	0.00	95.40
42 / 1 .	0.02	0.07	0.00	17.36	5.93	0.06	4.55	0.00	0.72	66.06	0.00	0.00	0.00	94.77
43 / 1 .	0.01	43.48	0.00	3.15	9.03	0.00	11.44	22.82	0.11	8.41	0.00	0.48	0.00	98.94
44 / 1 .	0.13	44.33	0.00	2.90	8.56	0.03	11.48	22.97	0.14	8.12	0.00	0.53	0.00	99.19
45 / 1 .	0.02	45.51	0.01	2.61	7.33	0.01	12.35	22.92	0.13	7.68	0.00	0.48	0.00	99.04
46 / 1 .	0.03	46.07	0.00	2.23	5.98	0.01	11.75	22.89	0.26	9.35	0.01	0.63	0.00	99.18
47 / 1 .	0.01	0.07	0.00	18.20	5.11	0.04	3.89	0.06	0.82	66.21	0.00	0.00	0.00	94.41
48 / 1 .	0.06	46.62	0.00	2.07	5.87	0.01	12.46	22.81	0.16	8.14	0.01	0.53	0.00	98.72

49 / 1 .	0.12	44.10	0.00	2.76	7.35	0.02	12.04	22.46	0.15	8.67	0.00	0.39	0.00	98.06
50 / 1 .	0.06	46.49	0.00	2.66	6.20	0.00	12.98	22.93	0.14	8.03	0.00	0.35	0.00	99.84
51 / 1 .	0.02	0.08	0.00	19.33	3.91	0.04	2.75	0.15	0.97	67.16	0.01	0.03	0.00	94.44
52 / 1 .	0.03	50.53	0.00	0.85	2.44	0.01	13.37	22.51	0.59	8.16	0.01	0.72	0.00	99.23
53 / 1 .	0.03	48.36	0.01	1.93	4.68	0.00	13.15	22.97	0.20	7.16	0.02	0.44	0.00	98.93
54 / 1 .	0.01	44.43	0.00	3.27	8.66	0.01	11.90	23.26	0.11	7.64	0.00	0.43	0.00	99.73
55 / 1 .	0.05	50.33	0.00	0.96	2.94	0.01	12.63	22.67	0.65	8.57	0.00	0.84	0.00	99.64
56 / 1 .	0.00	44.93	0.00	3.05	8.52	0.01	12.23	23.21	0.09	7.33	0.03	0.50	0.00	99.90
57 / 1 .	0.00	0.07	0.00	19.15	5.00	0.02	3.77	0.03	0.91	66.38	0.00	0.00	0.00	95.34
58 / 1 .	0.04	0.07	0.00	18.50	5.42	0.10	4.06	0.03	0.83	66.27	0.00	0.00	0.00	95.32
59 / 1 .	0.17	42.98	0.00	4.16	8.32	0.00	11.24	22.59	0.16	8.71	0.00	0.46	0.00	98.78
60 / 1 .	0.05	36.94	0.00	0.11	0.05	0.00	33.24	0.60	0.86	27.52	0.01	0.02	0.00	99.40
61 / 1 .	0.14	36.84	0.02	0.09	0.02	0.00	32.75	0.52	0.87	27.90	0.01	0.02	0.00	99.16
62 / 1 .	0.17	44.10	0.00	3.14	7.61	0.01	12.06	22.42	0.19	8.61	0.00	0.37	0.00	98.66
63 / 1 .	0.43	35.26	0.00	0.70	0.15	0.02	31.98	0.58	0.83	28.57	0.00	0.08	0.00	98.59
64 / 1 .	0.08	50.59	0.00	0.18	28.46	0.00	0.07	12.89	0.01	0.97	0.00	4.10	0.29	97.65
65 / 1 .	0.07	45.73	0.00	2.87	6.45	0.03	12.22	22.52	0.19	7.64	0.00	0.55	0.02	98.29
66 / 1 .	0.52	36.18	0.03	0.13	0.02	0.02	31.74	0.85	0.93	29.25	0.00	0.02	0.00	99.69
67 / 1 .	0.01	0.22	0.00	20.43	3.42	0.02	2.64	0.15	0.99	66.92	0.03	0.00	0.00	94.83
68 / 1 .	0.06	0.11	0.00	20.58	3.07	0.02	2.25	0.35	1.01	66.77	0.00	0.04	0.00	94.26
69 / 1 .	0.01	50.91	0.00	0.22	29.13	0.01	0.11	13.07	0.02	1.08	0.01	4.00	0.29	98.85
70 / 1 .	0.69	40.97	0.00	4.87	9.69	0.00	10.29	23.15	0.15	8.63	0.00	0.52	0.00	98.95
71 / 1 .	0.18	43.89	0.00	3.51	8.32	0.01	11.56	22.41	0.18	8.77	0.00	0.46	0.09	99.39
72 / 1 .	0.03	50.82	0.00	0.23	29.94	0.01	0.14	13.60	0.00	0.80	0.02	3.75	0.26	99.59
73 / 1 .	0.04	53.00	0.02	0.20	28.86	0.00	0.05	11.68	0.01	0.90	0.00	4.67	0.41	99.84
74 / 1 .	0.10	54.15	0.00	0.21	27.90	0.00	0.08	9.36	0.01	0.90	0.00	7.16	1.12	100.97
75 / 1 .	0.23	43.72	0.00	3.46	8.17	0.02	11.74	22.48	0.16	9.07	0.00	0.33	0.00	99.38

76 / 1 .	0.29	44.04	0.00	2.52	7.89	0.02	10.10	23.29	0.23	9.52	0.00	0.60	0.01	98.51
77 / 1 .	0.02	49.31	0.00	1.26	3.70	0.00	12.72	22.86	0.39	8.35	0.00	0.71	0.00	99.33
78 / 1 .	0.01	48.79	0.02	1.61	4.17	0.00	12.56	22.80	0.38	8.47	0.01	0.70	0.00	99.51
79 / 1 .	0.04	47.73	0.00	2.03	5.75	0.00	12.55	22.97	0.18	8.27	0.00	0.47	0.00	99.99
80 / 1 .	39.30	0.79	0.97	0.02	0.03	0.00	0.32	53.23	0.06	1.26	0.00	0.36	0.00	96.33
81 / 1 .	0.00	51.29	0.00	0.21	29.63	0.00	0.09	13.30	0.01	1.01	0.02	4.01	0.27	99.84
82 / 1 .	0.00	51.43	0.00	0.21	29.64	0.00	0.07	13.18	0.00	0.96	0.01	4.10	0.40	100.01
83 / 1 .	6.02	36.81	0.00	3.70	5.78	0.00	9.84	24.78	0.21	10.81	0.00	0.53	0.04	98.52
84 / 1 .	0.08	52.21	0.02	0.21	29.26	0.02	0.07	12.65	0.01	0.80	0.02	4.36	0.24	99.94
85 / 1 .	0.10	46.54	0.00	2.66	6.75	0.00	12.27	22.46	0.21	7.83	0.02	0.63	0.15	99.63
86 / 1 .	0.05	45.97	0.00	2.84	6.95	0.00	12.57	22.91	0.13	7.79	0.00	0.47	0.00	99.67
87 / 1 .	0.12	57.06	0.01	0.21	25.30	0.00	0.02	5.58	0.00	0.52	0.01	6.89	2.38	98.09
88 / 1 .	4.25	33.03	0.00	0.28	0.34	0.00	28.06	6.80	0.81	25.11	0.00	0.03	0.04	98.74
89 / 1 .	0.33	38.71	0.00	0.76	2.65	0.00	28.64	4.84	0.66	21.86	0.00	0.52	0.18	99.14
90 / 1 .	0.10	45.82	0.00	2.69	6.69	0.00	13.05	22.23	0.16	8.38	0.01	0.40	0.01	99.56
91 / 1 .	0.06	52.12	0.00	0.18	28.54	0.00	0.06	12.29	0.00	0.67	0.00	4.26	0.29	98.47
92 / 1 .	0.32	44.28	0.00	3.25	7.97	0.03	11.52	22.39	0.18	8.20	0.00	0.57	0.06	98.77
93 / 1 .	0.20	52.40	0.02	0.16	27.22	0.00	0.04	9.65	0.01	0.68	0.01	4.40	3.60	98.38
94 / 1 .	0.05	54.08	0.02	0.17	24.04	0.00	0.04	3.47	0.01	0.52	0.00	1.39	13.89	97.66
95 / 1 .	2.55	40.16	0.00	3.76	7.68	0.01	10.47	23.90	0.18	8.76	0.00	0.44	0.04	97.95
96 / 1 .	0.32	36.50	0.00	0.06	0.03	0.00	33.31	0.56	0.87	27.05	0.02	0.06	0.00	98.78
97 / 1 .	0.20	45.44	0.00	3.26	7.77	0.00	11.61	22.01	0.18	8.02	0.00	0.64	0.05	99.17
98 / 1 .	0.06	50.52	0.02	0.12	29.30	0.00	0.09	13.43	0.01	0.68	0.01	3.82	0.26	98.31
99 / 1 .	0.05	49.51	0.00	0.17	29.59	0.00	0.07	14.12	0.02	0.95	0.00	3.37	0.21	98.06
100 / 1 .	0.00	0.08	0.00	19.39	3.62	0.04	2.61	0.15	1.04	67.22	0.00	0.03	0.00	94.20

Appendix C- Cultivating an intermediary stock

As described in Chapter 3, to reduce nutrient carry-over from the enrichment medium into the minimal medium, an intermediary minimal medium was used. Figure iv. shows the relatively high cell density achieved in the enrichment media that was used to provide high cell density inoculums for the intermediary minimal medium.
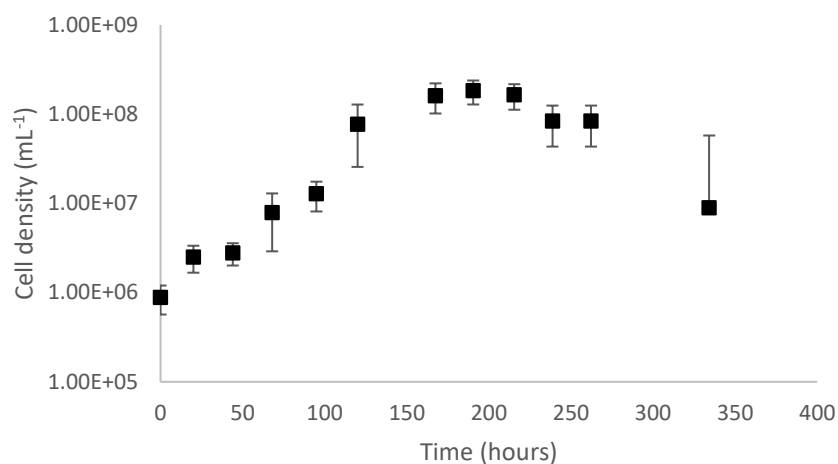
Figure A-5. Cell density plotted over time for the enrichment medium. Error represent the standard deviation in cell enumeration data.

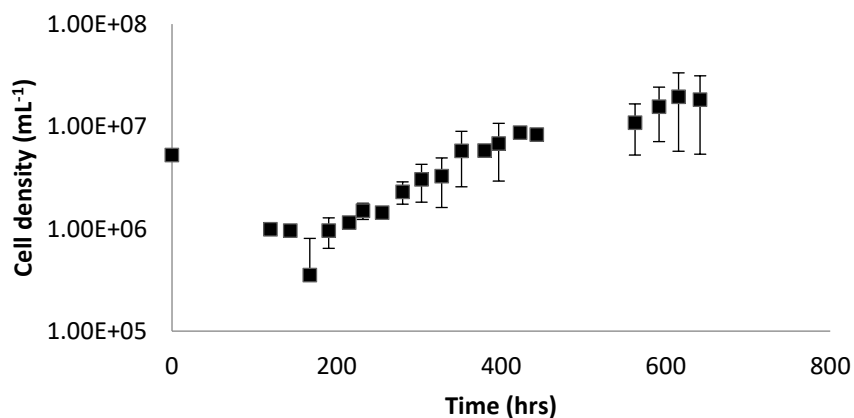Figure A-6. Cell density plotted over time for the intermediary medium inoculated from enrichment media. Error represent the standard deviation in cell enumeration data.

Cell enumeration of the intermediary minimal medium was carried out using the sybr green method described in Chapter 2 & 3, and at approximately 150 hours (Figure V), during the log phase, an inoculum was transferred into the experiment proper.

Appendix D- Primers used in this thesis

Table A-3. Details of the primers used in this thesis

| Primer	Sequence	Reference	Section(s)	Target gene
63f	5'-CAGGCCTAACACATGCAAGTC-3'	(Marchesi *et al.*, 1998)	2.2.3	16S rRNA
530r	5'GTATTACCGCGGCTGCTG-3'	(Muyzer *et al.*, 1993)	2.2.3	16S rRNA
27f	5'-AGAGTTTGATCCTGGCTCAG-3'	(Lane, 1991)	4.2.3	16S rRNA
1541r	5'-AAGGAGGTGATCCAGCCGCA-3'	(Summers *et al.*, 2015)	4.2.3	16S rRNA
COM1	5'-CAGCAGCCGCGGTAATAC-3'	(Olsson-francis *et al.*, 2015)	4.2.3	16S rRNA
COM2	CCGTCAATTCCTTTGAGTTT-3'	(Olsson-francis *et al.*, 2015)	4.2.3	16S rRNA
A341f	5'-CCTACGGGRSGCAGCAG-3'	(Baker *et al.*, 2003)	2.2.3	16S rRNA
A1204r	5'-TTMGGGGCATRCIKACCT-3'	(Baker *et al.*, 2003)	2.2.3	16S rRNA
28f	5'-GAGTTTGATCNTGGCTCAG-3'	(La Duc *et al.*, 2012)	2.2.3	16S rRNA
340wf	5'-CCCTAYGGGGYGCASCAG-3'	(Gantner *et al.*, 2011)	2.2.3	16S rRNA
534r	5'-GWATTACCGCGGCKGCTG-3'	(Baker *et al.*, 2003)	2.2.3	16S rRNA

Appendix E- BET plot and data
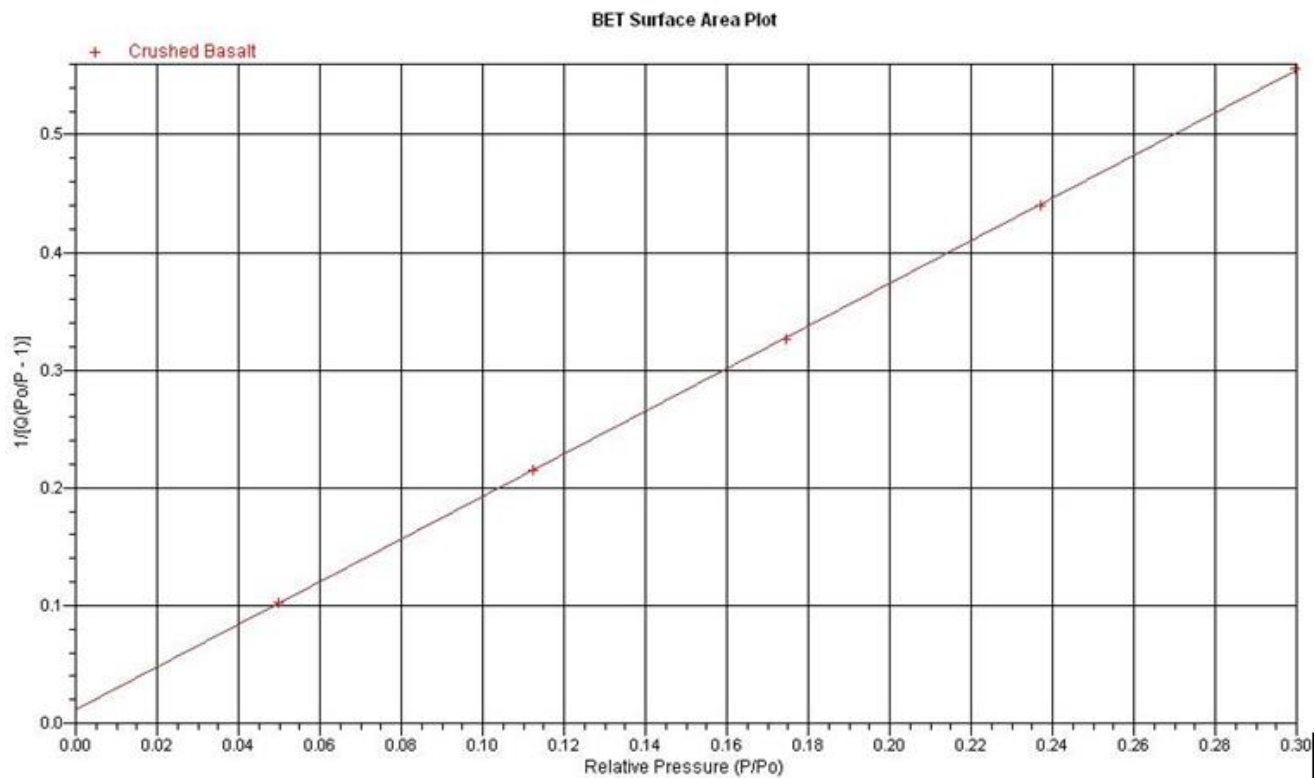
Figure A-7. BET plot for the terrestrial basalt used in Chapter 3

BET Surface Area: 2.3881 ± 0.0144 m^2/g

Slope: 1.811299 ± 0.010751 g/cm^3 STP

Y-Intercept: 0.011563 ± 0.002105 g/cm^3 STP

C: 157.650033

Qm: 0.5486 cm^3/g STP

Correlation Coefficient: 0.9999472

Molecular Cross-Sectional Area: 0.1620 nm^2

Appendix F- 16S rRNA contig sequences for isolates

E_01 *Acinetobacter johnsonii*

CTAATAGATGAGCCTAAGTCAGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGAC
GATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCG
CGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTTGGA
TTAATACTCTAGGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGC
AGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGT
AGGCGGCTTCTTAAGTCGGATGTGAAATCCCTGAGCTTAACTTAGGAATTGCATTCGATAC
TGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAG
AGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAATACTGACGCTGAGGTAC
GAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCT
ACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTG
GGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATAGTAAGAA
CTTTCCAGAGATGGATTGGTGCCTTCGGGAACTTACATACAGGTGCTGCATGGCTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTATTTG
CCAGCGGGTTAAGCCGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGG
GACGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGTCGGT
ACAAAGGGTTGCTACCTAGCGATAGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATT
GGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGCCGC
GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAATTTGTTGCACC
AGAAGTAGGTAGTCTAACCGCAAGGAGGACG

E_02 *Clostridium amygdalinum*

GCTCCCTCCTTGCGGTTGGGTCACTGACTTCGGGCGTTACCAACTCCCATGGTGTGACGGG

CGGTGTGTACAAGACCCGGGAACGTATTCACCGCGGCATTCTGATCCGCGATTACTAGCGA

TTCCAGCTTCATGTAGTCGAGTTGCAGACTACAATCCGAACTGAGACGTTATTTATGGGAT

TTGCTTCACCTCACGGCTTCGCTTCCCTTTGTTTACGCCATTGTAGCACGTGTGTAGCCCA

AATCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCAGGTTATCCCTGGCAG

TCTCCCCAGAGTGCCCAGCCGAACTGCTGGCTACTAAGGATAAGGGTTGCGCTCGTTGCGG

GACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCTAGAATG

CCCCGTAGGGAAGCCCTGGTTACAGGACGATCATTCCGATGTCAAGACTTGGTAAGGTTCT

TCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTT

TGAGTTTCATTCTTGCGAACGTACTCCCCAGGTGGAATACTTACTGCGTTAGCGACGGCAC

CGAAGAGCTTTGCTCCCCGACACCTAGTATTCATCGTTTACGGCGTGGACTACCAGGGTAT

CTAATCCTGTTCGCTCCCCACGCTTTCGAGCCTCAACGTCAGTTACAGTCCAGTAAGCCGCC

TTCGCCACTGGTGTTCCTCCTAATATCTACGCATTTCACCGCTACACTAGGAATTCCACTT

ACCTCTCCTGCACTCTAGCAACACAGTTTCCAAAGCAGTCCCGGGGGTTGAGCCCCGGGCTT

TCACTTCAGACTTGCATCGCCGTCTACGCTCCCTTTACACCCAGTAAATCCGGATAACGCT

TGCCCCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTCTTAGTCAGGTA

CCGTCATTTTCTTCCCTGCTGATAGACCTTTACATACCGAAATACTTCTTCAGTCACGCGG

CGTCGCTGGATCAGGGTTTCCCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGT

TTGGGCCGTGTCTCAGTCCCAATGTGGCCGGTCACCCTCTCAGGTCGGCTACTGATCGTCG

GCTTGGTGGGCCGTTACCTCACCAACTACCTAATCAGACGCGGGTCCATCTCATACCACCG

GAGTTTTTCACACTGTGCTATGCAGCACTGTGCGCTTATGCGGTATTAGCAGTCATTTCTA

ACTGTTATCCCCCTGTATGAGGCAGGTTACCCACGCGTTACTCACCCGTCCGCCGCTAA

E_03 *Bacillus toyonensis*

AATTCCTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACT
TCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTAC
CAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAG
AAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACATGGA
ATTCCACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGAGCC
GTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTTTACGCCCAATAATTCCGG
ATAACGCTTGCCACCTACGTATTACGCGGGCTGCTGAGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACTCTAG
AGATAGAGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATT
AAGTTGGGCACTCTAAGGTGACTGCCGGTGACAACCGGAGGAAGTGGGGATGACGTCAAA
TCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAGAGCTGCA
AGACCGCGAG

E_04 *Bacillus drentensis*

CTTCGCGTTGCTTCCAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCT
TTGAGTTTCAGCCTTGCGGCCGTACTCCCCAAGCAGAGTGCTTAATGCGTTTGCTGCAGCA
CTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGT
ATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGACCAGAAAGTC
GCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCAC
TTTCCTCTTCTGCACTCAAGTCCCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCT
TTCACATCAGACTTAAGAGACCGCCTGCGCGCGCTTTACGCCCAATAATTCCGGACAACGC
TTGCCACCTACGTATTACGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTCAGGT
ACCGTCAAGGTGCCGGCAGTTACTCCGGCACTTGTTCTTCCCTGACAACAGAGTTTTACGA

TCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGA

TTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCAC

CCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATG

CGCCGCGGGCCCATCTGTAAGTGATAGCCGAAACCATCTTTCAGCATAGCCTCATGAGAGG

CTGTGCATTATCCGGTATTAGCTCCGGTTTCCCGAAGTTATCCCAGTCTTACAGGCAGGTT

GCCCACGTGTTACTCACCCGTCCGCCGCTGACTTCAGGGAGCAAGCTCCCATCCGTCCGCTC

GACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCT

E_05 *Bacillus niabensis*

GTTTGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACTCATCGTTTACGGCGT

GGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTAC

AGACCACAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACA

CGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCACGGT

TGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGCGCGCTTTACGCCCAATAA

TTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGC

TTTCTGGTTAGGTACCGTCAAGGTACCAGCAGTTACTCTGGTACTTGTTCTTCCCTAACAA

CAGAACTTTACGACCCGAAGGCCTTCATCGTTCACGCGGCGTTGCTCCGTCAGACTTTCGT

CCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCA

GTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCA

CCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGATAGCCGAAACCATCTTTCAATTT

CGAACCATGCGGTTCGAAATGTTATCCGGTATTAGCTCCGGTTTCCCGGAGTTATCCCAAT

CTTACAGGCAGGTTACCCACGTGTTACTCAC

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