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Microbial iron reduction on Earth and Mars

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Doctor of Philosophy – The University of Edinburgh – 2014

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Declaration

This thesis has been solely composed by the candidate. Unless clearly stated, this work is the candidate's own. Where samples or results have been obtained through collaborations with other researchers, their precise contributions are made clear in the text as appropriate. The literature review builds upon two multi-authored review papers, both of which are first-authored by the candidate reflecting her substantial contribution. These publications are listed below and are bound at the back of the thesis:

1. Nixon, S.L., Cockell, C.S. and Tranter, M., 2012. Limitations to a microbial iron cycle on Mars. *Planetary and Space Sciences*, 72, 116-128. http://dx.doi.org/10.1016/j.pss.2012.04.003
2. Nixon, S.L., Cousins, C.R. and Cockell, C.S., 2013. Plausible microbial metabolisms on Mars. *Astronomy and Geophysics*, 54(1), 1.13-1.16. http://dx.doi.org/10.1093/astrogeo/ats034

This work has not been submitted for any other degree or professional qualification.

Signature: ..... Date: .....

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Abstract

The search for life beyond Earth is the driving force behind several future missions to Mars. An essential task in the lead-up to these missions is a critical assessment of the habitability for, and feasibility of, life. However, little research has been conducted on this issue, and our understanding of the plausibility for life on Mars remains unconstrained. Owing to the anoxic and iron-rich nature of Mars, microbial iron reduction (MIR) represents a compelling candidate metabolism to operate in the Martian subsurface, past and present. The objectives of this thesis are to address the feasibility of MIR on Mars by i) better defining the habitability of MIR on Earth, and ii) assessing the range and availability of organic electron donors in the subsurface of Earth and Mars.

Samples collected from Mars-relevant environments on Earth were used to initiate MIR enrichment cultures at 4°C, 15°C and 30°C. Results indicate MIR is widespread in riverbed and subglacial sediments but not sediments from desert or recent volcanic plains. The iron-reducing microorganisms in subglacial enrichments are at least psychrotolerant and in some cases psychrophilc. Culture-independent methods highlighted the changes in diversity between temperature conditions for subglacial sediments, and indicated that members of the prolific MIR *Geobacteraceae* family are common. The genera *Geobacter* and *Desulfosporosinus* are responsible for MIR in the majority of enrichments. Long-term anoxia and the availability of redox constituents are the major factors controlling MIR in these environments.

A MIR enrichment culture was unable to use shales and kerogens as the sole source of electron donors for MIR, despite the presence of known electron donors. Furthermore, MIR was inhibited by the presence of certain kerogens. The causes of inhibition are unknown, and are likely to be a combination of chemical and physical factors.

Experiments were conducted to assess the ability of three pure strains and a MIR enrichment to use non-proteinogenic amino acids common to carbonaceous meteorites as electron donors for MIR. Results demonstrate that γ-aminobutyric acid served as an electron donor for the enrichment culture, but no other amino acids supported MIR by this or other iron-reducing cultures. The D-form of chiral amino acids was found to exert a strong inhibitory effect, which decreased in line with concentration. Theoretical calculations using published meteoritic accretion rates onto the surface of Mars indicate that the build up

inhibitory amino acids may place important constrains on habitability over geologic time scales.

Contamination of a pure strain of *Geobacter metallireducens* with a strain of *Clostridium* revealed a syntrophic relationship between these microorganisms. Anaerobic heterotrophs are likely to play an important role in maintaining an available supply of electron donors for MIR and similar chemoorganic metabolisms operating in the subsurface.

This research indicates that MIR remains a feasible metabolism to operate on Mars providing a readily available redox couple is present. However, given the observed inhibition in the presence of bulk carbonaceous material and certain amino acids found in meteorites, the use of extraterrestrial carbonaceous material in the Martian subsurface for microbial iron reduction is questionable, and should be the focus of future research

Lay Summary

The purpose of this research is to guide the search for life on Mars by studying the limits of one plausible microbial metabolism on Earth, microbial iron reduction. Mars today is a cold, barren desert, and has been for much of its history. The surface is bathed in damaging radiation, and the soil contains chemicals that destroy organic material. Life as we know it cannot endure these conditions. Yet several lines of evidence indicate Mars was warmer and wetter at the time life began to proliferate on Earth. It is therefore possible that life prospered on Mars, and may have continued to do so underground as conditions at the surface worsened.

Mars lacks the oxygen depended upon by much of life on Earth. The search for life on Mars is therefore concerned with simple, single-cell 'microorganisms' that thrive in the absence of oxygen. On Earth many of these microorganisms gather the energy they require to prosper from chemical reactions in the environment. One such metabolism, microbial iron reduction, uses iron in rocks and soils to fuel its metabolism. On Earth this metabolism is prolific operating beneath the surface. Given that iron is more plentiful on Mars than on Earth, this is a compelling candidate metabolism for life on Mars, and the focus of this PhD research.

In this work I looked for evidence of this metabolism in 'extreme' environments on Earth that are similar to past and present environments on Mars. These included environments in Iceland with similar rocks to those on Mars, as well as the extremely arid Namib Desert, the acidic iron-rich Rio Tinto river in Spain, and environments beneath glaciers. I found this metabolism in river sediments and from beneath glaciers, but not from desert or recent volcanic environments in Iceland. In particular, this metabolism appeared to function in colder and more acidic environments than previously investigated. Therefore, from what we understand about environments on Mars, this metabolism remains a compelling candidate to operate on the Red Planet.

An addition focus of this work was to evaluate the range of 'food' (energy sources) for this metabolism to operate on Mars. The energy sources were tested in growth experiments with iron-reducing microorganisms, and included complex organic material, as well as individual amino acid compounds, both common to meteorites. These meteorites fell to the Martian surface early in the history of the planet, as they did on the Earth. It is thought that these meteorites remain buried beneath the surface of Mars, and may serve as a source

of energy for this and other microbial metabolisms. The results indicated, however, that not only did these materials not serve as energy sources, but they also inhibited growth. In other words, these potential energy sources instead appear to be toxic.

The overall conclusion of this work is that microbial iron reduction remains a feasible metabolism to occur on Mars (past and present), however caution should be exercised in the search for life on Mars. Specifically, the presence of potential energy sources beneath the surface may hinder and not help microbial life on Mars.

Chapter 1: Introduction

The present-day surface of Mars is considered inhospitable for life. However, there is a wealth of evidence indicative of a warmer and wetter, and hence more hospitable past. Such evidence raises the question of whether life has ever existed, or perhaps still does exist, on Mars. This search for life is the driving force behind a number of current and planned surface missions to Mars. For example, the joint European Space Agency and Roscosmos (Russian space agency) ExoMars rover, due for launch in 2018, has been specifically designed to search for signs of past and present life in the subsurface of Mars. An essential task in the lead up to this and other life detection missions is to better constrain the feasibility for plausible forms of microbial metabolism to operate on Mars, an area of research which has received limited attention to date.

This thesis attempts to address this critical knowledge gap by focusing on one plausible metabolism, microbial iron reduction (MIR). The underlying hypothesis is that MIR is a feasible microbial metabolism to operate in the subsurface of Mars, past or present. The assumptions underpinning this hypothesis are threefold. First, owing to the extreme radiation levels and strong oxidising agents at the surface of Mars it is assumed that any life, past or present, would reside in the subsurface. Second, given the almost complete lack of oxygen in the atmosphere, any life on Mars is assumed to be anaerobic. Finally, such anaerobic subsurface life is assumed to operate a chemosynthetic metabolism, acquiring energy by mediating chemical reactions in the environment. These assumptions, and the relevant background pertaining to them, are expanded upon in Chapter 2.

The overall goal of this thesis is to significantly further our understanding of the feasibility for life sustained by MIR on Mars, by addressing the limits of MIR on Earth. This research will be of relevance not only to those interested in the search for life on Mars, but also to those with an interest in the role of MIR in major global biogeochemical cycles on Earth.

1.1 Thesis scope and objectives

Although not the only plausible microbial metabolism that could operate on Mars, MIR is recognised as the most compelling owing to 1) the widespread abundance of ferric iron detected at the Martian surface, and 2) expected presence of carbonaceous material of

extraterrestrial origin within the subsurface. Such material is known to harbour organic compounds that serve as electron donors for MIR on Earth. Although this thesis only directly addresses MIR, the close association of the microbially-mediated reduction of both iron and sulphur render this thesis relevant to the feasibility of the latter. However, a dedicated assessment of the feasibility of microbial sulphate-reduction on Mars is beyond the scope of this thesis. Furthermore, an evaluation of general factors affecting habitability, such as the effect of radiation or availability of essential nutrients, is also beyond the scope of this thesis.

The objectives of this thesis are twofold: i) to better define the prevalence of MIR on Earth, and ii) to assess the range and availability of organic electron donors in the subsurface of Earth and Mars. These objectives are addressed by asking the following specific science questions:

Q1: Is MIR widespread in Mars-like environments on Earth?

Q2: Are iron-reducing microorganisms capable of using ancient recalcitrant carbonaceous material in the terrestrial subsurface as a source of electron donors for MIR?

Q3: Are iron-reducing microorganisms capable of using rare amino acids found in carbonaceous meteorites as electron donors for MIR?

1.2 Thesis outline

Chapter 2 provides the relevant context to this thesis in the form of a comprehensive and up to date literature review, culminating in the identification of specific knowledge gaps that relate to the scope of this thesis. A brief overview of methods common to all proceeding chapters is given in Chapter 3. The remainder of the thesis is aligned with the above specific science questions. In each of these stand-alone studies, the rationale and underlying hypotheses are given in the introduction, and conclusions and limitations are stated at the end.

Chapter 4 details a study conducted to address Q1, in which sediments from a number of Mars-relevant environments were used to initiate MIR enrichment cultures at various temperatures. The purpose of the study reported in Chapter 5 is to address Q2, in which

growth experiments were conducted using samples of shale and kerogen as the sole source of electron donors for MIR. The study in Chapter 6 directly addresses Q3 by testing the ability of a number of iron-reducing microorganisms to utilise nonproteinogenic meteoritic amino acids as electron donors for MIR. Chapter 7 reports on an apparent syntrophic relationship between anaerobic heterotrophs and iron-reducing microorganisms, brought to light when a pure culture of a well-characterised iron-reducing microorganism was found to be contaminated.

The findings of these independent studies are synthesised in a concluding discussion in Chapter 8, which also addresses the remaining knowledge gap and provides recommendations for future research directions.

Chapter 2: Literature review

This chapter features a thorough review of relevant literature, focusing the discussion on Mars as a potentially habitable environment, and the feasibility of microbial iron reduction (MIR) as a candidate microbial metabolism to operate in the subsurface of Mars. As previously noted, other microbial metabolisms are considered plausible (Nixon *et al*., 2013), however these are beyond the scope of this thesis and only MIR will be discussed. The exception to this is the microbial reduction of sulphate, which overlaps with MIR in the redox constituents utilised. This chapter also reviews relevant other work that addresses the plausibility of MIR on Mars, whether directly or indirectly, and concludes with a summary of the knowledge gaps. The following builds upon the review papers that were published during the course of this PhD (Nixon *et al*., 2012; Nixon *et al*., 2013; see Appendix)

2.1 Microbial iron reduction

A wide array of microorganisms acquire energy from mediating reduction-oxidation (redox) reactions in the environment. This mode of energy acquisition is achieved by harnessing electrons liberated from electron donor compounds (oxidation), which are passed down an electron transport chain and ultimately used to establish a proton gradient used to synthesise adenosine triphosphate (ATP; the universal energy molecule for all known life) (Madigan and Martinko, 2006). The electrons are passed on to a terminal electron acceptor compound, thereby leading to reduction. As the fourth most abundant element on Earth, iron has the capacity to support significant microbial populations through the mediation of iron oxidation and reduction. In the case of the latter, iron-reducing microorganisms typically exploit organic compounds as a source of electrons for the reduction of ferric-iron bearing minerals outside the cell.

Microorganisms capable of mediating iron reduction reactions are phylogenetically diverse, and belong to both the Bacteria and Archaea domains (see Table 2.1). The vast majority of these, however, are Bacteria, most of which belong to the delta-proteobacteria subdivision. The dominant family within this subdivision is *Geobacteraceae*, which encompasses the genera *Desulfuromonas*, *Desulfuromusa*, *Geoalkalibacter*, *Geopsychrobacter*, *Geothermobacter*, *Pelobacter* and *Geobacter*. Microorganisms belonging to this family dominate subsurface microbial communities in the anoxic zone (Rooney-

Varga *et al*., 1999; Snoeyenbos-West *et al*., 2000), and with the exception of *Pelobacter* species are capable of coupling the reduction of iron to the complete oxidation of organic compounds to CO_2 (Lovley, 2013).

A number of microorganisms are known to carry out the transfer of electrons to ferric iron without conserving energy from the process. In other words, iron reduction is carried out passively and not as a means to fuel metabolism (Lovley, 2013). These microorganisms include a number of fermentative bacteria, such as species of *Bacillus* and *Pseudomonas*, archaea (including methanogens) and perhaps most importantly numerous sulphate-reducing bacteria. A number of these sulphate-reducers have been found to host the intermediate electron carrier, cyctochrome c3, which in a number of microorganisms that do conserve energy from MIR acts as a ferric iron reductase (Lovley *et al*., 1993a). It has thus been suggested that these cytochromes inadvertently reduce ferric iron (Lovley *et al*., 1993b). An alternative hypothesis is that iron reduction is carried out by these microorganisms is an attempt to deplete ferric iron, thereby rendering conditions more favourable for energy acquisition through microbial sulphate reduction (Lovley, 2013).

The ability of an isolated microorganism to reduce iron is widely cited in the corresponding type strain paper, though whether this reaction supports growth or not (and hence is being utilised as a means to generate ATP) is often ambiguous. Table 2.1 was compiled as a result of a thorough literature search of microorganisms known to carry out MIR characterised to date, and care was taken to ensure only papers reporting *growth* through iron reduction were included.

Organism	Kingdom | Class | Family	Source	Optimal (and range) growth conditions T (°C) | pH | salinity (% w/v)
Acidiphilium cryptum[1]	*Bact* | *α-Proteobacteria* | *Acetobacteraceae*	Acidic coal mine lake sediments	35 (12-37) | 3.2 (2.1-5.8) | ND
Acidithiobacillus ferroxidans[2-4]	*Bact* | *βProteobacteria* | *Acidithiobacillaceae*	Acid, bituminous coal mine effluent	(10-37) | 2.5 (1.3-4.5) | ND
Aeromonas hydrophila[5-7]	*Bact* | *γProteobacteria* | *Aeromonadaceae*	Freshwater, sewage, microbial fuel cell	30 (5-30) | 7.0-9.0 (5.0-10.0) | (0.5-4.0)
Ardenticatena maritima[8]	*Bact* | *Chloroflexi* | *Ardicatenaceae*	Coastal hydrothermal field	55-70 (30-75) | 7.0 (5.5-8.0) | 1-2.0 (0-6.0)
Bacillus arsenicoselenatis[9]	*Bact* | *Bacilli* | *Bacillaceae*	Lake sediment, Mono Lake	20 | 8.5-10.0 | 6.0 (2.0-12.0)
Bacillus infernus[10]	*Bact* | *Bacilli* | *Bacillaceae*	Deep subsurface sediment	61 (45-60) | 7.3 (7.0-8.1) | ND
Carboxydocella manganica[11]	*Bact* | *Clostridia* | *Syntrophomonadaceae*	Terrestrial hydrothermal system	58-60 (26-70) | 6.5 (5.5-8.0) | 0.5 (0-2.0)
Carboxydothermus pertinax[12]	*Bact* | *Clostridia* | *Peptococcaceae*	Terrestrial hydrothermal system	65 (50-70) | 6.5 (4.6-8.6) | ND
Deferribacter autotrophicus[13]	*Bact* | *Deferribacteres* | *Deferribacteraceae*	Hydrothermal vent field	60 (25-75) | 6.5 (5.0-7.5) | 2.5 (1.0-6.0)
Deferribacter thermophilus[14]	*Bact* | *Deferribacteres* | *Deferribacteraceae*	Offshore oil field	60 (50-65) | 6.5 (5.0-8.0) | 2.0 (0-5.0)
Desulfitobacterium aromaticivorans[15]	*Bact* | *Clostridia* | *Peptococcaceae*	HC-contamiated aquifer	30 | 6.6-7.0 (6.5-7.5) | ND
Desulfitobacterium metallireducens[16]	*Bact* | *Clostridia* | *Peptococcaceae*	U-contaminated aquifer	30 (20-37) | 7.0 | ND
Desulfotalea arctica[17]	*Bact* | *δ-Proteobacteria* | *Desulfobulbaceae*	Arctic marine sediments	18 (−1.8-26) | 7.2-7.9 | 1.9-2.5
Desulfotalea psychrophila[17]	*Bact* | *δ-Proteobacteria* | *Desulfobulbaceae*	Arctic marine sediments	10 (−1.8-19) | 7.3-7.6 | 1.0
Desulfofrigus fragile[17]	*Bact* | *δ-Proteobacteria* | *Desulfobacteraceae*	Arctic marine sediments	18 (−1.8-27) | 7.0-7.4 | 1.0-2.5
Desulfofrigus oceanense[17]	*Bact* | *δ-Proteobacteria* | *Desulfobacteraceae*	Arctic marine sediments	10 (−1.8-16) | 7.0-7.5 | 1.5-2.5
Desulfosporomusa polytropa[18]	*Bact* | *Negativicutes* | *Veillonellaceae*	Lake sediment	28 (4-37) | (6.1-8.2) | (0-0.15)
Desulfosporosinus lacus[19]	*Bact* | *Clostridia* | *Peptococcaceae*	Lake sediment	(4-32) | (6.5-7.5) | ND
Desulfuromonas acetexigens[20,21]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Anoxic sewage	30-35 | 7.6-7.8 | ND
Desulfuromonas acetoxidans[22,23]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Marine sediments	30 (25-35) | 7.0-7.5 | ND
Desulfuromonas michiganensis[24]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	River sediment	25-30 | 7.0-7.5 | ND
Desulfuromonas palmitatis[21]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	HC-contaminated marine sediments	40 | {6.9} | ND
Desulfuromonas svalbardensis[25]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Arctic marine sediments	14 (−2-20) | 7.3 (6.5-7.5) | ND
Desulfuromusa bakii[26,27]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Marine and freshwater sediments	25-30 (8-32) | 6.7-7.4 (6.2-7.8) | ND
Desulfuromusa ferrireducens[25]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Arctic marine sediments	14-17 (−2-23) | 7.0-7.3 (6.5-7.9)| ND
Desulfuromusa kysingii[26,27]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Marine sediment	32 (4-35) | 6.5-7.9 (5.8-8.25) | ND

Organism	Kingdom | Class | Family	Source	Optimal (and range) growth conditions T (°C) | pH | salinity (% w/v)
Desulfuromusa succinoxidans[26,78]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Marine sediment	32 (4-35) | 6.5-7.9 (5.8-8.25) | ND
Ferribacterium limneticum[28]	*Bact* | *βProteobacteria* | *Rhodocyclaceae*	Mine-impacted lake sediment	{25} | {7.0} | ND
Ferrimonas balearica[29]	*Bact* | *γProteobacteria* | *Ferrimonadaceae*	Marine sediment	(42) | (6.0-9.0) | (0.5-7.5)
Ferroglobus placidus[30,31]	*Arch* | *Archaeoglobi* | *Archaeoglobaceae*	Marine hydrothermal system	85 (65-95) | 7.0 (6.0-8.5) | 1.8-2.0 (0.5-4.5)
Fontibacter ferrireducens[32]	*Bact* | *Cytophagia* | *Cyclobacteriaceae*	Microbial fuel cell	30 (15-37) | 7.0 (6.5-8.5) | 1.0 (0-5.0)
Geoalkalibacter ferrihydriticus[33]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Lake sediment	35 (18-39) | 8.6 (7.8-10.0) | 0 (0-5.0)
Geoalkalibacter subterraneus[34]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Onshore oilfield	40 (30-50) | 7.0 (6.0-9.0) | 2.0 (0.1-10.0)
Geobacter argillaceous[35]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Subsurface sediment	30 (10-36) | 6.2-6.8 (5.8- 7.4) | ND
Geobacter bemidjiensis[36]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	HC-contaminated subsurface sediment	30 (15-37) | 7.0 | ND
Geobacter bremensis[37]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Subsurface sediment	30-32 | 5.5-6.7 | ND
Geobacter chapelli[38,39]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Deep subsurface sediments	25 | 7.0 | ND
Geobacter daltonii[40]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	U- and HC-contaminated sediment	30 | 6.7-7.3 (6.0-8.0) | 0 (0-0.7)
Geobacter grbiciae[38,39]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	River sediment	{30} | {6.7-7.0} | ND
Geobacter hydrogenophilus[38,39]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	HC-contaminated aquifer	35 | 6.5 | ND
Geobacter lovleyi[41]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Subsurface sediment	35 (10-40) | 6.5-7.2 | ND
Geobacter luticola[42]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Lotus field sediment	30-37 (20-40) | 7.0 (6.5-7.5) | 0 (0-0.5)
Geobacter metallireducens[43,44]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	River sediment	30-35 | 6.7-7.0 | ND
Geobacter pelophilus[37]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Subsurface sediment	30-32 | 6.7-7.0 | ND
Geobacter pickeringii[35]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Subsurface sediment	30 (10-36) | 6.6-7.2 (5.8-8.0) | ND
Geobacter psychrophilus[36]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Aquifer groundwater	17-30 (4-30) | 7.0 (6.0-9.0) | (1.0-3.0)
Geobacter sulfurreducens[45]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	HC-contaminated surface sediment	35 | {6.8} | ND
Geobacter toluenoxydans[15]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	HC-contaminated aquifer	25-32 | 6.6-7.0 (6.6-7.5) | ND
Geobacter uraniireducens[46]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	U-contaminated subsurface sediment	32 (10-34) | 6.5-7.0 (6.0-7.7) | ND
Geoglobus ahangari[87]	*Arch* | *Archaeoglobi* | *Archaeoglobaceae*	Marine hydrothermal system	88 (65-90) | 7.0 (5.0-7.6) | 1.9 (0.9-3.8)
Geopsychrobacter electrodiphilus[48]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Microbial fuel cell	22 (4-30) | {6.8} | ND
Geothermobacter ehrlichii[49]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Marine hydrothermal system	55 (35-65) | 6.0 (5.0-8.0) | 1.9 (0.5-5.0)

Organism	Kingdom | Class | Family	Source	Optimal (and range) growth conditions T (°C) | pH | salinity (% w/v)
Geothermobacterium ferrireducens[50]	*Bact* | *Thermodesulfobacteria* | *Thermodesulfobacteriaceae*	Terrestrial hydrothermal system	85-90 (65-100) | 6.8-7.0 | 0-0.05 (0-0.75)
Geothrix fermentans[51]	*Bact* | *Holophage* | *Holophagaceae*	HC-contaminated subsurface sediment	35-40 | {7} | ND
Geovibrio ferrireducens[52]	*Bact* | *Deferribacteraceae* | *Deferribacteraceae*	Surface sediment	35 | {7.0} | 0 (0-2.0)
Melioribacter roseus[53]	*Bact* | *Ignavibacteria* | *Melioribacteraceae*	Terrestrial hydrothermal sediment	52-55 (35-60) | 7.5 (6.0-8.7) | 0.6 (0-6.0)
Pantoea agglomerans[54]	*Bact* | *γProteobacteria* | *Enterobacteriaceae*	Near-shore surface sediment	30 (5-40) | 6.0-7.2 (6.0-8.5) | 0.5 (0-5.0)
Pelobacter carbinolicus[55,56]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Sewage, subsurface, marine sediments	35 (15-40) | 6.5-7.2 (6.0-8.0) | (0-2.0)
Pelobacter propionicus[27,55]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Subsurface sediment	33 (4-45) | 7.0-8.0 (6.5-8.4) | ND
Pelobacter venetianus[27,55]	*Bact* | *δ-Proteobacteria* | *Geobacteraceae*	Subsurface and marine sediments	33 (10-40) | 7.0-7.5 (5.5-8.0) | ND
Pyrobaculum islandicum[58-61]	*Arch* | *Thermoprotei* | *Thermoproteaceae*	Terrestrial hydrothermal sediment	100 (74-102) | (5.0-9.0) | (0-0.8)
Pyrobaculum aerophilum[62,63]	*Arch* | *Thermoprotei* | *Thermoproteaceae*	Marine hydrothermal system	100 (75-104) | 7.0 (5.8-9.0) | 1.5 (0.0-3.6)
Rhodoferax ferrireducens[64]	*Bact* | *βProteobacteria* | *Comamonadaceae*	Marine sediment	25 (4-30) | 7.0 (6.7-7.1) | ND
Shewanella alga[65-68]	*Bact* | *γProteobacteria* | *Shewanellaceae*	Estuarine sediments	30 (10-40) | 7.45 | (3.0-6.0)
Shewanella arctica[69]	*Bact* | *γProteobacteria* | *Shewanellaceae*	Arctic marine sediments	20 (4-28) | 7.5 (4.5-9.0) | 0.5-1.0 (0.5-3.5)
Shewanella frigidimarina[70,71]	*Bact* | *γProteobacteria* | *Shewanellaceae*	Antarctic sea ice	20-22 (<0-30) | (6.0-9.5) | 0 (0-9.0)
Shewanella gelidimarina[70]	*Bact* | *γProteobacteria* | *Shewanellaceae*	Antarctic sea ice	15-17 (<0-23) | {7.4-7.8} | (1.0-6.0)
Shewanella livingstonensis[71]	*Bact* | *γProteobacteria* | *Shewanellaceae*	Antarctic marine water	(4-20) | (6.0-9.5) | 0
Shewanella olleyana[72]	*Bact* | *γProteobacteria* | *Shewanellaceae*	Estuarine sediments	20-22 (2-30) | {7.0} | 2.3-3.5 (2.0-6.4)
Shewanella oneidensis[73,77]	*Bact* | *γProteobacteria* | *Shewanellaceae*	Lake sediment	30 | {7.0-7.2} | ND
Shewanella pealeana[78]	*Bact* | *γProteobacteria* | *Shewanellaceae*	Nidamental gland of a squid	25 (4-30) |7.0 (6.0-8.0) | 2.9 (0.7-4.4)
Shewanella profunda[79]	*Bact* | *γProteobacteria* | *Shewanellaceae*	Marine sediment	25-30 (4-37) | 7.0 (6.5-8) | 0.5 (0-6.0)
Shewanella saccharophilia[80]	*Bact* | *γProteobacteria* | *Shewanellaceae*	River sediment	30-35 (25-35) | 7.6 | (0-4.0)
Sulfurospirillum barnesii[81,82]	*Bact* | *ϵ-Proteobacteria* | *Campylobacteraceae*	Se-contaminated sediment	33 | 7.5 | 0.05
Tepidimicrobium ferriphilum[83]	*Bact* | *Clostridia* | *Clostridiaceae*	Terrestrial hydrothermal sediment	50 (26-62) | 7.5-8.0 (5.5-9.5) | (0-3.5)
Thermincola ferriacetica[84]	*Bact* | *Clostridia* | *Peptococcaceae*	Terrestrial hydrothermal sediment	57-60 (45-70) | 7.0-7.2 (5.9-8.0) | 0 (0-3.5)
Thermoanaerobacter siderophilus[85]	*Bact* | *Clostridia* | *Thermoanaerobacteraceae*	Terrestrial hydrothermal sediment	69-71 (39-78) | 6.3-6.5 (4.8-8.2) | (0-3.5)

Organism	Kingdom | Class | Family	Source	Optimal (and range) growth conditions T (°C) | pH | salinity (% w/v)
Thermolithobacter ferrireducens[86]	*Bact* | *Thermolithobacteria* | *Thermobacteraceae*	Terrestrial hydrothermal sediment	73 (50-75) | 7.1-7.3 (6.5-8.5) | ND
Thermoterrabacterium ferrireducens[87]	*Bact* | *Clostridia* | *Thermoanaerobacteraceae*	Terrestrial hydrothermal sediment	65 (50-74) | 6.0-6.2 (5.5-7.6) | (0-1.0)
Thermotoga maritima[88]	*Bact* | *Thermotogae* | *Thermotogaceae*	Geothermally heated sea floor	80 (55-90) | 6.5 (5.5-9.0) | 2.7 (0.25-3.75)
Thermovenabulum gondwanense[89]	*Bact* | *Clostridia* | *Thermoanaerobacterales*	Terrestrial hydrothermal sediment	65 (50-70) | 7.0 (6.0-9.0) | ND

Table 2.1: Growth parameters and environment of origin for all microorganisms reported to date. Where growth parameters were not reported, { } represent growth conditions recommended by DSMZ. Refs: [1] Küsel *et al*., 1999, [2] Drobner *et al*., 1990, [3] Das *et al*., 1992, [4] Pronk *et al*., 1992, [5] Knight and Blakemore, 1998, [6] Pham *et al*., 2003, [7] Vivekanandhan *et al*., 2003, [8] Kawaichi *et al*., 2013, [9] Blum *et al*., 1998, [10] Boone *et al*., 1995, [11] Slobodkina *et al*., 2012, [12] Yoneda *et al*., 2012, [13] Slobodkina *et al*., 2009, [14] Greene *et al*., 1997, [15] Kunapuli *et al*., 2010, [16] Finneran *et al*., 2002, [17] Knoblauch *et al*., 1999, [18] Sass *et al*., 2004, [19] Ramamoorthy *et al*., 2006, [20] Finster *et al*., 1994, [21] Coates *et al*., 1995, [22] Pfennig and Biebl, 1976, [23] Roden and Lovley, 1993, [24] Sung *et al*., 2003, [25] Vandieken *et al*., 2006, [26] Liesack and Finster, 1994. [27] Lonergan *et al*., 1996, [28] Cummings *et al*., 1999, [29] Rosselló-Mora *et al*., 1995, [30] Hafenbradl *et al*., 1996, [31] Tor and Lovley, 2001, [32] Zhang *et al*., 2013, [33] Zavarzina *et al*., 2006, [34] Greene *et al*., 2009, [35] Shelobolina *et al*., 2007, [36] Nevin *et al*., 2005, [37] Straub and Buchholz-Cleven, 2001, [38] Coates *et al*., 1996, [39] Coates *et al*., 2001, [40] Prakash *et al*., 2010, [41] Sung *et al*., 2006, [42] Viulu *et al*., 2013, [43] Lovley and Phillips, 1988, [44] Lovley *et al*., 1993a, [45] Caccavo *et al*., 1994, [46] Shelobolina *et al*., 2008, [47] Kashefi *et al*., 2002b, [48] Holmes *et al*., 2004, [49] Kashefi *et al*., 2003, [50] Kashefi *et al*., 2002a, [51] Coates *et al*., 1999, [52] Caccavo *et al*., 1996, [53] Podosokoskoya *et al*., 2013, [54] Francis *et al*., 2000, [55] Schink, 1984, [56] Lovley *et al*., 1995, [57] Schink and Stieb, 1983, [58] Huber *et al*., 1987, [59] Vargas *et al*., 1998, [60] Kashefi and Lovley, 2000, [61] Feinberg *et al*., 2008, [62] Völkl *et al*., 1993, [63] Lovley, 2013, [64] Finneran *et al*., 2003, [65] Simidu *et al*., 1990, [66] Caccavo *et al*., 1992, [67] Nozue *et al*., 1992, [68] Roden and Zachara, 1996, [69] Kim *et al*., 2012, [70] Bowman *et al*., 1997, [71] Bozal *et al*., 2002, [72] Skerratt *et al*., 2002, [73] Myers and Nealson, 1990, [74] Venkateswaran *et al*., 1999, [75] Kosta and Nealson, 1995, [76] Roden *et al*., 2000, [77] Neal *et al*., 2003, [78] Leonardo *et al*., 1999, [79] Toffin *et al*., 2004, [80] Coates *et al*., 1998, [81] Laverman *et al*., 1995, [82] Stolz *et al*., 1999, [83] Slobodkin *et al*., 1999, [84] Zavarzina *et al*., 2007, [85] Slobodkin *et al*., 1999, [86] Sokolova *et al*., 2007, [87] Slobodkin *et al*., 1997, [88] Huber *et al*., 1986, [89] Ogg *et al*., 2010.

2.1.1 Electron donors and acceptors known to support MIR

The range of electron donors known to support MIR is vast, and spans fatty acids, carbohydrates and aromatic hydrocarbons (Lloyd, 2003). Some iron-reducing microorganisms are capable of using hydrogen as an electron donor, however a carbon source is still required to support growth. In a small number of species, carbon dioxide (CO_2) can serve as the sole source of carbon; hence these microorganisms can carry out autotrophic iron reduction (e.g., *Geoglobus ahangari*, Kashefi *et al*., 2002b). Elemental sulphur oxidation can also support MIR in environments characterised by a low pH (Lovley, 2013). The full range of electron donors and acceptors known to support MIR by characterised iron-reducing microorganisms to date is summarised in Table 2.2.

The mostly widely used electron donors by iron-reducing microorganisms are the carboxylate ions acetate and lactate, and the most readily used terminal electron acceptor is soluble iron, typically chelated as iron(III)-citrate. This combination of donors and acceptors represents the most commonly used redox couple for growing iron-reducing microorganisms in the laboratory. However, iron-reducers proliferate in the natural environment by using insoluble forms of ferric iron, most prevalent in the form of oxide and oxyhydroxide minerals in soils and sediments. These minerals range in crystallinity, from poorly-crystalline (amorphous) ferrihydrite ($Fe_2O_3.0.5H_2O$), to highly crystalline magnetite (Fe_3O_4) and hematite (Fe_2O_3). Of all the insoluble forms of iron known to serve as electron acceptors, ferrihydrite is most commonly used. Although less abundant than crystalline phases in the natural environment, the reduction of ferrihydrite is the most thermodynamically favourable of the ferric iron oxides. In fact it is the only iron oxide in which reduction is more thermodynamically favourable than reduction of sulphate, of which many iron-reducing microorganisms are capable (Neal *et al*., 2003).

		Acetobacteraceae sp.[1]	*Acidithiobacillaceae* sp.[2-4]	*Aeromonadaceae* sp.[5-7]	*Archaeoglobaceae* sp.[8-10]	*Ardenticatenaceae* sp.[11]	*Bacillaceae* sp.[12,13]	*Campylobacteraceae* sp.[14,15]	*Clostridiaceae* sp.[16]	*Comamonadaceae* sp.[17]	*Cyclobacteriaceae* sp.[18]	*Deferribacteraceae* sp.[19-21]	*Desulfobacteraceae* sp.[22]	*Desulfobulbaceae* sp.[22]	*Desulfuromonadaceae* sp.[23-28]	*Enterobacteriaceae* sp.[29]	*Ferrimonadaceae* sp.[30]	*Geobacteraceae* sp.[31-50]	*Holophagaceae* sp.[51]	*Melioribacteraceae* sp.[52]	*Peptococcaceae* sp.[50,53-56]	*Rhodocyclaceae* sp.[57]	*Shewanellaceae* sp.[58-73]	*Syntrophomonadaceae* sp.[74]	*Thermoanaerobacteraceae* sp.[75-77]	*Thermodesulfobacteriaceae* sp.[78]	*Thermolithobacteraceae* sp.[79]	*Thermoproteaceae* sp.[80-85]	*Thermotogaceae* sp.[85,86]	*Veillonellaceae* sp.[28,87-89]
e− DONORS																														
Hydrocarbons	**MF**																													
toluene*[90-94]	C_7H_8				−							−			−			+	−		+		−			−				
o-xylene*[90]	C_8H_{10}																	−			+									
Alcohols and Phenols																														
methanol	CH_4O								−	−		−			−			+	−		−	−	−		−	−				
ethanol*[95]	C_2H_6O	+							−	−		−	+	+	+			+	−		−	−	−		−	−				+
propanol*[95]	C_3H_8O								+	−		−	+	+	+			+			−				−					+
isopropanol*[95]	C_3H_8O								−			−						+				−			−					
propanediol	$C_3H_8O_2$																								+					
glycerol*[96]	$C_3H_8O_3$	+		+	+				−	−		−	+	+	−			+	−		−		+		+	−				−
butanol*[95]	$C_4H_{10}O$									−		−	+	+	+			+			−				−					+
butandiol	$C_4H_{10}O_2$											−						+												
phenol*[90,92,93]	C_6H_6O				+							−			−			+	−		+	−	−		−	−				
sorbitol*[97]	$C_6H_{14}O_6$											−			−						−				+					−
m-cresol*[94]	C_7H_8O																	+			−									
p-cresol	C_7H_8O				−													+			+									
benzyl alcohol	C_7H_8O				−													+			+									
p-hydroxybenzalcohol	$C_7H_8O_2$																	+												
choline	$C_9H_{19}NO_7$														+						+									−

		Acetobacteraceae sp.[1]	*Acidithiobacillaceae* sp.[2-4]	*Aeromonadaceae* sp.[5-7]	*Archaeoglobaceae* sp.[8-10]	*Ardenticatenaceae* sp.[11]	*Bacillaceae* sp.[12,13]	*Campylobacteraceae* sp.[14,15]	*Clostridiaceae* sp.[16]	*Comamonadaceae* sp.[17]	*Cyclobacteriaceae* sp.[18]	*Deferribacteraceae* sp.[19-21]	*Desulfobacteraceae* sp.[22]	*Desulfobulbaceae* sp.[22]	*Desulfuromonadaceae* sp.[23-28]	*Enterobacteriaceae* sp.[29]	*Ferrimonadaceae* sp.[30]	*Geobacteraceae* sp.[31-50]	*Holophagaceae* sp.[51]	*Melioribacteraceae* sp.[52]	*Peptococcaceae* sp.[50,53-56]	*Rhodocyclaceae* sp.[57]	*Shewanellaceae* sp.[58-63]	*Syntrophomonadaceae* sp.[74]	*Thermoanaerobacteraceae* sp.[75-77]	*Thermodesulfobacteriaceae* sp.[78]	*Thermolithobacteraceae* sp.[79]	*Thermoproteaceae* sp.[80-85]	*Thermotogaceae* sp.[85,86]	*Veillonellaceae* sp.[28,87-89]
Aldehydes and Ketones																														
acetoin	$C_4H_8O_2$																	+												
benzaldehyde*[94]	C_7H_6O				+													+			+									
p-hydroxybenzaldehyde	$C_7H_6O_2$				+													+			–									
Carboxylates																														
formate	CHO_2^-	–			+			+	–	–		+	+	+	–			+	–		+	+	+	+	–	–	+			+
oxalate	$C_2O_4^{2-}$											–						+												
acetate*[98]	$C_2H_3O_2^-$	–		–	+	+			–	+		+	+	–	+	+		+	+	+	+	+	+		–	–				+
glycolate	$C_2H_3O_3^-$																				+									
malonate	$C_3H_2O_4^{2-}$																	+												
pyruvate	$C_3H_3O_3^-$	–		+	+			+	–	+		+	+	+	+			+			+		+	+	+	–				+
propionate*[98]	$C_3H_5O_2^-$				+				–	+		+		+	+	–		+	+		–	–	–		–	–				+
lactate	$C_3H_5O_3^-$	–			–		+	+	–	+		+	+	+	+	+	+	+	+		+	+	+	+	+	–				+
glycerate*[95]	$C_3H_5O_4^-$																								+					
fumarate*[99]	$C_4H_2O_4^{2-}$	+			+			+	–			+	+	+	+			+	+		+	–	+		–	–				+
maleate*[100]	$C_4H_2O_4^{2-}$																								–					+
succinate*[101]	$C_4H_4O_4^{2-}$				+					+		+		–	+	–		+	+		+	–	+	+	–	–				+
malate*[100]	$C_4H_4O_5^{2-}$	+			+		+		–	+		+	+	+	+			+			+				–	–				+
tartrate	$C_4H_4O_6^{2-}$																	+			–									
oxalacetate	$C_4H_5O_5^{2-}$																													+
butyrate*[98]	$C_4H_7O_2^-$				+				–	–		–	+	–	–	–		+			–		–	+		–				–

		Acetobacteraceae sp.[1]	*Acidithiobacillaceae* sp.[2-4]	*Aeromonadaceae* sp.[5-7]	*Archaeoglobaceae* sp.[8-10]	*Ardenticatenaceae* sp.[11]	*Bacillaceae* sp.[12,13]	*Campylobacteraceae* sp.[14,15]	*Clostridiaceae* sp.[16]	*Comamonadaceae* sp.[17]	*Cyclobacteriaceae* sp.[18]	*Deferribacteraceae* sp.[19-21]	*Desulfobacteraceae* sp.[22]	*Desulfobulbaceae* sp.[22]	*Desulfuromonadaceae* sp.[23-28]	*Enterobacteriaceae* sp.[29]	*Ferrimonadaceae* sp.[30]	*Geobacteraceae* sp.[31-50]	*Holophagaceae* sp.[51]	*Melioribacteraceae* sp.[52]	*Peptococcaceae* sp.[50,53-56]	*Rhodocyclaceae* sp.[57]	*Shewanellaceae* sp.[58-73]	*Syntrophomonadaceae* sp.[74]	*Thermoanaerobacteraceae* sp.[75-77]	*Thermodesulfobacteriaceae* sp.[78]	*Thermolithobacteraceae* sp.[79]	*Thermoproteaceae* sp.[80-85]	*Thermotogaceae* sp.[85,86]	*Veillonellaceae* sp.[28,87-89]
isobutyrate*[98]	$C_4H_7O_2^-$									–		–						+			–									
valerate*[98]	$C_5H_9O_2^-$				+					–		+	+	–		–		+			–					–				
isovalerate*[98]	$C_5H_9O_2^-$											–						+												
caproate	$C_6H_{11}O_2^-$									–			+	–	–			–			–									–
citrate	$C_6H_5O_7^{3-}$	+			–		+					+			–	–		+			–	–	–	–		–				+
benzoate	$C_7H_5O_2^-$	–			+				–	+		–			–			+	–		+	+	–		–	–				–
p-hydroxybenzoate*[102,103]	$C_7H_5O_3^-$				+													+												
salicylate	$C_7H_5O_3^-$																	+												
p-anisate	$C_8H_7O_3^-$																	+												
caprylate	$C_8H_{15}O_2^-$											–			–			+												
t-cinnamic acid	$C_9H_7O_2^-$				+																									
caprate	$C_{10}H_{19}O_2^-$											–	+	–																
laurate	$C_{12}H_{23}O_2^-$											–			+															
palmitate	$C_{16}H_{31}O_2^-$				+							–	+	–	+			+	+				–			–				
stearate	$C_{18}H_{35}O_2^-$				+							–			+			+								–				
Sugars and Carbohydrates																														
xylose*[103]	$C_5H_{10}O_5$	+							–			–											–	–	+					
fructose	$C_6H_{12}O_6$	+					+		–			–			–			+			–		–		+					–
glucose*[103]	$C_6H_{12}O_6$	+							–		+	–			–			–	–		–	–	+		+					–
mannose*[103]	$C_6H_{12}O_6$								–			–													+					
N-acetyl-D-glucosamine	$C_8H_{15}NO_6$																				+									

		Acetobacteraceae sp.[1]	*Acidithiobacillaceae* sp.[2-4]	*Aeromonadaceae* sp.[5-7]	*Archaeoglobaceae* sp.[8-10]	*Ardenticatenaceae* sp.[11]	*Bacillaceae* sp.[12,13]	*Campylobacteraceae* sp.[14,15]	*Clostridiaceae* sp.[16]	*Comamonadaceae* sp.[17]	*Cyclobacteriaceae* sp.[18]	*Deferribacteraceae* sp.[19-21]	*Desulfobacteraceae* sp.[22]	*Desulfobulbaceae* sp.[22]	*Desulfuromonadaceae* sp.[23-28]	*Enterobacteriaceae* sp.[29]	*Ferrimonadaceae* sp.[30]	*Geobacteraceae* sp.[31-50]	*Holophagaceae* sp.[51]	*Melioribacteraceae* sp.[52]	*Peptococcaceae* sp.[50,53-56]	*Rhodocyclaceae* sp.[57]	*Shewanellaceae* sp.[58-73]	*Syntrophomonadaceae* sp.[74]	*Thermoanaerobacteraceae* sp.[75-77]	*Thermodesulfobacteriaceae* sp.[78]	*Thermolithobacteraceae* sp.[79]	*Thermoproteaceae* sp.[80-85]	*Thermotogaceae* sp.[85,86]	*Veillonellaceae* sp.[28,87-89]
cellobiose	$C_{12}H_{22}O_{11}$								–			–													+					
maltose	$C_{12}H_{22}O_{11}$								–			+						+							+					
sucrose	$C_{12}H_{22}O_{11}$								–			–												+	+					
glycogen	$C_{24}H_{42}O_{21}$																				+									
starch	$(C_6H_{10}O_5)n$							+	–			–						+			–		–	–	+					
Amino Acids and Proteins																														
glycine*[103-110]	$C_2H_5NO_2$				–				–			–	+	+	–			+							–	–				–
alanine*[103-110]	$C_3H_7NO_2$				–				–			–	+	+	–			+							–	–				+
serine*[103-106]	$C_3H_7NO_3$				+								+	+	–			+			–					–				–
aspartate*[103-107,109]	$C_4H_6NO_4^-$				–													+								–				+
asparagine	$C_4H_8N_2O_3$				+							–						+								–				
glutamate*[103-108,110]	$C_5H_8NO_4^-$	+			–							–						+			–					–				+
proline*[103-106,108]	$C_5H_9NO_2$				–				–			+			–			+								–				+
glutamine	$C_5H_{10}N_2O_3$				+													+												
betaine	$C_5H_{11}NO_2$								–						–			–			+									–
valine*[100,103,106-108]	$C_5H_{11}NO_2$								+																					
methionine*[103]	$C_5H_{11}NO_2S$																	+												
histidine*[103]	$C_6H_9N_3O_2$				–							–						+			–					–				
isoleucine*[103]	$C_6H_{13}NO_2$				+													–								–				
arginine*[103]	$C_6H_{14}N_4O_2$				+				–									+			–				–	–				
casamino acids	undefined								+			+			–			+			+		+		+					

		Acetobacteraceae sp.[1]	*Acidithiobacillaceae* sp.[2-4]	*Aeromonadaceae* sp.[5-7]	*Archaeoglobaceae* sp.[8-10]	*Ardenticatenaceae* sp.[11]	*Bacillaceae* sp.[12,13]	*Campylobacteraceae* sp.[14,15]	*Clostridiaceae* sp.[16]	*Comamonadaceae* sp.[17]	*Cyclobacteriaceae* sp.[18]	*Deferribacteraceae* sp.[19-21]	*Desulfobacteraceae* sp.[22]	*Desulfobulbaceae* sp.[22]	*Desulfuromonadaceae* sp.[23-28]	*Enterobacteriaceae* sp.[29]	*Ferrimonadaceae* sp.[30]	*Geobacteraceae* sp.[31-50]	*Holophagaceae* sp.[51]	*Melioribacteraceae* sp.[52]	*Peptococcaceae* sp.[50,53-56]	*Rhodocyclaceae* sp.[57]	*Shewanellaceae* sp.[58-73]	*Syntrophomonadaceae* sp.[74]	*Thermoanaerobacteraceae* sp.[75-77]	*Thermodesulfobacteriaceae* sp.[78]	*Thermolithobacteraceae* sp.[79]	*Thermoproteaceae* sp.[80-85]	*Thermotogaceae* sp.[85,86]	*Veillonellaceae* sp.[28,87-89]
peptone	undefined				+				+			+				–		+			+			+	+	–		+		
tryptone	undefined								+			+						+			–			+	+					
yeast extract	undefined				+	+			+	–		+			–	–		+	+		+		+		+	–		+		+
Other																															
hydrogen	H_2	+		–	±			+		–		+		+	+	+		+	–		±	–	+	+	±	±	+	+	+	+
carbon monoxide	CO																				±			–						
sulphur (elemental)	S^0		+															–							–					
e− ACCEPTORS																															
soluble Fe(III)		+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	–		+		+
amorphous Fe(III)					+	+			+	+		+	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
crystalline Fe(III)					–							–						–			+		+			–		–		

Table 2.2: Electron donors and acceptors tested on all known iron-reducing microorganisms. Microorganisms are listed by family, and represent all strains listed in Table 2.1. Key: + utilised, – not utilised, ± utilised with inorganic C source, *compound detected in carbonaceous chodrites. Note: the combined data reported for individual strains are such that a positive result overrides any negative result reported for another species of the same family. Refs: [1] Küsel *et al*., 1999, [2] Das *et al*., 1992, [3] Drobner *et al*., 1990, [4] Pronk *et al*., 1992, [5] Knight and Blakemore, 1998, [6] Pham *et al*., 2003, [7] Vivekanandhan *et al*., 2003, [8] Hafenbradl *et al*., 1996 [9] Kashefi *et al*., 2002b, [10] Tor and Lovley, 2001, [11] Kawaichi *et al*., 2013, [12] Blum *et al*., 1998, [13] Boone *et al*., 1995, [14] Laverman *et al*., 1995, [15] Stolz *et al*., 1999, [16] Slobodkin *et al*., 2006, [17] Finneran *et al*., 2003, [18] Zhang *et al*.,

2013, [19] Caccavo *et al*., 1996 [20] Greene *et al*., 1997, [21] Slobodkina *et al*., 2009, [22] Knoblauch *et al*,. 1999, [23] Coates *et al*., 1995, [24] Finster *et al*., 1994, [25] Pfennig and Biebl, 1976, [26] Roden and Lovley, 1993, [27] Sung *et al*., 2003, [28] Vandieken *et al*., 2006, [29] Francis *et al*., 2000, [30] Rosselló-Mora *et al*., 1995, [31] Caccavo *et al*., 1994, [32] Coates *et al*., 1996, [33] Coates *et al*., 2001, [34] Greene *et al*., 2009, [35] Holmes *et al*., 2004, [36] Kashefi *et al*., 2003, [37] Lovley and Phillips, 1988, [38] Lovley *et al*., 1993a, [39] Lovley *et al*., 1995, [40] Nevin *et al*., 2005, [41] Prakash *et al.*, 2010, [42] Schink and Stieb, 1983, [43] Schink, 1984, [44] Shelobolina *et al*., 2007, [45] Shelobolina *et al*., 2008, [46] Straub and Buchholz-Cleven, 2001, [47] Sung *et al*., 2006, [48] Viulu *et al*., 2013, [49] Zavarzina *et al*., 2006, [50] Kunapuli *et al*., 2010, [51] Coates *et al*., 1999, [52] Podosokoskaya *et al*., 2013, [53] Finneran *et al*., 2002, [54] Ramamoorthy *et al*., 2006, [55] Yoneda *et al*., 2012, [56] Zavarzina *et al*., 2007, [57] Cummings *et al*., 1999, [58] Bowman *et al*., 1997, [59] Bozal *et al*., 2002, [60] Caccavo *et al*., 1992, [61] Coates *et al*., 1998, [62] Kim *et al*., 2012, [63] Kosta and Nealson, 1995, [64] Leonardo *et al*., 1999, [65] Myers and Nealson, 1990, [66] Neal *et al*., 2003, [67] Nozue *et al*.,1992 [68] Roden and Zachara, 1996, [69] Roden *et al*., 2000, [70] Simidu *et al*, 1990, [71] Skerratt *et al*., 2002, [72] Toffin *et al*., 2004, [73] Venkateswaran *et al*., 1999, [74] Slobodkina *et al*., 2012, [75] Ogg *et al*., 2010, [76] Slobodkin *et al*., 1997, [77] Slobodkin *et al*., 1999, [78] Kashefi *et al*., 2002a, [79] Sokolova *et al*., 2007, [80] Feinberg *et al*., 2008, [81] Huber *et al*., 1987, [82] Kashefi and Lovley, 2000, [83] Lovley, 2013, [84] Völkl *et al*., 1993, [85] Vargas *et al*., 1998, [86] Huber *et al*., 1986, [87] Liesack and Finster, 1994, [88] Lonergan *et al*., 1996, [89] Sass *et al*., 2004, [90] Bandurski and Nagy, 1976, [91] Levy *et al*., 1973, [92] Sephton *et al*., 1998, [93] Sephton *et al*., 2000 [94] Sephton and Gilmour, 2001, [95] Jungclaus *et al*., 1976, [96] Pizzarello, 2006, [97] Cooper *et al*., 2001, [98] Yuen *et al*., 1984, [99] Cronin *et al*., 1993, [100] Lawless *et al*., 1974, [101] Pizzarello *et al*., 2001, [102] Briggs, 1963, [103] Kaplan *et al*., 1963, [104] Ponnamperuma, 1972, [105] Cronin and Pizzarello, 1983, [106] Cooper and Cronin, 1995, [107] Óro *et al*., 1971, [108] Kvenvolden *et al*., 1970, [109] Cronin *et al*., 1979 and [110] Engel *et al*., 1990.

Studies testing the ability of iron-reducing microorganisms to use crystalline iron oxides as a terminal electron acceptor are few. Kosta and Nealson (1995) observed an increase in cell numbers and production of ferrous iron when *Shewanella oneidensis* was provided with magnetite as the sole electron acceptor and formate or lactate as the electron donor, though they found that direct contact between cells and the iron oxide was essential. They point out that the reduction of magnetite is not favourable above pH 6, which may explain negative results reported for other strains in laboratory tests conducted at pH 7 (Kosta and Nealson, 1995). Nevertheless, the ability of iron-reducing microorganisms to reduce magnetite is interesting given that this mineral is often the end product of ferrihydrite reduction in the environment (Lovley, 2013).

A study testing the ability of *S. alga* to utilise crystalline ferric oxides as terminal electron acceptors reported reduction of goethite spanning a range of surface areas, in addition to hematite powder, with a corresponding increase in cell number (Roden and Zachara, 1996). Their results indicated that surface area plays a large role in how readily these crystalline iron oxides are reduced, with rates of MIR greater with high surface area synthetic goethite compared to low surface area goethite. They attribute this to the availability of active sites for the microorganisms to attach to, and hypothesised that sorption of Fe^{2+} reduces the availability of active sites more readily with oxides of lower surface areas. The importance of this sorption effect was further demonstrated with *S. alga* in a study by Urrutia *et al*. (1998), where introducing aqueous Fe^{2+}-complexants, which prevented the sorption of Fe(II) onto the ferric oxide mineral surface, enhanced rates of goethite reduction. Further support for this effect was demonstrated by Roden and Urrutia (1999), where semi-continuous cultures, allowing for the periodic removal of Fe(II), enhanced the reduction of goethite by *S. alga* two- to three-fold in comparison to batch cultures.

A study by Zachara *et al*. (1998) found that MIR by *S. oneidensis* of natural and synthetic crystalline ferric oxides was not primarily controlled by particle size, but instead by crystalline disorder and microheterogeneities. In their study, natural forms of iron oxide minerals were equally or more readily reduced than their synthetic counterparts, despite their association with non-reducible mineral phases (Zachara *et al*., 1998). In contrast to these studies, Cutting *et al*. (2009) tested the use of ferric iron oxides of varying crystallinities on a strain of *Geobacter*, which are found to be more abundant than *Shewanella* in subsurface anoxic environments (Lovley, 2013). Experiments were conducted with synthetic forms of hematite, goethite, lepidocrocite, ferrihydrite, akaganeite and schwertmanite provided as terminal electron acceptors, in which acetate was supplied as the electron donor. It was found that all ferric oxide minerals were reduced to some extent by *G. sulfurreducens*,

though the amount of MIR with more crystalline phases such as goethite and hematite was substantially less than for poorly crystalline phases, such as ferrihydrite (Cutting *et al*., 2009).

A great many studies have demonstrated the ability of numerous iron-reducing microorganisms to reduce ferrihydrite for energy conservation (see Table 2.2). Despite the relatively fewer studies testing more crystalline phases, it is evident that a range of naturally occurring ferric-iron oxides of varying crystallinities can serve as terminal electron acceptors for MIR. Nonetheless, this review demonstrates that there is a heavy bias towards strains of *Shewanella*.

2.1.2 Biogeography and growth parameters of MIR on Earth

The vast majority of all known iron-reducing isolates grow optimally at temperatures of around 30°C (see Table 2.1). These isolates typically originate from terrestrial subsurface, river or lake sediments, or from marine sediments. However, some characterised iron-reducers can be classified as extremophiles. For instance, a number of iron-reducing microorganisms originate from terrestrial and marine hydrothermal systems, and corresponding optimum growth temperatures span the moderately thermophilic range of 50-65°C (such as *Tepidimicrobium ferriphilum*, Slobodkin *et al*., 2006; and *Thermovenabulum gondwanense*, Ogg *et al*., 2010) to the hyperthermophilic range of 80-100°C (for example *Geoglobus ahangari*, Kashefi *et al*., 2002b; and *Pyrobaculum aerophilum*, Finneran *et al*., 2003). Additionally, the Archaeal iron-reducers all fall into the hyperthermophilic category which, given their deep branching phylogeny, has been suggested as evidence that MIR could have been one of the earliest forms of respiration on the early Earth (Vargas *et al*., 1998). This hypothesis is reinforced by the ability of some deep branching bacteria and archaea, such as *Geothermobacterium ferrireducens* (Kashefi *et al*., 2002a), to respire ferric iron autotrophically, using hydrogen and inorganic carbon (CO or CO_2) serving as electron donor and carbon sources, respectively (Lovley, 2013).

On the opposite end of the temperature scale, a small number of cold-adapted (psychrotolerant) microorganisms capable of energy conservation through MIR have been characterised. These microorganisms grow optimally below 15°C, and were isolated from Arctic marine sediments (Knoblauch *et al*., 1999; Vandieken *et al*., 2006). However, a larger number of microorganisms, whose optimal growth temperatures exceed temperatures

that qualify as psychrophilic ('cold-loving'), can tolerate temperatures near or below freezing (e.g., *Pelobacter propionicus*, Schink, 1984; Lonergan *et al*., 1996).

The range of pH environments tolerated by iron-reducing microorganisms is comparatively less extreme, with almost all strains exhibiting optimum growth at or near pH 7. The exceptions to this rule include the acidophilic microorganisms *Acidiphilium cryptum* (Küsel *et al*., 1999) and *Acidithiobacillus ferrooxidans* (Drobner *et al*., 1990, Das *et al*., 1992, Pronk *et al*., 1992, Kelly and Wood, 2000), with respective optima of 3.2 and 2.5. The latter microorganism carries out iron oxidation under aerobic conditions, but is known to reduce iron coupled to the oxidation of elemental sulphur under anoxic conditions at low pH (Drobner *et al*., 1990; Das *et al*., 1992; Pronk *et al*.,1992). At the alkali end of the spectrum, a similarly small number of iron-reducers exhibit optimal growth greater than pH 8, including *Bacillus arsenicoselenatis* (Blum *et al*., 1998) and *Geoalkalibacter ferrihydriticus* (Zavarzina *et al*., 2006).

The current knowledge of growth parameters and environmental conditions of MIR (Table 2.1) hints towards a metabolism that proliferates in benign environments, characterised by circumneutral pH values and temperatures close to 30°C. However, the restricted pH and, to a lesser extent, temperature conditions known to sustain iron-reducing microorganisms may in part demonstrate bias resulting from two technological applications; firstly the use of iron-reducing microorganisms as biological agents in the clean up of contaminated environments, and secondly their application in microbial fuel cells. With respect to former, their ability to metabolise compounds hazardous to human health lends iron-reducing microorganisms to be used as bioremediation agents (Finneran *et al*., 2002; Anderson *et al*., 2003). These hazardous compounds include hydrocarbons, and metals and metalloids such as uranium and selenium (Lloyd, 2003). In all cases, some iron-reducing microorganisms can render the hazardous substance in question less hazardous, either through their oxidation to smaller and non-toxic organic compounds (in the case of organic electron donors), or from the reduction of soluble terminal electron acceptors to their reduced insoluble forms. In the case of microbial fuel cells, some iron-reducing microorganisms are exploited to harness the transfer of electrons from organic waste to carbon anodes, in order to generate electricity (e.g., *Geobacter sulfurreducens*, Bond and Lovley, 2003; Nevin *et al*., 2008).

It has become increasingly more apparent that iron-reducing microorganisms play a significant role in the functioning of natural ecosystems, namely through the degradation of organic matter and the availability and form of iron-bearing substrates (Lovley, 2013). Furthermore, evidence points to MIR as one of the earliest microbial metabolisms on Earth

(Vargas *et al*., 1998). Yet iron-reducing microorganisms remain one of the least studied of all the chemosynthetic microorganisms (Lovely, 2013), which has left knowledge gaps regarding the limits to microbial iron reduction on Earth, and the feasibility of MIR on Mars, past and present.

2.2 Habitable Mars?

The present day surface of Mars is a highly inhospitable environment. Mean daily temperatures in equatorial regions average 215 K, and pressure is a mere 6 millibars (Carr, 1996). These conditions render the surface equal to the triple-point of water; hence liquid water is unstable. Mars has an atmosphere less than 1% as thick as Earth's, containing little to no ozone, which provides negligible protection from harmful ultraviolet (UV) radiation (Cockell and Raven, 2004). Furthermore, the lack of a substantial global magnetic field allows ionising radiation to penetrate to the surface and near surface environments (Dartnell *et al*., 2007). Collectively, these forms of radiation cause damage to organic compounds (ten Kate *et al*., 2005), and simulations have indicated microbial cells would survive such radiation for only tens of minutes to a few hours (Schuerger *et al*., 2006). Evidence from the Magnetic Field Experiment / Electron Reflectrometer instrument aboard the Mars Global Surveyor orbiter suggests Mars once had a magnetic field until around 4 billion years ago, when Mars's internal dynamo appears to have ceased (Acuña *et al*., 1999). This led to the erosion of a once thicker atmosphere by ionising solar radiation (Chassefière and Leblanc, 2004). The consequential high doses of radiation at the surface are thought to have resulted in the presence of strong chemical oxidising agents in the soil (Zent and McKay, 1994), such as perchlorate salts that were detected at the northern plains of Mars by the Wet Chemistry Laboratory onboard the Phoenix Lander (Hecht *et al*., 2009).

Despite the cold barren desert conditions that prevail on Mars today, there is a wealth of evidence indicating the existence of liquid water on the surface of Mars. Ample geomorphological evidence from orbital spacecraft over the last few decades has revealed familiar meandering river channels, valley networks and fluvial depositional features, such as deltas and alluvial fans, common to Earth (Baker, 2001; 2006). Further evidence suggests the presence of lakes within impact craters (Malin and Edgett, 2003), and *in situ* rover investigations have reported sedimentary features that further support orbital evidence (e.g.

Squyres *et al*., 2004). Most recently, the Mast Camera onboard Mars Science Laboratory revealed rounded pebbles and sand grains indicative of sustained flowing water on the order of several kilometers across the ancient Martian landscape (Williams *et al*., 2013).

Perhaps more compelling than this geomorphological evidence of a wetter Mars is the wealth of mineralogical evidence. The present-day surface of Mars is dominated by unweathered primary volcanic minerals, such as plagioclase feldspar, pyroxene and olivine (Christensen *et al*., 2008). However, global orbital investigations have uncovered widespread regions of hydrated minerals, indicative of the presence of liquid water at the time of formation. These can be grouped into three classes, namely hydrated phyllosilicates, hydrated sulphates, and in some cases ferric oxides (Bibring and Langevin, 2008).

Phyllosilicates are clay minerals that form from the interaction of nominally anhydrous volcanic minerals with water (Guggenheim and Martin, 1995). This interaction results in the incorporation of hydroxyl (OH) or water (H_2O) molecules in their structure (Ehlmann *et al*., 2011). The OMEGA spectrometer onboard the Mars Express orbiter has unambiguously detected a diverse family of phyllosilicates at the surface of Mars, of which nontronite was the most abundant (Poulet *et al*., 2005, Bibring *et al*., 2005). Higher resolution spectroscopic data from the CRISM instrument on the Mars Reconnaissance Orbiter expanded the known inventory of this mineral class (Mustard *et al*., 2008; Carter *et al*., 2013), the diversity and abundance of which indicates widespread liquid water activity in a variety of alteration processes (Mangold *et al*., 2007). On Earth these minerals are more typical of circumneutral aqueous environments (Ehlmann *et al*., 2009).

Sulphate minerals have been detected from orbit by OMEGA (Bibring *et al*., 2005; Gendrin *et al*., 2005; Langevin *et al*., 2005; Bibring *et al*., 2007) and CRISM instruments (Bishop *et al*., 2009; Farrand *et al*., 2009; Wray *et al*., 2009), and *in situ* by Mars Exploration Rovers, Spirit (Morris *et al*., 2006b) and Opportunity (Klingelhöfer *et al*., 2004; Squyres *et al*., 2006; Ruff *et al*., 2008), and the Mars Science Laboratory Curiosity rover (Bish *et al*., 2013; McLennan *et al*., 2014). The presence of sulphates has been attributed to the existence of acidic ambient waters, and their occurrence on opposite sides of the planet is taken as evidence that acidic ambient waters were common at the time these minerals formed (Squyres *et al*., 2006; Bibring *et al*., 2007; Morris and Klingelhöfer, 2008).

Unlike phyllosilicates and sulphate minerals, the presence of ferric oxides on Mars is not necessarily evidence of aqueous conditions upon formation. However, the association of crystalline hematite with sedimentary rocks supports their formation through precipitation from iron-rich standing bodies of water (Christensen *et al*., 2000, 2001). Furthermore, orbital

detection of these crystalline hematite deposits associated with sulphate minerals, which are unambiguously aqueous in origin, is considered evidence of an extensive acidic aqueous system (Bibring *et al*., 2007). The Opportunity rover at Meridiani Planum detected and analysed crystalline hematite in the form of small spherules, which on Earth are indicative of the presence of liquid water. The spectral signatures of these hematitic spherules on Mars firmly support an aqueous origin (Christensen *et al*., 2004; Brückner *et al*., 2008; Ruff *et al*., 2008).

Collectively, detection of these alteration minerals are considered compelling evidence of widespread aqueous activity on Mars, and are supported by the detection of similar mineral classes detected in Martian meteorites (Gooding, 1992; Bridges and Grady, 2000; Bridges *et al*., 2001; Beard *et al*., 2013). The presence of liquid water on or near the surface of Mars is critical for habitability, but our understanding of the history of the planet indicates such conditions have long since vanished.

Our current understanding of the geological history of Mars stems from studying the density and sizes of observable impact craters on the surface, coupled with dating Martian meteorites (Hartmann and Neukum, 2001). The history of Mars has thus been separated into three major geological epochs. The oldest of these is the Noachian period, dating back to the formation of the Hellas impact crater approximately 4.1 to 3.8 billion years (Gyr) ago (Hartmann and Neukum, 2001). Not much is known about the history of Mars prior to this point, though it is thought that Mars accreted and differentiated within tens of millions of years of formation, and hosted a magnetic field (Carr and Head, 2010). Areas of the Martian surface that date back to the Noachian indicate that this period was characterised by high rates of impact cratering, erosion, and valley formation. The latter is evidenced by numerous terrains of this age dissected by valley networks (Carr and Head, 2010). It was also during this period that the aforementioned phyllosilicate minerals formed through the aqueous alteration of the parent volcanic mineralogy (Bibring *et al*., 2006). When coupled to the prevalence of valley networks in Noachian terrains, it is thought that these minerals are evidence of at least episodic periods of warm surface conditions and precipitation that ceased to occur in later epochs (Carr and Head, 2010).

The Noachian period prevailed until around 3.7 Gyr ago, at which point conditions changed globally and the period known as the Hesperian began (Hartmann and Neukum, 2001). Characterised by continued volcanism, it was during this epoch that extensive lava plains formed which buried previously extensive areas of Noachian phyllosilicates (Ehlmann *et al*., 2011). Major outflow channels were created from large water floods, and the canyon

systems were formed (Carr and Head, 2010). It is thought that the loss of much of Mars' CO_2-rich atmosphere occurred during the Hesperian, giving rise to the much colder and drier conditions of present-day Mars (Bibring and Langevin, 2008). The mineralogy of this period is dominated by regions of hydrous sulphate minerals, which began to form towards the end of the Noachian period. Unlike the phyllosilicates, which could have formed either at the surface of in the subsurface of Mars (Bibring *et al*., 2006; Ehlmann *et al*., 2011), sulphate minerals form through the precipitation of sulphate-rich water bodies; thus their formation must have occurred in aqueous environments at the surface. These sulphate minerals are more extensive than the phyllosilicates, and are thought to have formed from the large quantities of sulphur liberated through the volcanic activity of this epoch (Bibring *et al*., 2006). Upon contact with water, this sulphur would have oxidised to form sulphuric acid, which is thought to have created the acidic conditions necessary to form the jarosite detected at the surface today (Klingelhöfer *et al*., 2004; Morris *et al*., 2006a, 2006b; Farrand *et al*., 2009).

Approximately 3 Gyr ago global conditions changed to the extremely arid and cold surface environment characteristic of the Amazonian that continues to present day (Hartmann and Neukam, 2001). Surface geology from this period exhibits extremely low rates of erosion (Carr and Head, 2010), and the spectral signature of ferric iron present everywhere on the surface indicates a transition to global oxidation (Poulet *et al*., 2007). Given the lack of evidence of hydrated ferric oxides, this oxidation is not thought to be aqueous, driven instead by oxidants in the atmosphere (Bibring *et al*., 2006; Bibring and Langevin, 2008).

Of these three periods in Martian history, the Noachian is considered the most conducive to life (Bibring *et al*., 2006; Bibring and Langevin, 2008; Murchie *et al*., 2009; Ehlmann *et al*., 2011; Beard *et al*., 2013). From this point surface conditions became progressively more inhospitable, with the majority of Mars' history dominated by the harsh conditions seen on the surface today. However, recent evidence from rover missions indicates that habitable conditions prevailed for hundreds of millions of years through the Noachian and early Hesperian. Arvidson *et al* (2014) reported results from the Opportunity rover of analysis of sedimentary rocks at the rim of Noachian-aged Endeavour crater, estimated to be in the region of 4 billion years old. These fine-grained layered sedimentary rocks, containing spherules that exhibit concentric internal structures and cross cut by fractures, constitute the earliest evidence of water activity detected by Mars rovers (Arvidson *et al*., 2014). The Curiosity rover, which landed in Gale crater in August 2012, recently identified signs of a crater lake dating back to the early-Hesperian period (Grotzinger *et al*., 2014). This crater is

inferred to have formed at the Noachian-Hesperian boundary, approximately 3.7 billion years ago (Thomson *et al*., 2011). Similarly fine-grained rocks were identified, and the mineralogy attributed to the presence of a near-neutral, low salinity body of water, with an estimated duration of hundreds to tens of thousands of years (Grotzinger *et al*., 2014). Both environments are considered habitable, and indicate the likely presence of similar conditions over a period of hundreds of millions of years at a time when microbial life proliferated on Earth.

Given the transition to progressively harsher surface conditions after the early Hesperian, life would need to seek refuge in the subsurface to survive. A subsurface mode of living could not rely on photosynthetic pathways, nor would aerobic metabolisms operate given the mere trace quantities of oxygen present in the Martian atmosphere. As such, the most plausible candidate metabolisms to operate in the subsurface of Mars are driven by redox metabolisms (Nixon *et al*., 2013). Although the mineralogy on Mars is such that Hesperian-aged sulphate minerals provide potential energy for microbially-mediated sulphate-reduction, the significant enrichment of iron and resulting ferric compounds on Mars has driven this thesis to focus on microbially-mediated reduction of iron.

2.3 The feasibility of MIR on Mars

Iron is everywhere more abundant in the Martian crust relative to Earth (Boynton *et al*., 2008; Brückner *et al*., 2008). This raises the question of whether Mars could support life through iron redox reactions. The following review assesses the presence of redox couples on Mars that could support MIR.

2.3.1 Sources of electron donors on Mars

2.3.1.1 Organic electron donors

Organic compounds are prevalent in the solar system, and are delivered to planetary bodies in the form of meteorites and interplanetary dust particles (Flynn, 1996). The class of meteorites that contain organic material is the carbonaceous chondrites, which typically

contain up to 2 % by weight organic compounds (Sephton, 2002). Due to the thinner atmosphere and lower surface gravity, the quantity of meteoritic material delivered to the surface of Mars is thought to be an order-of-magnitude higher than on Earth. Furthermore, entry velocities (and hence impact temperatures) are lower, which results in more of the meteorite remaining intact than an equivalent impact on Earth. It is estimated that 8.6×10^6 kg of meteoritic material is delivered to the surface of Mars each year. Of this, 2.4×10^6 kg is unaltered organic matter (Flynn, 1996). It is therefore a surprise that neither Viking or Phoenix lander missions, equipped with instruments to detect organic compounds at ppb to ppm levels, failed to detect any organics at the Martian surface (Biemann *et al*., 1977; Klein, 1978; Sutter *et al*., 2009; Ming *et al*., 2009). The Viking gas chromatography mass spectrometers (GCMS) in particular should have detected numerous organic compounds, since these lander instruments were capable of releasing volatiles even from non-volatile compounds (Sephton *et al*., 1998; Benner *et al*., 2000). It was therefore suggested that the lack of organic compounds at the surface is due to the presence of strong chemical oxidising agents, which on Earth are known to completely oxidise organic compounds to carbon dioxide and water (Klein, 1979). This hypothesis is strongly supported by the subsequent detection of perchlorate salts in Martian soils by the Phoenix lander (Hecht *et al*., 2009).

On Earth, the production of perchlorate is an atmospheric process, resulting from the reaction of chlorine species with oxygen. The result is a globally-distributed inventory, though accumulation is favoured in arid environments (Kounaves *et al*., 2010, Catling *et al*., 2010). The production of perchlorate on Mars is thought to be similar (Catling *et al*., 2010). Furthermore, concentrations in Martian soils are thought to be higher relative to those on Earth, given the relatively more arid conditions that have prevailed throughout most of Mars' history (Navarro-González *et al*., 2010). The high oxidation state (+7) of the chlorine atom in the perchlorate molecule renders it a strong oxidising agent, though it is very stable unless exposed to acidity or heat (Parker, 2009). It is therefore thought that when Martian soil samples were heated during the Viking Biology experiments, any organic compounds present would have been oxidised by the unstable perchlorates, resulting in the lack of detection reported (Ming *et al*., 2009). This phenomenon was demonstrated in an experiment using Mars analogue soils from the Atacama Desert, in which soils containing 32 ± 6 ppm organic carbon were mixed with 1% magnesium perchlorate, and subsequently analysed using an instrument similar to the Viking GCMS (Navarro-González *et al*., 2010). Their analysis showed that almost all the organics previously present in the soil had decomposed to carbon dioxide and water, leading them to hypothesise that the Martian soil samples

analysed by the Viking GCMS instruments contained between 0.5 and 6.5 ppm organic carbon, oxidised by 0.1% or less perchlorate salts.

Based on the lack of evidence of widespread geological alteration of ancient Martian terrain, it has been suggested that the oxidising layer present at the surface is only as deep as the chemical oxidants can diffuse (Carr and Clow, 1981; Kanavarioti and Mancinelli, 1990). It is below this layer that exogenous organic material delivered to the Martian surface throughout geological history is expected to reside (Kanavarioti and Mancinelli, 1990).

Of the organic material found in carbonaceous chondrites, the majority (more than 70%) is solvent-insoluble macromolecular carbon, analogous to kerogens on Earth (Hayes, 1976; Becker *et al*., 1999; Botta and Bada, 2002). The remainder consists of solvent-soluble compounds, including aliphatic and aromatic hydrocarbons (Olson *et al.*, 1976; Cronin and Pizzarello, 1990), sugars (Cronin *et al*., 1993), carboxylic acids (Cronin *et al*., 1993), amino acids (Cronin and Pizzarello,1983; Ehrenfreund *et al*., 2001) and nucleobases (Martins *et al*., 2008), amongst others (Sephton, 2002). A number of these compounds are known to serve as electron donors for MIR on Earth, as identified in Table 2.2. For example, a large number of iron-reducing microorganisms conserve energy by coupling the reduction of poorly crystalline iron oxide to the oxidation of acetate, the carboxylate anion of acetic acid, one of many compounds identified in the Murchison meteorite (Yuen *et al*., 1984). Amino acids are known to be prevalent in the organic fraction of carbonaceous chondrites, and span essential amino acids such as alanine and glycine (e.g. Kaplan *et al*., 1963; Cronin and Pizzarello, 1983) to relatively rare compounds in terrestrial environments, such as α-aminoisobutyric acid (Kvenvolden *et al*., 1971). This is interesting since *Geopsychrobacter electrodiphilius*, along with other members of the *Geobacteraceae* family, are known to use amino acids as electron donors for MIR (Table 2.2). Despite this overlap, there are many more organic compounds that have been identified in carbonaceous meteorites than have been tested as electron donors for MIR. As such, the inventory of potential electron donors of extraterrestrial origin buried beneath the Martian surface is likely to be much greater.

A recent study by Steele *et al*. (2012) reported the detection of macromolecular organic carbon in ten out of the eleven Martian meteorites studied, and their association with magmatic minerals indicates that such reduced carbon is nearly ubiquitous in Martian basalts. Similar material was identified in the NWA 7034 Martian meteorite, radiometric dating of which indicates it formed in the early Amazonian, the only such specimen of this age (Agee *et al*., 2013). The mechanism of formation of this carbonaceous material is thought to be precipitation of reduced carbon speciation during crystallisation, hence this crustal reservoir of abiotic carbon dates back to very early in the history of Mars (Steele *et*

al., 2012). This deep store of organic carbon may contribute to the inventory of electron donors on Mars, though it remains to be seen whether macromolecular carbon in such materials as shales and kerogens represent a usable source of electron donors on Earth.

Organic compounds may have formed at the surface of Mars *in situ*. Such endogenous production of organics is thought to represent an important source of organic compounds on the early Earth, and the mechanisms of formation include inputs into the planetary atmosphere by spark discharges (Miller, 1953), shock waves (Bar-Nun *et al*., 1970), ultraviolet light (Sagan and Khare, 1971) and high energy particles emulating cosmic rays (Kobayashi *et al*., 1997, 2001). As demonstrated by Miller (1953), a spark discharge applied to a highly reducing atmosphere led to the production of amino acids. Owing to our advanced understanding of planet formation and resulting atmospheric composition, it now seems that the atmosphere of early Earth was only mildly reducing than was simulated for Miller's landmark experiment, and hence the potential yield of endogenous organics is correspondingly less (Heinrich *et al*., 2007). Nonetheless, their contribution to the terrestrial prebiotic organic inventory is considered important (Chyba and Sagan, 1992).

It is thought that the early atmospheres of Earth and Mars were similar, comprising a mixture of nitrogen, hydrogen, carbon monoxide and carbon dioxide (Abelson, 1965; Heinrich *et al*., 2007). It is therefore possible that the in situ production of organic compounds also occurred on ancient Mars. However, there are a number of reasons to suggest the quantity of organics produced on Mars was much less significant than on Earth. Firstly, owing to its much smaller mass, Mars more readily loses hydrogen to space, which leaves the atmosphere less reducing, and hence less conducive for endogenous production. In one experiment, the presence of large amounts of hydrogen proved to be crucial in the endogenous production of amino acids (Abelson, 1965). Additionally methane and ammonia, which also operate as atmospheric reducing agents, are thought to have readily oxidised in the Martian atmosphere (Abelson, 1965). Secondly, the atmospheric erosion caused by large impacts and the loss of a global magnetic field is thought to have stripped away any sufficiently reducing atmosphere that Mars may once have possessed (Melosh and Vickery, 1989).

2.3.1.2 Inorganic electron donors

Organic compounds are not the only potential source of electron donors for MIR on Mars. In the event that the organic inventory expected from meteoritic infall is severely depleted or lacking, inorganic compounds may present an alternative source of electron donors for MIR. As previously discussed, a number of iron-reducing microorganisms can utilise molecular hydrogen for iron reduction. Hydrogen is liberated in the process of serpentinisation, which occurs as a result of the hydration of mafic minerals (Quesnel *et al*., 2009). Ample evidence for the existence of this precursor mineralogy has been identified on Mars (Hoefen *et al*., 2003; Poulet *et al*., 2007; Koeppen and Hamilton, 2008), in addition to the *in situ* detection by the Spirit rover at Gusev Crater (McSween *et al*., 2004). Due to the higher iron content in Martian olivine compared to that on Earth, it is expected that greater volumes of hydrogen would be liberated in any serpentinisation processes on Mars (Schulte *et al*., 2006). More recently, rocks bearing serpentine itself have been identified at the Martian surface (Ehlmann *et al*., 2010). However, in order for serpentinisation to represent a present-day source of molecular hydrogen for MIR the process must take place in the subsurface, out of reach of current orbital and rover detection capabilities.

Organic compounds used for MIR also serve to satisfy the need for cellular carbon, a universal requirement for all known life. In contrast, the use of molecular hydrogen as an electron donor for MIR hinges on the presence of an additional carbon source. Although organic carbon sources are favoured or required by hydrogen-utilising iron-reducers, an inorganic carbon source can suffice for some (see Table 2.2). However, hydrogen produced by serpentinisation on Earth is known to form methane in the presence of carbon dioxide (Kelley *et al*., 2005). This process was proposed as an explanation for the recent observations of methane in the Martian atmosphere (Oze and Sharma, 2005; Attreya *et al*., 2007), though the lack of methane detection by the Curiosity rover renders these observations questionable (Webster *et al*., 2013). Nonetheless, methane is known to serve as a carbon source for other chemosynthetic microorganisms, namely sulphate-reducing and denitrifying microorganisms (Michaelis *et al*., 2002; Thalasso *et al*., 1997). Therefore it is feasible that the same is true for MIR. The ability of iron-reducers to utilise methane as a carbon source remains an unanswered question, however if it proved to satisfy this requirement it may represent a carbon source for hydrogen-driven MIR in serpentinising regions of the Martian subsurface.

2.3.2 Sources of terminal electron acceptors

Despite iron existing primarily in the ferrous state in the parent basaltic rocks, Mars hosts a plethora of ferric-iron bearing minerals, as summarised in Table 2.3. Many of these minerals overlap with those tested and identified as terminal electron acceptors for characterised iron-reducing microorganisms on Earth. The crystalline iron oxides hematite, magnetite and goethite have all been identified at the Martian surface, of which the former dominates (Christensen *et al*., 2001; Klingelhöfer *et al*., 2004; Morris *et al*., 2006a, 2006b; Christensen *et al*., 2008; Brückner *et al*., 2008; Ruff *et al*., 2008). Widespread outcrops of crystalline gray hematite, an oxide reduced by some iron-reducing microorganisms (albeit with questionable ability to support growth, Cutting *et al*., 2009) have also been detected at various regions of Mars (e.g., Christensen *et al*., 2001, 2004). Furthermore, the poorly crystalline iron oxides that most readily serve as electron acceptors in natural environments on Earth also exist on Mars. Nanophase ferric oxides are ubiquitous on the Martian surface, and their crystallinity is thought to be short-ordered, similar to ferrihydrite (Ehmann, 2011; personal communication). The ferric oxide-hydroxide akaganeite, after which the iron-reducer *Geobacter akaganeitreducens* was named (Straub *et al*., 1998), was recently detected in a number of locations at Gale Crater by the Curiosity rover (Bish *et al*., 2013; McLennan *et al*., 2014; Ming *et al*., 2014). Additionally, ferrihydrite has been identified in Martian meteorites, which represent the only subsurface samples of Mars analysed to date (Gooding *et al*., 1991). It is therefore evident that terminal electron acceptors known to support MIR exist on Mars.

Few studies have been conducted in which iron-bearing phyllosilicates are tested as terminal electron acceptors for MIR, though the structural iron in such minerals is known to be redox-active (Gorski *et al*., 2012a, 2012b). Wu *et al*. (2012) extracted Fe(III)-bearing smectite and illite from bulk subsurface sediments, and found them to support MIR. More recently Benzine and colleagues (2013) enriched microorganisms from subsurface sediments that were found to be capable of metabolising structural iron in Fe-bearing phyllosilicates. In their study, a strain of *Geobacter bremensis* was found to grow by the reduction of a Fe-smectite as an electron acceptor, with acetate as an electron donor. Therefore it is feasible that ferric-iron-bearing phyllosilicates on Mars, such as nontronite, represent an additional viable source of electron acceptors for subsurface MIR.

Class	Mineral	Formula	Source	Refs
Iron oxides	Hematite	Fe_2O_3	orbit	2, 3, 4, 9
			rover	5, 6, 7, 8
			meteorite	1
	Maghemite	Fe_2O_3	meteorite	13
	Magnetite	Fe_3O_4	orbit	10, 11, 12
			rover	6, 7, 8
	Goethite	FeO(OH)	orbit	14
			rover	6, 8
			meteorite	11
	Lepidocrocite	FeO(OH)	meteorite	11
	Ferrihydrite	$Fe_2O_3 \cdot 0.5H_2O$	meteorite	1
	Akaganeite	$Fe_{7.}6Ni_{0\cdot4}O_{6.4}(OH)_{9.7}$	rover	10, 12, 15
	Nanophase ferric oxide	unknown	orbit	16
			rover	7, 8
Iron sulphates	Jarosite	$KFe_3(SO_4)_2(OH)_6$	orbit	14
			rover	5, 7, 8
	Ferricopiapite	$Fe_{2/3}Fe_4(SO_4)_6(OH)_2 \cdot 20H_2O$	orbit	18
Phyllosilicates	Nontronite	$Na_{0.3}Fe_2Si_3AlO_{10}(OH)_2 \cdot 4H_2O$	orbit	19, 20, 21
	Chamosite	$Fe^{(2+)}{}_3Mg_{1.5}AlFe^{(3+)}{}_{0.5}Si_3AlO_{12}(OH)_6$	orbit	20
	Hisingerite	$Fe_2Si_2O_5(OH)_4 \cdot 2(H_2O)$	orbit	10

Table 2.3: **Ferric iron minerals detected on the surface of Mars to date.** Refs: [1] Gooding *et al*., 1991, [2] Christensen *et al*., 2000, [3] Christensen *et al*., 2003, [4] Christensen *et al*., 2004, [5] Klingelhöfer *et al*., 2004, [6] Klingelhöfer *et al.*, 2005, [7] Morris *et al*., 2006a, [8] Morris *et al*., 2006b, [9] Fraeman *et al*., 2013, [10] Bish *et al*., 2013, [11] Rull *et al*., 2004, [12] McLennan *et al*., 2014, [13] Agee *et al*., 2013, [14] Farrand *et al*., 2009, [15] Ming *et a*l., 2014, [16] Poulet *et al*., 2007, [17] Milliken *et al*., 2008, [18] Bishop *et al*., 2009, [19] Bibring *et al*., 2005, [20] Poulet *et al*., 2005, [21] Ehlmann *et al*., 2009.

2.4 Relevant other work

Aside from the review papers that resulted from this research (Nixon *et al*., 2012, 2013), only two publications directly linkMIR with the search for life on Mars (Nealson and Cox, 2002; Weber *et al*., 2006a). However there are significantly more studies presenting evidence of MIR in environments considered analogous to Mars, past and present.

Río Tinto is a naturally acidic ecosystem, emanating from the Iberian Pyritic Belt in Spain. Characteristics of the Río Tinto system include the constantly acidic pH (mean of 2.3), high concentrations of heavy metals, and most relevant to Mars are the high concentrations of ferric iron and sulphates found in its waters (Amilis *et al*., 2007). The majority of the microbial diversity of Río Tinto relates to acidophilic members of the iron cycle, most notably species of *Leptospirillum*, *Acidiphilium* and the strain *Acidithiobacillus ferrooxidans* (formerly *Thiobacillus ferroxidans*) (Lopez-Archilla *et al*., 2001; González-Toril *et al*., 2003; García-Moyano *et al*., 2007). The latter is known to reduce iron using elemental sulphur in anoxic conditions (Pronk *et al*., 1992). It is common for concentrations of ferric iron at acid mine drainage sites to exceed concentrations of sulphate by several orders of magnitude, and thus represents a widely available electron acceptor for microbial metabolisms (Druschel *et al*., 2004; Coupland and Johnson, 2008).

Through the application of culture-independent techniques, Sánchez-Andrea and colleagues (2011) identified the presence of microorganisms capable of MIR at low pH values in anaerobic sediments from Río Tinto. A culture-based follow-up study of the same sediments neglected to enrich for iron-reducing microorganisms, though sulphate reduction was observed and molecular identification indicates this was carried out by strains of *Desulfomaculum* and *Desulfosporosinus* (Sánchez-Andrea *et al*., 2012). One species of the latter, *D. lacus*, is known to grow from MIR (Ramamoorthy *et al*., 2006), and alongside *A. ferrooxidans* represents a likely candidate for MIR in this environment.

Subglacial environments, once thought too cold and dark to harbour life, are now known to support diverse communities of microorganisms. Given that Mars hosts vast bodies of ice locked up in the polar caps, in addition to evidence of smaller glaciers at lower latitudes today (Holt *et al*., 2008; Plaut *et al*., 2009) and in the past (Clifford, 1987; Head *et al*., 2006; Head *et al*., 2010), these environments are considered analogous to Mars (Skidmore *et* al., 2000; Christner *et al*., 2008; Fisher and Schulze-Makuch, 2013). Microbial communities have been studied in subglacial sediment and outflow samples from Arctic

(Skidmore *et al*., 2000, 2005; Wadham *et al*., 2004; Bhatia *et al*., 2006; Kaštovská *et al*., 2007; Yde *et al*., 2010; Boyd *et al*., 2010, 2011; Stibal *et al*., 2012; Mitchell *et al*., 2013), Antarctic (Mikucki and Priscu, 2007; Lanoil *et al*., 2009; Stibal *et al*., 2012), and valley glaciers (Sharp *et al*., 1999; Campen *et al*., 2003; Foght *et al*., 2004; Gaidos *et al*., 2004; Buzzini *et al*., 2005; Marteinsson *et al*., 2013).

Phylogenetic analysis of 16S DNA from subglacial sediments indicates the presence of iron-reducing microorganisms in a number of geographically-distinct subglacial environments. Sequences affiliated with *Rhodoferax ferrireducens*, a psychrotolerant iron-reducing isolate, were detected in samples from beneath Bench Glacier, Alaska (Skidmore *et al*., 2005), Robertson Glacier, Canada (Mitchell *et al*., 2013) and the Kamb Ice Stream, West Antarctica (Lanoil *et al*., 2009). Strains closely related to *Geopsychrobacter electrodiphilus* were identified in Blood Falls subglacial outflow, draining from the Taylor Glacier in the Dry Valleys of Antarctica (Mikucki and Priscu, 2007), and strains affiliated with *Geobacter* were recovered from beneath the Vatnajökull ice cap in Iceland (Marteinsson *et al*., 2013).

To date only two studies have attempted to enrich for these iron-reducers to assess their viability. In the first instance Foght and colleagues (2004) successfully cultured ferric-iron reducing bacteria from sediments retrieved from beneath two temperate glaciers in New Zealand, but noted they were few in number. More recently, Mikucki and Priscu (2007) reported iron reduction in the enrichment of Blood Falls samples after nine months of incubation at near-freezing temperatures. Isolation and subsequent phylogenetic analysis indicated a close relation to the strain *Shewanella frigidimarina*, originally isolated from Antarctic sea ice (Mikucki and Priscu, 2007; Bowman *et al*., 1997).

At the other end of the temperature scale, Yellowstone National Park (herein referred to as Yellowstone) hosts hundreds of hydrothermal springs considered analogous to impact- or volcanically-generated hydrothermal activity on Mars, a plausible explanation for the presence of a number of hydrous minerals detected at the Martian surface (Christensen *et al*., 2000; Poulet *et al*., 2005; Morris *et al*., 2006a; Bishop *et al*., 2009; Ehlmann *et al*., 2009; Carter *et al*., 2013).

Although not the only hydrothermal analogue environment, Yellowstone is by far the most studied. A number of these studies have focused on individual persistent pools, such as Obsidian Pool, which is characterised by slightly acidic pH and temperatures of around 80°C (Meyer-Dombard *et al*., 2005). It is from this pool that the hyperthermophilic autotrophic iron-reducer *Geothermobacterium ferrireducens* was isolated (Kashefi *et al*., 2002a), and was later detected in Bison Pool, characterised by a similar temperature but

relatively alkaline pH (7-9.5) (Meyer-Dombard *et al*., 2005). *Thermolithobacter ferrireducens*, another autotrophic iron-reducing microorganism, was isolated from Calcite Spring in Yellowstone, where temperatures are similarly high (~70°C) and pH is near-neutral (Sokolova *et al*., 2007). MIR was observed in sediments from the iron-rich moderately-warm (45-50°C) Chocolate Pots, and phylogenetic analysis revealed close association of strains to the well-known iron-reducing bacterium *Geobacter metallireducens* (Fortney *et al*., 2014).

With the exception of the few studies in which iron-reducing microorganisms have been isolated and characterised, there is a strong bias towards using culture-independent phylogenetic tools in many studies of MIR in Mars-relevant environments. The result is a limited understanding of the viability of iron-reducers identified. It thus remains to be seen whether MIR is an active microbial metabolism in many of these settings.

2.5 The knowledge gap

Despite the strong argument for the presence of MIR redox couples on Mars, there are a number of limitations to our knowledge that impede our ability to constrain habitability. These fall into two categories, firstly limitations due to a lack of experimental data of Earth-based systems (Earth-based limitations; EBLs), and secondly limitations caused by a lack of knowledge of Mars itself (Mars-based limitations; MBLs), which are summarised below. The EBLs form the basis of the following chapters of this thesis, and individual chapters are indicated in parentheses accordingly.

1. The majority of isolated iron-reducing microorganisms to date originate from environments characterised by moderate or high temperatures. Although MIR has been observed and iron-reducers identified in more Mars-relevant environments, such studies are in the minority. Given that Mars is a cold volcanic planet, and has been for the majority of its past, there remains a gap in our understanding of the prevalence and viability of MIR in similar environments on Earth (EBL; Chapter 4).
2. Despite a strong argument for the presence of meteoritic and possibly endogenous organic material on Mars, the presence of these materials is yet to be unambiguously documented (MBL).

3. In spite of the above MBL, it remains to be seen whether organic compounds in bulk carbonaceous materials on Earth (such as shales and kerogens) are sufficiently abundant and accessible to serve as electron donors for MIR. These materials collectively represent more than 90% of organic material in the terrestrial subsurface; therefore the availability of this material for use in microbial metabolism has implications for our understanding of the global carbon cycle. Furthermore, since the carbonaceous fraction of these materials is somewhat similar to that in carbonaceous meteorites, they represent good terrestrial analogues with which to gain insight into the potential accessibility of electron donors on Mars (EBL; Chapter 5).
4. There are many more compounds that have been detected in carbonaceous chondrites than have been tested as electron donors for MIR. Therefore the range of potentially available electron donors on Mars remains unconstrained (EBL; Chapter 6).

Chapter 3: General methodology

Each of the following chapters includes methods unique to each set of experiments, however a number of methods are common to all. These are outlined below.

3.1 Preparation of anaerobic growth medium

Growth media were prepared aerobically according to each growth medium recipe, and purged to remove dissolved oxygen either in bulk (at least 15 minutes per L) or in individual serum vials (7 minutes per 10 mL) with N_2/CO_2 (80:20), prior to autoclaving at 121°C for 20 minutes. After autoclaving and prior to inoculation, the pH of growth media was checked by measuring the pH of a 100-150 µl subsample. The pH of growth media was adjusted up or down to the required value by means of gassing with N_2 or N_2/CO_2, respectively. For sterile anaerobic medium in serum vials, 0.2 µm syringe filters attached to sterile needles were used to prevent contamination from this step.

3.2 Anaerobic techniques

Experiments were conducted using serum vials (or hungate tubes), closed with butyl rubber stoppers and crimp sealed with aluminium caps (or screw tops). The headspace in inoculated vials was always N_2/CO_2. All manipulations were carried out using sterile needles and syringes, flushed with oxygen-free N_2 gas. Stoppered vials were flame-sterilised with ethanol prior to needle insertion. Needles and syringes were flushed with anoxic gas using a gassing cannula in close proximity to a Bunsen burner. Prior to use the cannula was flame sterilised as far as the cotton-wool filter within the syringe barrel.

Amendments to media of electron donors/acceptors, reducing agents or other substrates were made as described above from sterile anoxic stocks. Stocks were made aerobically and transferred to 100 mL or 10 mL serum vials by means of filter-sterilising through a 0.2 µm syringe filter, and purged with N_2 (7 minutes per 10 mL). All stocks were stored in the dark.

3.3 Determination of iron concentration

Microbial iron reduction was monitored by measuring the production of HCl-extractable Fe(II) over time using the Ferrozine assay (Stookey, 1970). To measure the concentration of Fe(II) in a sample, 100 µl was anaerobically extracted using a sterile needle and syringe, added to 4.9 ml of HCl (0.5 M) and left to digest for 1 hour. A 50 µl subsample of this sample was transferred to a cuvette containing 2.45 ml Ferrozine solution (1 g ferrozine [5,6-Diphenyl-3-(2-pyridyl)-1,2,4-triazin-4,4'-disulfonic acid disodium salt hydrate] and 11.96 g HEPES per 1 L distilled water, pH corrected to 7), mixed thoroughly with a pipette, and absorbance measured at 562 nm using a spectrophotometer (Helios alpha, Thermo Fisher Scientific, Waltham, MA, USA). The concentration of ferrous iron was calculated using the linear regression equation from a calibration graph, on which the absorbance of solutions of $FeSO_4.7H_2O$ (1 to 50 mM) at 562 nm were plotted. These standards were prepared in exactly the same way as experimental samples.

Ferrozine solution was stored in the dark at 4°C, and kept for no longer than 3 weeks. A calibration was conducted for every new batch of ferrozine solution made. In each calibration, standards were made fresh. The most concentrated standard (50mM) was made by dissolving 0.139 g $FeSO_4.7H_2O$ in 10 ml 0.5M HCl to prevent oxidation to Fe^{3+}. The remaining standards were made by diluting this solution to 20 mM, 10 mM, 5 mM and 1 mM using distilled water.

3.4 Preparation of 2-line ferrihydrite

Ferrihydrite was synthesised as described in Straub *et al*. (2005), whereby ferric chloride is neutralised dropwise with sodium hydroxide. Specifically, a 0.4 M solution of $FeCl_3$ was prepared using distilled water, and placed on a stirring plate and stirred vigorously. A calibrated pH electrode was placed into this solution, and the pH adjusted slowly by adding NaOH (1M) drop by drop using a burette. Care was taken to ensure the pH remained stable at pH 7 for 30 minutes before ferrihydrite synthesis was assumed to be complete.

The resulting precipitate was left to settle over night, before being transferred to 250 mL centrifuge bottles. After topping up to a total volume of approximately 230 mL and

weight-balancing, the ferrihydrite was concentrated down by centrifugation at 12,000 rpm for 20 minutes (Sorvall RCB5 centrifuge with GSA rotor, Sorvall, UK). The pellet was resuspended in 200 ml distilled water and re-centrifuged using the same settings in order to wash the ferrihydrite of sodium and chloride ions. This process was repeated until the conductivity of the supernatant was less than that of a fresh 0.01% (wt/vol) solution of NaCl. At this point, the ferrihydrite was concentrated down once more and resuspended in an adequate volume of distilled water to obtain a 1 M suspension. This concentration was determined using the ferrozine assay, in which five subsamples of 100 µl were acidified in 4.9 ml HCl (0.5 M) with the addition of 200 µl fresh 6.25M hydroxylamine-hydrochloride, and left to digest overnight in order to completely reduce all ferric to ferrous iron. These digested replicates were diluted 100-fold in distilled water (50 µl sample in 950 µl distilled water) before immediately transferring a 50 µl subsample to 2.45 ml ferrozine assay for measurement of absorbance at 562 nm. Concentrations were determined as before using a calibration curve, and averaged across replicates.

Ferrihydrite was stored in suspension at 4°C. A 2 mL aliquot of this suspension was dried overnight and powdered in a pestle and mortar, before confirmation of mineralogy using XRD.

Ferrihydrite was often added from sterile anoxic stock in experiments. Such a stock was prepared by transferring a well mixed 80 mL aliquot of the suspension to a clean 100 mL serum vial, sealing with a butyl rubber stopper and aluminium crimp-seal, and purging for 10 minutes with oxygen-free N_2. The anoxic stock was stored at 30°C for 24 hours to activate any spores which may have been present in the deionised water, then autoclaved, and subsequently stored in the dark at 4°C. Ferrihydrite stocks (oxic or anoxic) were used for no longer than 6 months, since ferrihydrite transforms to the more crystalline iron oxides hematite and/or goethite over time (Straub *et al*., 2005).

3.5 Cell enumerations

Samples of 250 µl were fixed in 100 µl 25% glutaraldehyde for 1 hour, then stored at –20°C until use. For cell enumeration, between 100-200 µl of this fixed sample was stained with SYBR-Gold (Invitrogen, Paisley, UK) in the dark for 15 minutes. For cultures grown on ferrihydrite, fixed samples were treated with 4 ml filtered oxalate reagent (28 g/L ammonium oxalate and 15 g/L oxalic acid, pH 3) prior to staining in order to dissolve the

iron oxide (Roden and Zachara, 1996). Stained samples were then filtered through a 0.2 µm filter membrane, and excess stain was removed by passing an additional 2 ml sterile distilled water through. Filters were mounted on glass slides, with a drop of CitiFluor™ antifade (Citifluor, London, UK) on the inverted cover slip. Cells contained within a 10x10 eyepiece graticule in 20 randomly selected fields of view were enumerated by epifluorescence microscopy (Leica DM 4000 B, Leica Microsystems, Milton Keynes, UK). If fewer than 200 cells were counted after 20 fields of view, counting continued in as many more as necessary until this figure was reached.

Cells per ml sample were calculated by extrapolating the average number of cells per filter.

3.6 DNA extraction

DNA was extracted from microbial enrichment cultures using the FastDNA® SPIN kit for Soil (MP Biomedicals, Illkirch, France), according to the manufacturer's instructions. Unless otherwise stated, 500 µl of sample was used for each DNA extraction.

In some cases it was necessary to introduce a step to reduce the effect of adsorption onto clay matrices within samples. This was achieved by incorporating the protocol of Direito and others (2012), which substitutes the FastDNA SPIN kit phosphate buffer with an equal volume of 1M Sodium Phosphate buffer in 15% molecular-grade ethanol and corrected to pH 8.0. Samples were incubated in a heating block at 80°C for 40 minutes following the lysing step. All subsequent processing was the same as in the manufacturer's protocol.

In some cases, DNA was also extracted from the initial bulk sediments. Ten grams of wet sediment was weighed out in a laminar flow hood, and DNA extracted using PowerMax® Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions.

3.7 16S rRNA sequencing and data analysis methodology

All DNA samples were sequenced for Bacterial 16S ribosomal RNA (rRNA) using 454 Pyrosequencing (Research and Testing Laboratories, Austin, TX, USA). Following 16S rRNA PCR amplification and sequencing, the resulting sequence reads from each sample are subject to rigorous analysis by Research and Testing Laboratories. Broadly, this analysis can be split into two stages; 1) the quality checking and denoising stage, and 2) the diversity analysis stage. The first of these can be further broken down into four individual steps. The first step is quality trimming, which attempts to clean up potentially poor read ends in the sequence data. This is achieved by assessing the quality scores assigned to bases in the sequencing process. The quality score for each base is summed to a running total and divided by the number of bases read in by the sequencer at any one point. Once all base quality scores from a sequence have been summed and divided, the resulting average is compared to a threshold value. Unless the overall average for any given sequence is greater than the threshold value, the sequence read is trimmed back to the last base that allowed the read to fall below this threshold. The second step in the quality checking and denoising stage is clustering. Using the trimmed read data from the previous step, this process attempts to classify all reads into clusters using the USEARCH application (Edgar, 2010). The criterion applied to this process is a 4% divergence, and all reads that have failed to exhibit a similar or exact match elsewhere on the region are removed. The output from this step is then inputted to the chimera-checking step, which attempts to remove chimeric sequences that arise when an aborted sequence extension is erroneously amplified during the pre-sequencing PCR step. Possible chimeric sequences in the data are identified using the *de novo* method of the UCHIME programme (Edgar *et al*., 2011). The resulting data output is a classification of all sequence reads as possibly chimeric or definitely not chimeric. Any sequences flagged up as the former are removed. The final step in this first stage is denoising. Here, base pair errors are corrected and bad sequences are removed in the data outputted from all previous quality checking steps. Bases are added to, corrected or deleted from sequences according to the combination of quality score and relative match to the consensus sequence. For sequences in which bases are changed or added, a new quality score is generated based on the lower median value of all other base quality scores in that sequence. The corresponding quality scores of removed bases are also removed from the data.

The sequences that pass the above quality checks are carried forward to the diversity analysis stage. It is during this step that taxonomic identification of sequences takes place. To achieve this, USEARCH is employed to cluster sequences into Operational Taxonomic Units (OTU) with 100% identity. Using a distributed .NET algorithm that utilises BLASTN+ (KrakenBLAST, www.krakenblast.com), the seed sequence of each cluster is queried against a database of high quality sequences compiled from the National Center for Biotechnology Information (NCBI). Sequences are then resolved to taxonomic levels based on the BLASTN+ derived sequence identity percentage. Species level identification is assigned to sequences with greater than 97% identity, genus level is assigned to between 95% and 97% identity, family level to between 90% and 95% identity, order level to between 85% and 90% identity, class level to between 80% and 85% identity, and phylum level to between 77% and 80% identity. All sequences characterised by a match of less than 77% are discarded.

Data used for analysis in the following chapters was taken from the Counts files at various levels of taxonomic identification, and any sequences with equal similarity to more than one taxon were omitted.

3.8 PCR amplification

The success of DNA extractions was determined through gel electrophoresis after amplification of the 16S rRNA through polymerase chain reaction (PCR). In most cases the 16S rRNA gene was amplified using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and UN1492R (5'-TAC GGT TAC CTT GTT ACG ACT T-3') (Lane, 1991). A typical 50 µl volume PCR mixture would contain 1 µl DNA template, each primer at a concentration of 0.4 µM, deoxynucleoside triphosphates (dNTPs) at 200 µM, 1.5 mM $MgCl_2$, 5 µl 10 X PCR buffer, 5 µl bovine serum albumin (BSA), and 0.5 µl *Taq* polymerase. With this primer set, template DNA was initially denatured at 94°C for 4 minutes, followed by 30 cycles of denaturing (30 seconds at 94°C), annealing (30 seconds at 55°C), and extension (60 seconds at 72°C), and a final extension at 72°C for 5 minutes.

In some cases, a different universal bacterial 16S rRNA primer set was used; 357F (5'-CCT ACG GGG AGG CAG CAG-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer *et al*., 1993). The PCR reaction mix was kept as described above. For this primer set, template DNA was initially denatured at 94°C for 4 minutes, followed by 35 cycles of

denaturing (30 seconds at 94°C), annealing (30 seconds at 54°C) and extension (30 seconds at 72°C), and a final extension at 72°C for 5 minutes.

A subset of DNA extractions were screened for the presence of strains of the *Geobacteraceae* family using the primers Geo-494F (5'-AGG AAG CAC CGG CTA ACT CC-3'; Holmes *et al*., 2002) and Geo-825R (5'-TAC CCG CRA CAC CTA GT-3'; Anderson *et al*., 1998). Using the same proportions of PCR reagents as outlined above, template DNA was initially denatured at 94°C or 5 minutes, followed by 35 cycles of denaturing (20 seconds at 94°C), annealing (20 seconds at 51°C), and extension (30 seconds at 72°C), and a final extension at 72°C for 7 minutes.

Denaturing Gradient Gel Electrophoresis (DGGE) was employed to qualitatively assess the phylogenetic diversity of some samples. For this, relevant DNA extracts were amplified using a GC-enriched forward primer (F357GC: 5'- CGC CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGG CCT ACG GGA GGC AGC AC-3') with the universal R518 primer. Per 50 µl PCR reaction, 2 µl of DNA template was mixed with primers at a final concentration of 0.8 µM and 25 µl GoTaq™ mastermix (Promega, Wisconsin, USA). The PCR reaction mix was subject to the same thermocycler conditions as for the 357F and 518R primer set, outlined above.

Regardless of the extraction method, primer set employed or the end purpose, all DNA and PCR products were analysed by gel electrophoresis. Between 5 µl and 8 µl of PCR product was mixed with 1 µl to 2 µl loading buffer and transferred to wells in a 1% agarose gel containing 35 µl/L SYBR Safe DNA dye (Invitrogen, Paisley, UK), submerged in TAE buffer within a gel electrophoresis tank. The success of PCR amplification was assessed by ultraviolet transillumination (Syngene G-Box, Syngene, Cambridge, UK). In all cases a 1 kb DNA molecular weight marker (Hyperladder™, Bioline, London, UK) was run along side DNA or PCR products, in addition to positive, negative and blank controls as appropriate.

Chapter 4: MIR in Mars-relevant environments on Earth

4.1 Introduction

In order to assess the feasibility for MIR on Mars, it is essential to better understand the prevalence of active iron-reducing microorganisms in Mars-relevant environments on Earth. Although the field of MIR has grown substantially over the last few decades, research has primarily been driven by the need to implement iron-reducing microorganisms in the bioremediation of metal- and hydrocarbon-contaminated environments (e.g. Coates *et al*., 1999, Anderson *et al*., 2003). Therefore the current census of MIR on Earth is not reflective of environments on Mars, past or present, and hence our ability to constrain habitability for MIR remains restricted.

The overall goal of this biogeographical survey is to broaden the census of environments in which MIR may operate to incorporate those with relevance to Mars. This goal is addressed by drawing upon several environments with Mars-like characteristics, including various locations in Iceland, the Namib Desert, Río Tinto, and numerous subglacial environments. Specific aims underpinning this study are twofold: 1) to better assess environments on Mars as potential habitats for MIR, and 2) to broaden our understanding of the prevalence and importance of MIR on Earth, with emphasis on the predominance of the most prolific family of iron-reducing microorganisms reported to date, *Geobacteraceae*. The hypothesis tested in this study is that MIR is widespread in these environments and is carried out by a diverse array of taxa, which extend beyond the *Geobacteraceae* family. To test this hypothesis, MIR enrichment cultures were initiated using sediments from the above Mars-relevant environments, and the following questions asked:

i. Does MIR occur? This is a pertinent question owing to the lack of attention paid to more extreme environments that share common features with Mars; hence the extent of MIR in these settings remains unknown.
ii. Do ranges in growth temperatures reflect those of the original environment? Most studies to date have enriched for MIR in environments characterised by a mesophilic temperature range, and enrichments are typically conducted at 30°C. Colder

environments have been especially neglected. This question addresses whether MIR is adapted to the temperatures of the host environemens.

iii. Are species of the *Geobacteraceae* family present? This family has long been thought of as the most prolific group of iron-reducing microorganisms in subsurface environments. By examining MIR in relatively extreme environments, this study offers an opportunity to challenge this view.

iv. Which microorganisms are responsible for MIR? If members of the *Geobacteraceae* family are not dominant in these environments, this question serves to identify which microorganisms are.

4.2 Field sites

The environments chosen for this study all share the common characteristic in that to some extent they resemble environments on Mars, as summarised in Table 4.1. Samples used to initiate enrichments are grouped 4 ways. The first group originates from various environments in Iceland, spanning acidic hydrothermal systems, recent volcanic systems, and iron-rich streams. In contrast, the second group comprises sediments collected from the Namib desert, spanning a desiccation transect in the stable gravel plains, to the mobile dunes of the Namib Sand Sea, and the bed of the Kuiseb River that separates the two. The third, smallest group was collected from the Río Tinto system in Spain. The fourth group consists of subglacial sediments from geographically distinct glacial environments, including Svalbard, Norway, Greenland, Antarctica and Iceland. All samples used for the initiation of MIR enrichments are summarised in Table 4.2.

Location	Analogue Features
Iceland	Basaltic mineralogy Cold *Analogue for volcanic landforms in early/middle Mars history*
Namib Desert	Hyper arid Dune seas and gravel plains *Analogue for present-day Mars deserts*
Rio Tinto	Natural acid rock drainage system Fe- and S-dominant mineralogy *Analogue for early river channels on Mars*
Subglacial	Cold *Analogue for liquid water in cold environments on Mars*

Table 4.1: Mars-relevant attributes of field sites.

Field Site	Sample	Description
Iceland	FimmV	Regolith from 2010 summit
	FimmCC	Waterlogged ash from centre of recent cinder cone
	FimmBB	Sediment, side of footpath dubbed 'bubbling biomat'
	FimmAH	Sediment from near the base of an ash pile
	FimmUR	Sediment containing oxidised iron from under a rock
	FimmIF	Sediment from interface of rock, ash and snow
	FimmHA	Hot ash from beneath 2010 summit
	FimmSS	Sediment from snowfield edge, dubbed 'subsurface sludge'
	Gp	Sediment from periphery of a hydrothermal pool in the Geysir
	Gv	Sediment above vent feeding a hydrothermal pool, Geysir
	K	Sediment from bed of acidic stream at Seltún sping, Krýsuvík
	H	Sediment, pebbles and water from stream at Hvalfjördur
	B	Waterlogged sediment from iron-rich stream near Borgarnes
Namib	C14-0	Sediment from W-most point of C14 transect
	C14-30	Sediment from 30 km along C14 transect
	C14-60	Sediment from 60 km along C14 transect
	C14-90	Sediment from 90 km along C14 transect
	C14-120	Sediment from E-most point, 120 km along C14 transect
	SPS02	Brine and biomat from saline spring
	RM	Sediment from pile of rusty metal
	MOP	Sediment from base of a mineal ore pile
	BD	Sand from between two dunes of Namib Sand Sea
	ARB	Sediment from ancient river bed in Namib Sand Sea
	KR	Waterlogged sediment from bed of ephemeral Kuiseb River
Río Tinto	US	Upstream water and sediment from near source of the river
	BM	Biomat and sediment from standing body of water
Subglacial	E	Basal sediment from Engabreen glacier, Norway
	R	Upthrust basal sediment from Russell glacier, Greenland
	L	Upthrust basal sediment from Leverett glacier, Greenland
	FL	Basal sediment from ice cave in Leverett glacier, Greenland
	LW	Sediment-laden basal ice from Lower Wright, Antarctica
	F	Upthrust basal sediment from Finsterwalderbreen, Svalbard
	AB1	Upthrust basal sediment from Austre Brøggerbreen, Svalbard
	AB2	Upthrust basal sediment from Austre Brøggerbreen, Svalbard
	ABp	Proglacial sediment from Austre Brøggerbreen, Svalbard
	AL1	Upthrust basal sediment from Austre Lovénbreen, Svalbard
	AL2	Upthrust basal sediment from Austre Lovénbreen, Svalbard
	ALp	Proglacial sediment from Austre Lovénbreen, Svalbard
	P	Proglacial sediment from Pedersbreen, Svalbard
	Ss	Basal sediment from ice cave in Solheimajökull, Iceland
	Sp	Progacial sediment from Solheimajökull, Iceland

Table 4.2: Details of sediment samples used to initiate MIR enrichments.

4.2.1 Iceland

Iceland is a volcanic island. Its mineralogy is basalt-dominated, and hence shares many similarities to the mineralogy of Mars. However a wide variety of environments exist on Iceland, encompassing a dramatic range of temperature and pH. A number of these were sampled in an attempt to capture this variety.

Several samples were collected from, or proximal to, the recent lava flow at Fimmvörðuháls (Fimm; 63°38’N, 19°26’W), the result of the March/April 2010 fissure eruption of Eyjafallajökull. The Fimm pass is a 2 km ice-free saddle between the ice-capped Eyjafallajökull and Mýrdalsjökull volcanoes. The eruption emanated from a 300 m radial fissure comprising 15 lava fountains, and produced a lava flow covering an area of 1.3 km^2, with a mean thickness of 20 m (Thordarson *et al*., 2011). The composition of the expelled magma was mildly alkali, and olivine- and plagioclase-bearing (Gunnlaugsson *et al*., 2011). Using an ethanol-sterilised trowel, samples were collected from a range of locations in this region, including from the top of the volcano (FimmV), from waterlogged sediments of a cinder cone (FimmCC), and hot ash (FimmHA). In each case, sediments were transferred in bulk to sterile polythene sample bags.

Situated 110 km from Reykjavik, the Geysir high temperature geothermal field resides in a shallow valley running from North to South on the southern coast of Iceland. Samples were collected from sediments in a small hydrothermal pool near the main Geysir (G; 64°18’N, 20°18’W). The temperature measured at this site was 95°C, and the pH was 8.1. The first sample was collected from waterlogged sediment at the periphery of the pool (Gp), and the second from the soil directly above the vent from which the boiling water originated (Gv). Samples were collected using sterile 50 ml falcon tubes, which were completely filled with sediment and the lids sealed with parafilm. Each falcon tube was tightly packed in individual sterile polythene sample bags to minimise aeration of samples.

Located on the Reykjanes peninsula of South-West Iceland, the Krýsuvík geothermal area is one of five active geothermal areas in this region. The area consists of mainly acid surface alteration, characterised by montmorillonite, haematite, pyrite, goethite, opaline silica and elemental sulphur (Markusson and Stefansson, 2011). Sediment was collected from the bed of an outflow stream emanating from a hot spring at Seltún (K; 63°53’N, 22°03’W). The pH measured at this location was 2.2, and the temperature 50.5°C. Sediment was collected in a sterile 50 ml falcon tube and the lid sealed with parafilm, as before.

Sediments were collected from the bed of a small stream draining into the north side of Hvalfjördur (H) in western Iceland (64°20'N, 21°51'W). The stream cuts through hydrothermally altered basalts comprising 3-4 million year old plateau basalts (Ehlmann *et al*., 2012). At the time of sampling, the pH of the stream water was 7.9, and the temperature 12°C. Small pebbles dominated the streambed sediment, and in contrast to previous sampling efforts it proved difficult to obtain a sediment sample. The resulting sample therefore comprised what little waterlogged sediment that could be accessed, in addition to small pebbles and stream water. As before, the sample was collected in a sterile 50 ml falcon tube and the lid sealed with parafilm.

The final sampling site in Iceland was an iron-rich stream near the town of Borgarnes (B; 64°29'N, 21°31'W). The microbial ecology of sediments and biomats from this stream has been previously investigated (Cockell, 2011) and suggested the presence of a diverse microbial iron cycle. Therefore this site was thought of as a positive control, since it was thought highly likely that enrichments would demonstrate active MIR. Samples were collected from waterlogged sediments at the edge of the stream using an ethanol-sterilised trowel and transferred to sterile polythene bags.

In the case of G, K, H and B, duplicate sediment samples were collected for the dual purposes of initiating enrichments and sequencing the 16S rRNA genes within the sediment.

4.2.2 Namib Desert

Dating back 8 million years, the Namib is the oldest desert in the world (Eckardt and Drake, 2011). Situated in South East Africa, the Namib Desert stretches 200 km along the coast, and ranges in width from 50-150 km (Henschel and Lancaster, 2013). Average annual rainfall in the Namib is 25 mm, earning it the classification of hyper-arid. Rainfall is extremely varied in space and time, though in general it increases from West to East (Eckardt *et al*., 2013). Fog represents a substantial contribution to the moisture budget of the desert, especially in the coastal region in the west. A number of organisms that dwell in the Namib thrive on this fog, particularly the lichens and microorganisms that form biological soil crusts (Warren-Rhodes *et al*., 2013). Owing to the Mars-like landscape of dunes, aeolian features and extreme aridity, this environment has been recognised as a Mars analogue in a recent review of terrestrial analogues by Preston and Dartnell (2014). Of these attributes it is

the extreme aridity of the Namib that makes it a relevant environment in which to assess MIR in this study.

The desert incorporates four distinct areas, namely North Namib, South Namib, Central Plains and Central Dunes (including the Namib Sand Sea). Samples were collected from a number of locations spanning both the gravel plains and Sand Sea dunes of the central region. In the case of the gravel plains, sediments were sampled from various locations along a 120 km desiccation transect running from East to West along the C14 road within the Namib-Naukluft National Park towards Walvis Bay (C14-0 - C14-120) (Stomeo *et al*., 2013; Warren-Rhodes *et al*., 2013). A sample was retrieved from a saline spring (SPS02), common features in the gypsous beds of the gravel plains of this region (Watson, 1979). Additional anomalous settings were also sampled, such as a pile of rusty metal (RM), a mineral ore pile (MOP), and a fog-dominant area of the gravel plains (FOG). Samples were also retrieved from an area between mobile dunes (BD; 23°47’S, 15°05’E) of the Namib Sand Sea south of the gravel plains. Given how aerated the mobile sands were, it was anticipated that no MIR would be detected in these sediments; hence this sample was considered a negative control. An additional sample was retrieved from a dried up ancient riverbed (ARB; 23°84’S, 15°20’E) in an area enclosed by Sand Sea dunes. In contrast, the bed of the ephemeral Kuiseb River, which separates the gravel plains from the mobile dunes, was rich in organic matter owing to frequent visits from local wildlife. Furthermore, at the time of sampling, sediments remained waterlogged from the wet season. It was therefore deemed likely that MIR was occurring, and hence the sample retrieved from here (KR; 23°34’S, 15°02’W) was considered a positive control amongst the Namib Desert sediments collected.

4.2.3 Río Tinto

Owing to its association with the Iberian Pyrite Belt in Spain, the Río Tinto system is a naturally acidic river harboring a diverse array of acidophilic microorganisms. Considered an analogue site for early Mars (Preston and Dartnell, 2014), this environment has been extensively studied for its geology/mineralogy (Sáez *et al*., 1996; Buckby *et al*., 2003; Sánchez-España *et al*., 2005; Fernández-Remolar and Knoll, 2008) and microbiology (Lopez-Archilla *et al*., 2001; González-Toril *et al*., 2003; García-Moyano *et al*., 2007). Although culture-independent methods have identified the presence of microorganisms

capable of MIR (Sánchez-Andrea *et al*., 2011), the process of active MIR from Río Tinto has yet to be demonstrated in culture.

Two samples were collected from Río Tinto using sterile hungate tubes and an ethanol-sterilised spatula. The first was retrieved upstream (US) near to the source of the river, and comprised mostly of water. The second was collected from a downstream location, where the river water had been dammed and biomats were floating by the water's edge. This second sample (BM) was a mixture of this biomat and the waterlogged sediment from the edge of the water body. In both cases, sterile hungate tubes were completely filled and stoppered with thick butyl bungs and sealed on with screw tops.

4.2.4 Subglacial sediments

Subglacial samples serve as analogues to Mars in that the environments they originate from are perennially cold, dark and often nutrient-poor. Not only are the physical characteristics relevant to the prevailing conditions throughout much of the history of Mars, subglacial environments are also directly relevant to the potential habitability of past and present Martian subglacial environments.

Samples from numerous glaciers have been used in this study, and span two major collaborations with external research groups. Six samples were acquired from the Bristol Glaciology Centre, and were collected by members of that research group over a number of years and stored at the Low Temperature Experimental Facility (LOWTEX) at the University of Bristol. These sediment samples originate from Engabreen in Norway, Finsterwalberbreen in Svalbard, Russell Glacier and Leverrett Glacier in Greenland, and Lower Wright Glacier in Antarctica. The second collaboration, with researchers at Aberystwyth University, led to the acquisition of samples from Austre Lovénbreen, Austre Brøggerbreen and Pedersbreen in Svalbard. The final sample of this batch was collected on the same trip as the aforementioned Iceland samples, and originates from Solheimajökull. Each sample site is discussed in more detail below.

Engabreen (E; 66°41'N, 13°46'E) is a temperate glacier of the western Svartisen Icecap in northern Norway. The underlying bedrock is metamorphic, dominated by schists and gneisses with calcite-filled cracks (Jansson *et al*., 1996). The bedrock and basal ice of this glacier is accessible through a system of underground tunnels leading to the glacier bed from the Svartisen Subglacial Laboratory. This basal ice was sampled by implementing hot

water drilling, and sediment-laden ice was chain sawed from the resulting cavity 200 m below the glacier surface (Stibal *et al*., 2012).

Russell (R; 67°03'N, 50°10'W) and Leverett (L; 67°03'N, 50°07'W) are neighboring land-terminating outlet glaciers. Together they constitute a large discharge lobe emanating from the western Greenland Ice Sheet (GIS); Leverett is the southern offshoot of the larger Russell glacier. Both are polythermal (cold and warm-based regions at the ice-bed interface), but warmer conditions dominate, as evidenced by accelerated glacial flow at the onset of the melt season (Sundal *et al*., 2011). The underlying bedrock is similarly metamorphic, dominated by Archaean gneiss, which was subsequently reworked in the early Proterozoic (Henrikson *et al*., 2000) and during numerous Holocene readvances, leading to the incorporation of organic matter (Ten Brink and Weidick, 1974). At Russell Glacier, samples were obtained from upthrusted subglacial sediment near the terminus. The outermost surface of the ice was removed using a chain saw before subsampling the remaining sediment-laden ice (Stibal *et al*., 2012). Two subglacial samples were collected from Leverett; L was chain sawed from a pressure ridge at the glacier margin, believed to be subglacial in origin, and FL ('Fresh' Leverett) was collected from the basal sediments at the ice-bed interface within an ice cave during a more recent field season using a flame-sterilised spatula. At the time of collection the latter sediment (FL) was assumed to be freshly melted (Hawkings, 2013; personal communication).

Lower Wright (LW; 77°25'S, 163°0'E) is a cold-based glacier draining westwards from the Wilson Piedmont Glacier in the McMurdo Dry Valleys region of Antarctica. The glacier currently terminates in the permanently ice-covered Lake Brownworth (Stibal *et al*., 2012). The underlying bedrock is dominated by granite-gneisses (Hall and Denton, 2002), however during the last few centuries numerous glacial advances have overridden and reworked lake sediments, evidence of which was found in the presence of algal matter in subglacial sediment (Wadham *et al*., 2012). The sampling site featured exposed frozen sediment sandwiched between layers of pure glacial ice at the interface of the ice-covered lake and the terminal moraines of the glacier. Blocks of this debris-laden ice were chain sawed out (Stibal *et al*., 2012).

Finsterwalderbreen (F; 77°28'N, 15°18'E) is a polythermal glacier on the southern side of Van Keulenfjorden, Spitsbergen, Svalbard (Wadham and Nuttall, 2002). Similar to the Greenland glaciers, the thermal regime of this glacier is largely warm-based (Hodson and Ferguson, 1999). The major underlying geology is mainly sedimentary, comprising sandstone, siltstone, shale, limestone and dolomites (Dallmann *et al*., 1990). Basal sediment

was chain sawed from a pressure ridge close to the glacier margin (Telling, 2014; personal communication).

Austre Brøggerbreen (AB; 78°89’N, 11°85’E), Austre Lovénbreen (AL; 78°53’N, 12°04’E) and Pedersbreen (P; 78°86’N, 12°29’E) are adjacent polythermal valley glaciers located in the Kongsfjord region of Northwest Spitsbergen, Svalbard. Similar to Finsterwalderbreen, the underlying bedrock is predominantly sandstone, shale and limestone (Hodson and Ferguson, 1999; Svendsen *et al*., 2002). In the case of AB and AL sediments were retrieved from upthrusted basal ice at the glacier margins, in addition to a sample from the glacial forefield of each glacier. A forefield sample from P was also obtained. All samples were collected using a flame-sterilised spatula, and stored in 50 ml sterile falcon tubes.

Solheimajökull (63°35’N, 19°20’W) is a south-flowing temperate outlet glacier of the Mýrdalsjökull ice cap in southern central Iceland. Unlike the other field sites, the geology of the valley in which Solheimajökull flows is mainly basaltic in origin, associated with deposits of palagonite (weathered basalt glass) (Carswell, 1983). A subglacial sediment sample (Ss) was retrieved from the bed of an ice cave at the height of the 2012 melt season, using a flame-sterilised spatula. An additional proglacial sample (Sp) was collected from the glacier forefield. It was not possible to refrigerate the samples in the field, though samples were stored at 4°C immediately upon return to the University of Edinburgh.

Samples E, R, L, LW and R were transported frozen from the field site to LOWTEX at stored at –30°C until used. Samples were prepared at the LOWTEX facility prior to their use in this study. Specifically, sediment-laden basal ice was placed in a laminar-flow hood in pre-furnaced glass beakers and the outer layer of each sample was removed by washing with sterile deionised water. Samples were covered in furnaced aluminium foil and transferred to the anaerobic chamber, where they were allowed to thaw under 100% nitrogen atmosphere. The liberated sediment from basal ice samples was subsampled into sterile serum vials, crimp-sealed with thick butyl rubber stoppers and aluminium caps, and removed from the chamber. The headspace of each was immediately flushed with N_2 gas for 1 minute using sterile needles attached to sterile 0.2 µm syringe filters, before being transferred to the University of Edinburgh where they were stored at 4°C for a short period before enrichments were initiated.

Samples AL and AB were kept at ~0.1°C on site, and frozen at –80°C on return to Aberystwyth University. They were subsequently sent to the University of Edinburgh in polystyrene cool boxes containing ice blocks, where sediments were stored at 4°C until required.

4.3 Materials and methods

4.3.1 Initiation of enrichments

All enrichments were set up in triplicate in order to incubate at 4°C, 15°C and 30°C. In some cases a further replicate was prepared to incubate at 50°C, and in the case of the Borgarnes sediment an enrichment was also established at room temperature (BRT; this enrichment later became the inoculum for the experiments in Chapter 5 and 6). Although it was not possible to weigh sediment upon initiation of enrichments, the quantity of sediment was kept approximately equal within each set of replicates and across all sediments. Enrichment cultures were initiated in the anaerobic chamber by adding sediment into sterile serum vials containing sterile anoxic freshwater basal medium (pH 6.8-7.0). It should be noted that the pH of all media was the same prior to addition of inoculating sediment, that is to say the pH of enrichments may have fluctuated after addition of sediment, and the Río Tinto enrichments were not initiated in acid-modified medium. To keep conditions within the chamber as sterile as possible during this process, ethanol was used to wipe down all surfaces and fan boxes were turned off. Gloves were washed with ethanol in between enrichments, and sediment was transferred to serum vials using sterile disposable forceps. Serum vials were crimp-sealed inside the chamber. The basal medium contained (in grams per litre of deionised water): $NaHCO_3$ (2.5), NH_4Cl (0.25), $NaH_2PO_4.H_2O$ (0.06), KCl (0.2), and vitamins (10 ml) and trace elements (10 ml). The vitamin mixture contained (in mg l^{-1} deionised water): biotin (2.0), folic acid (2.0), pyridoxine-hydrochloride (10.0), riboflavin (5.0), thiamine (5.0), nicotinic acid (5.0), pantothenic acid (5.0), vitamin B-12 (0.1), *p*-aminobenzoic acid (5.0) and thioctic acid (5.0). The trace element mixture contained (in g l^{-1} deionised water): nitrilotriacetic acid (1.5), $MgSO_4$ (3.0), $MnSO_4.H_2O$ (0.5), NaCl (1.0), $FeSO_4.7H_2O$ (0.1), $CaCl_2.2H_2O$ (0.1), $CoCl_2.6H_2O$ (0.1), $ZnCl_2$ (0.13), $CuSO_4.5H_2O$ (0.01), $AlK(SO_4)_2.12H_2O$ (0.01), H_3BO_3 (0.01), $NaMoO_4$ (0.025), $NiCl_2.6H_2O$ (0.024) and $NaWO_4.2H_2O$ (0.025). Vitamin and trace element mixes were stored at 4°C in the dark.

The headspace of all vials was flushed with N_2/CO_2 (80:20) through a 0.2 µm syringe filter using a gas station. Enrichments were amended from sterile anoxic stocks with acetate (5 or 10 mM) and lactate (5 or 10 mM) as the electron donors and ferrihydrite (approximately 50 or 100 mM) as the terminal electron acceptor using sterile needles and syringes. $FeCl_2$ was added (1.3 - 2 mM) as a mild reducing agent.

4.3.2 Incubation and determination of MIR

Enrichments were incubated in the dark, and periodically measured for production of ferrous iron using the ferrozine assay (see Chapter 3). Enrichments were considered positive for MIR where an increase in HCl-extractable ferrous iron of ≥5 mM was measured after at least 40 days of incubation. This concentration was chosen as a nominal figure that greatly exceeded the typical error of the ferrozine assay, as measured in calibration procedures where triplicate ferrozine measurements are conducted on each single calibration standard. Positive enrichments were inoculated into fresh medium of the same composition, and any that did not give rise to production of ≥5 mM ferrous iron in this second generation after 40 days were no longer considered positive. Enrichments established from a subset of subglacial sediments (E, R, L, FL, LW and F) were subject to more in-depth study, in which transfer into fresh medium for the first subculture was done in duplicate.

4.3.3 DNA extraction

DNA was extracted from sediments collected from G, K, H and B, and from positive enrichments (see Chapter 3 for DNA extraction methods). In all cases, DNA was extracted from the second-generation enrichment cultures, and in the case of the duplicated subglacial enrichments, duplicates were pooled prior to DNA extraction.

4.3.4 Geobacteraceae-specific PCR

To deduce whether DNA belonging to species of the *Geobacteraceae* family was present in positive enrichments, 16S genes were amplified using *Geobacter*-specific PCR primers, as outlined in Chapter 3.

4.3.5 Bacterial 16S rRNA sequencing

DNA from a subset of positive controls, and the sediment samples from G, K, H and B, were sequenced for Bacterial 16S rRNA. The Shannon's index of diversity (H) was calculated for genus-assigned taxa in enrichment sequence data, according to the following equation:

$$H = -\Sigma p_i \ln p_i$$

where p_i is the proportion of individuals measured in species i.

4.3.6 Denaturing Gradient Gel Electrophoresis (DGGE)

The bacterial phylogenetic diversity of a subset of positive glacial enrichments was qualitatively assessed by DGGE. These enrichments include some that were subject to 16S rRNA sequencing (F, L and LW 4°C) in addition to their 15°C counterparts (L and LW). The 4°C proglacial enrichments P, ALp and ABp were also assessed with DGGE. DNA from L 4°C was used as a molecular marker after preliminary 16S rRNA sequencing results indicated that this enrichment captured the greatest phylogenetic diversity. The extracted DNA used for DGGE was the same as that used for *Geobacter*-specific PCR and/or bacterial 16S rRNA sequencing. Extracted DNA was amplified by PCR using a primer set including a GC-enriched forward primer, and the products checked by agarose gel electrophoresis (see Chapter 3 for details). The Bacterial 16S amplicons were run on a 8% (wt/vol) acrylamide gel with a 30 to 55% denaturant gradient (where 100% represents 7 M urea and 40% formamide) in 1X TAE buffer (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA [pH 8.0]) at 60°C. The gel was run for 4 hours at 200 V and then stained with SYBR-Gold, before visualising resolved PCR products by UV transillumination.

4.4 Results

4.4.1 Detection and rates of MIR

Results for the positive or negative detection of MIR in subcultured enrichment cultures at each temperature are summarised in Table 4.3. All data represent a diagnostic of MIR based on production (or lack of) ferrous iron in second-generation enrichment cultures of 5 mM or above after at least 40 days. It is important to state that in the case of Iceland, Namib and Río Tinto enrichments, 30°C first generation enrichment cultures were incubated for substantially longer than those incubated at 4°C and 15°C, in addition to all subglacial/proglacial enrichment cultures. Final Fe^{2+} concentrations were measured after an average of 240 days in the case of the former, compared to 40-85 days for all other enrichments. This difference can be attributed to an initial experimental design implemented early in the research project in which all samples were to be enriched for MIR at 30°C only. It was only after the initiation of subglacial/proglacial enrichments at lower temperatures some time after these early 30°C enrichments were initiated that it was decided that all samples should be independently initiated at 4°C, 15°C and in some cases 50°C. By this point, time restrictions were such that enrichments could not be incubated for similarly long periods, and a shorter incubation period was adopted accordingly.

Of the 41 enrichments initiated from Icelandic sediments, MIR was detected in only 4. All but one of these was from enrichments initiated with Borgarnes stream sediment (B), in which MIR was detected at 4°C, 15°C and 30°C. The fourth positive enrichment was that of K at 30°C. It is not possible to assess potential temperature adaptations for this sample owing to the lack of 4°C and 15°C enrichments. No enrichments established at 50°C tested positive over the two generations. High Fe^{2+} production of 51.45 mM and 38.17 mM was measured over a period of 46 days in the first generation enrichments of Gp and Gv, respectively. However neither gave rise to more than 3 mM Fe^{2+} production in the second generation; hence they are listed as negative for MIR.

Field Site	Sample	4°C	15°C	30°C	50°C
Iceland	FimmV	–	–	–	–
	FimmCC	–	–	–	
	FimmBB	–	–	?	
	FimmAH	–	–	?	
	FimmUR	–	–	–	
	FimmIF	–	–	?	
	FimmHA	–	–	–	–
	FimmSS	–	–	–	
	Gp	–	–	–	–
	Gv	–	–	–	–
	K			+	
	H	–	–	–	
	B	+	+	+	
Namib Desert	C14-0	–	–	–	
	C14-30	–	–	–	
	C14-60	–	–	–	
	C14-90	–	–	–	
	C14-120	–	–	–	
	SPS02	–	+	–	
	RM	–	–	–	
	MOP	–	–	–	
	BD	–	–	–	
	ARB	–	–	–	
	KR	+	+	+	
Río Tinto	US	–	–	+	
	BM	+	+	?	
Subglacial	E	+	–	–	
	R	+	–	–	
	L	+	+	–	
	FL	+	+	–	
	LW	+	+	–	
	F	+	–	–	
	AB1	–	–	–	
	AB2	–	–	–	
	ABp	+	–	–	
	AL1	–	–	–	
	AL2	–	–	–	
	ALp	+	–	–	
	P	+	–	–	
	Ss	–	–	–	
	Sp	–	–	–	

Table 4.3: Occurrence of MIR in enrichment cultures. Enrichments were considered positive (+) where an increase in Fe^{2+} concentration ≥5 mM was measured within 40 days of subculturing to a second generation. Initial enrichments that gave rise to positive MIR, but were not subcultured to a second generation, are marked '?' to indicate possible MIR.

Similar to the Iceland enrichments, only 4 of the 33 Namib enrichments signified positive for MIR. All but one of these (SPS01 15°C) was from enrichments established from Kuiseb river sediment at 4°C, 15°C and 30°C. No MIR was evident in the 4°C or 30°C SPS02 enrichments, despite a measurement of 26.92 mM over 231 days in the first-generation 30°C enrichment. Although the incubation period for this first enrichment was significantly longer than that of the negative second generation (49 days), the Fe^{2+} concentration of 5.33 mM after 34 days of this initial enrichment would still be deemed positive. Particularly high Fe^{2+} production of 40.38 mM was detected in the 30°C initial KR enrichment after 231 days of incubation, and represents the highest ferrous iron concentration of all initial 30°C enrichments established in the same batch.

Of the 6 Río Tinto enrichments established, 3 were classed as positive. Two of these are Biomat (BM) enrichments at 4°C and 15°C, and the third is the Upstream (US) enrichment at 30°C. The initial 30°C enrichment for the BM sample gave rise to a ferrous iron concentration of 11.90 mM after 223 days, but was not subcultured to a second-generation enrichment; it is thus possible that the 30°C enrichment would be positive if a second-generation culture had been initiated. After 51 days, ferrous iron in the 4°C or 15°C enrichments initiated with the US sample was measured as 0.22 mM and –0.80 mM, respectively. The concentration in the initial 30°C US enrichment measured 8.32 mM after 223 days. Although no measurement was taken early on in the first enrichment, Fe^{2+} concentration measured after 49 days of the second-generation enrichment was 5.07 mM, indicative of MIR.

Enrichments initiated from subglacial and proglacial sediment samples gave rise to the highest number of positive results. A total of 12 out the 45 enrichments initiated were sufficiently productive to be classed positive for MIR. Nine of these represent enrichments incubated at 4°C, and the remaining 3 were incubated at 15°C. No enrichments incubated at 30°C were positive for MIR.

Particularly high ferrous iron concentrations were detected in the 15°C Leverett (L) enrichment, in which 22.58 mM was measured after 49 days in the first-generation enrichment, and 37.75 ± 0.002 mM after 58 days in the second-generation culture. A concentration of 45.46 mM Fe^{2+} was measured after 84 days in the initial 4°C ALp enrichment culture, though the second-generation concentration was much lower at 5.13 mM after 42 days.

A negative change in ferrous iron concentration was measured in a number of initial enrichments. Changes of –62.25 mM and –60.44 mM Fe^{2+} were detected over 47 days in 4°C and 15°C Sp enrichments, respectively. In contrast, the change in Fe^{2+} concentration in

the initial 30°C Sp enrichment was measured as 27.82 mM, though this was over a period of 223 days. A negative change in Fe^{2+} concentration was similarly seen in a number of enrichments initiated with sediments collected from the Fimmvörðuháls pass. For instance, after 47 days substantial decreases in ferrous iron concentrations were measured in FimmCC 4°C (–37.13 mM) and 15°C (–28.08 mM), FimmBB 4°C (–15.72 mM) and 15°C (–9.81 mM), FimmAH 4°C (–12.94 mM) and 15°C (–18.31 mM), and FimmUR 4°C (–15.83 mM) and 15°C (–10.07 mM). No negative change in Fe^{2+} was measured in the corresponding 30°C enrichments of these samples, which were incubated for 223-285 days. Other negative changes of similar magnitude were detected in the first-generation 4°C F enrichment (–15.32 mM over 49 days) and the first-generation 30°C enrichment of P (–24.98 mM over 84 days).

A small number of initial 30°C Iceland enrichments gave rise to an increase in ferrous iron of more than 5 mM, but were not carried forward to a second-generation enrichment. The concentration of Fe^{2+} in FimmBB 30°C was 13.57 mM after 223 days. FimmAH and FimmIF 30°C enrichments both gave rise to ferrous iron concentrations of 6.78 mM Fe^{2+} after 285 days. That these were not subcultured to a second generation renders the question of whether they were positive for MIR unanswered. Cases in which MIR was detected in the first and only enrichment culture are marked with a question mark in Table 4.3, accordingly.

In contrast, a number of samples were carried forward to second generation despite testing negative for MIR in the initial enrichment. The majority of these were initiated from subglacial sediments, which were subject to more rigorous testing than other samples. It was therefore decided that all enrichments would be carried forward regardless of the outcome of the initial enrichment, with a view to generating more data either way. A subset of these subglacial enrichments was carried over in duplicate, data for which are presented in Figure 4.1. The two enrichments that were not subglacial in origin, but for which this is also true, are SPS02 15°C and K 30°C. In both these cases a second-generation enrichment was initiated due to the change in colour of the reddish-brown ferrihydrite to black. Despite relatively low values of ferrous iron at the end of initial enrichments, the observed phenomenon was deemed worthy of further study. Indeed, both subcultures yielded a positive second-generation enrichment. Specifically, after incubation for a period of 223 days, the 30°C initial K enrichment yielded an increase of only 2.24 mM Fe^{2+}. Yet the increase of ferrous iron after just 49 days of the second-generation enrichment was 8.99 mM. In the case of the initial 15°C SPS02 enrichment, an incubation period of 51 days yielded an increase of just 3.32 mM Fe^{2+}, but incubation of 42 days for the second-generation enrichment gave rise to a final Fe^{2+} concentration of 12.08 mM.

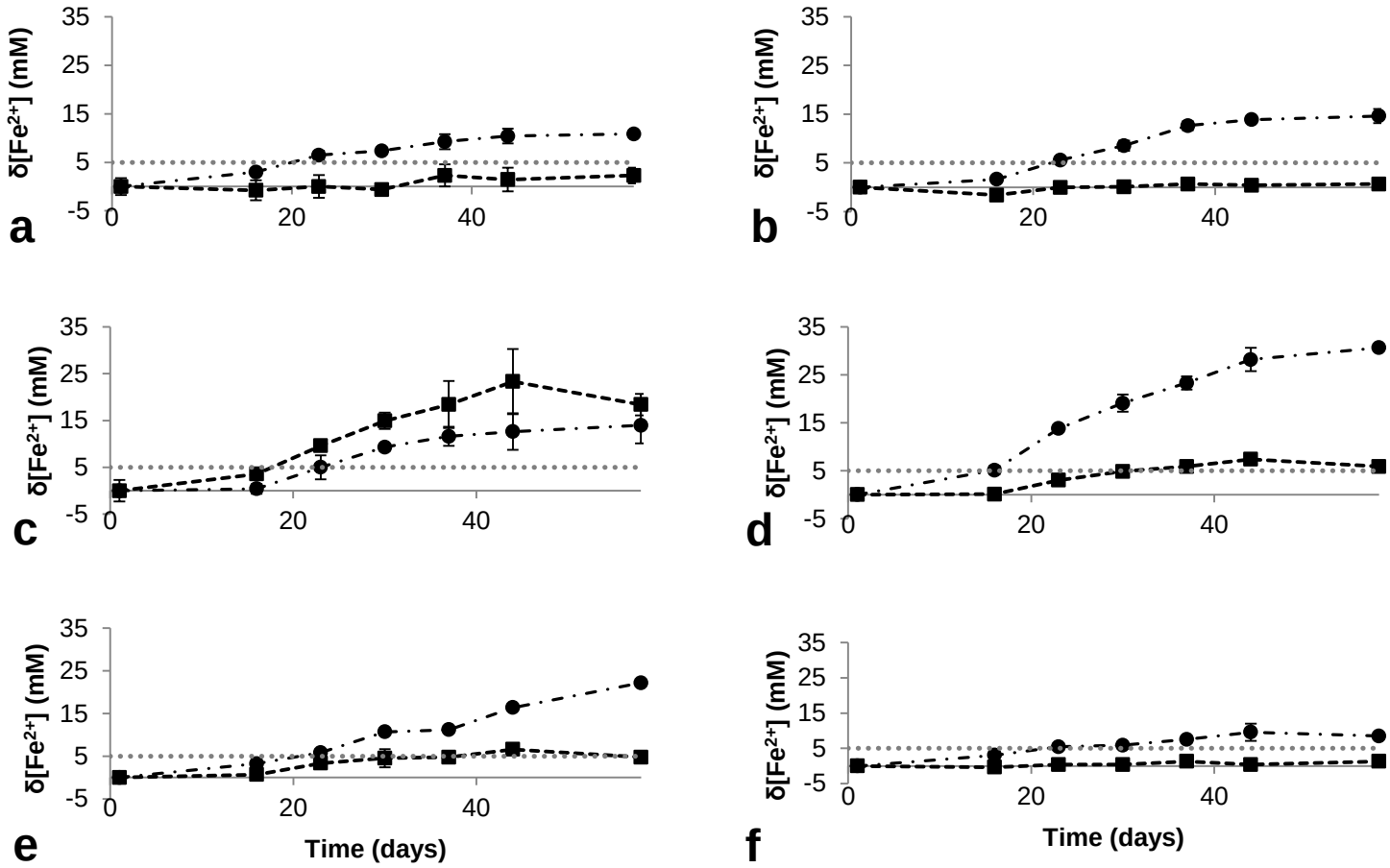

Figure 4.1: MIR in subglacial enrichments over time. Data, expressed as change in Fe^{2+} concentration (mM) over time, correspond with second-generation 4°C (circles, dot-dashed line) and 15°C (squares, dashed line) Engabreen (a), Russell (b), Leverett (c), Fresh Leverett (d), Lower Wright (e) and Finsterwalderbreen (f) enrichments. Error bars represent standard of duplicate measurements. Dotted line denotes 5 mM cut-off above which MIR is considered positive.

More dramatic changes were observed between initially negative subglacial enrichments and proceeding subcultures. The negative concentration of Fe^{2+} of –15.32 mM in the F 4°C enrichment was followed by a measurement of 8.49 ± 0.60 mM in the duplicated second-generation enrichment. Similarly, –4.36 mM Fe^{2+} was measured after 84 days incubation of ABp 4°C, followed by a concentration of 8.49 mM Fe^{2+} in the second-generation enrichment. Other instances of an initially negative first- but subsequently positive second-generation enrichment include 4°C E, 15°C FL and 15°C LW. Enrichments initiated at 4°C with samples AB1, AL1, and at 15°C with E, AB1, AL1 and ALp tested negative in both first- and second-generation enrichments. Only one 30°C subglacial enrichment was carried over to a second-generation, namely L. The initial enrichment gave rise to a concentration of 9.97 mM Fe^{2+} after 79 days, but the second-generation enrichment yielded only 0.95 mM Fe^{2+} after 42 days.

The change in Fe^{2+} concentrations measured in positive enrichments over both generations were normalised for total enrichment volume and the incubation period over which they were monitored. The resulting rates of MIR are shown in Figure 4.2, expressed as change in concentration of Fe^{2+} (μM) per ml of enrichment per day for each temperature. The highest rates of MIR at 4°C (Figure 4.2a) are evident in the first generation enrichments of ALp (67.65 μM ml^{-1} day^{-1}) and B (65.90 μM ml^{-1} day^{-1}), and the duplicated second generation FL enrichment (52.82 ± 0.56 μM ml^{-1} day^{-1}). At 47.40 μM ml^{-1} day^{-1} the rate of MIR in the first-generation 15°C B enrichment is the highest, followed by the second-generation L enrichments, at 44.40 μM ml^{-1} day^{-1} (Figure 4.2b).

There are several enrichments in which rates of MIR in the first-generation were negative (concentration of Fe^{2+} reduced over time) but the second-generation was positive for MIR. This is evident in the 4°C enrichments of F (first-generation rate of –15.63 μM ml^{-1} day^{-1}, second-generation 14.64 μM ml^{-1} day^{-1}) and ABp (–6.49 μM ml^{-1} day^{-1} followed by 25.27 μM ml^{-1} day^{-1}), and in 15°C enrichments of FL (–0.03 μM ml^{-1} day^{-1} followed by 13.15 μM ml^{-1} day^{-1}) and LW (–2.80 μM ml^{-1} day^{-1} followed by 19.53 μM ml^{-1} day^{-1}). Rates of all 30°C positive first-generation enrichments are low, ranging from 1.26 μM ml^{-1} day^{-1} in K, to and 3.73 μM ml^{-1} day^{-1} in US.

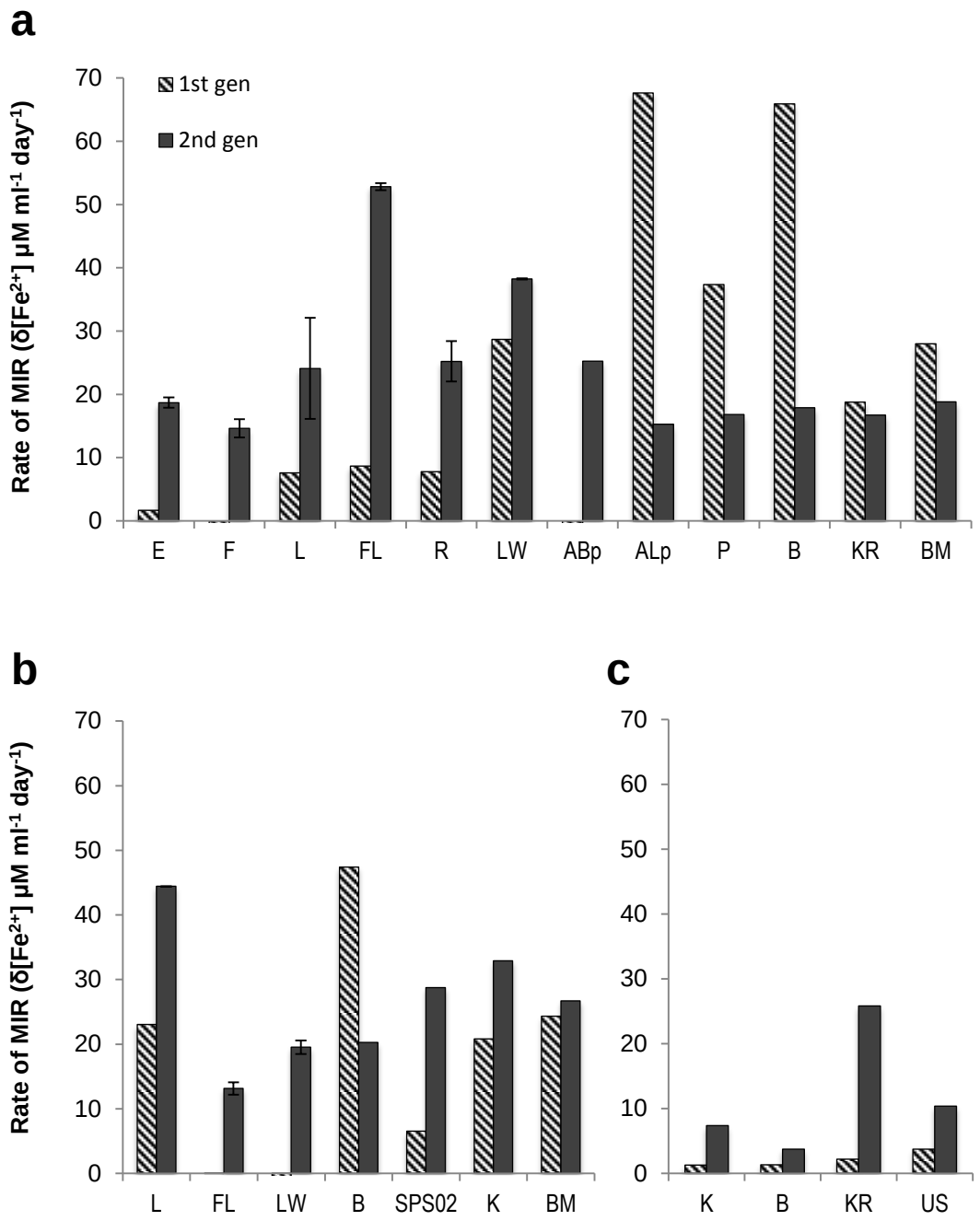

Figure 4.2: Rates of MIR in positive enrichments. Data are expressed as daily production of Fe^{2+} (µM) per ml of enrichment. First (striped) and second (solid grey) generations are displayed for enrichments established at 4°C (a), 15°C (b) and 30°C (c). Error bars represent standard deviation of duplicated second-generation E, F, L, FL, R and LW enrichments.

B enrichments are the only ones to qualify as positive for MIR at all three temperatures. At 4°C and 15°C, the daily rate of Fe^{2+} production in the first-generation enrichment is more than double that of the second. This is not the case in the 30°C enrichments, although the first-generation was incubated for significantly longer than the second and all B enrichments initiated at other temperatures. This is also the case for the other 30°C enrichments in Figure 4.2c, though the rate of MIR in the KR first-generation enrichment is similar to that of the second. Similarly little difference between first- and second-generation rates of MIR are evident in KR 4°C and BM 15°C.

4.4.2 Presence of Geoacteraceae

The majority of positive enrichments were tested for the presence of species of *Geobacteraceae* using a *Geobacter*-specific PCR primer set (see Chapter 3). Some enrichments were sequenced for 16S rRNA in addition to *Geobacter*-specific PCR, and for some 16S sequencing was carried out alone. Results showing the positive and negative detection of species belonging to the *Geobacteraceae* family are shown in Table 4.4. Of the 16 enrichments that were assessed in this way, three do not appear to harbour *Geobacteraceae*. Of these, LW and P represent the only sets in which more than one enrichment was assessed in this way. In both cases, the presence of *Geobacteraceae* species is evident in only one of each set; LW 4°C harbours *Geobacter* but LW 15°C does not, whereas *Geobacteraceae* are present in P 15°C but not P 4°C enrichment. E 4°C also appears not to contain *Geobacteraceae*, as deduced from 16S rRNA data.

Both 16S rRNA and *Geobacter*-PCR data are available for four samples, KR 30°C, LW 4°C, BM 15°C and F 4°C. In the case of BM and F the data are contradictory. In both, *Geobacteraceae* were evident when the PCR products were imaged on a gel, yet none were detected in the 16S rRNA data. Some 16S rRNA sequences from LW 4°C equally matched *Geobacteraceae* and *Clostridiaceae* at family-level, and *Geobacter* and *Clostridium* at genus-level, rendering this data inconclusive (marked '?' in Table 4.4).

Fieldsite	Sample	4°C	15°C	30°C
Namib	SPS02		Y[§]	
	KR			Y[§]*
Río Tinto	BM		Y[§] N*	
Subglacial	E	N*		
	R	Y*		
	L	Y*	Y[§]	
	FL	Y*	Y[§]	
	LW	Y[§]?*	N[§]	
	F	Y[§] N*		
	ABp	Y[§]		
	ALp	Y[§]		
	P	N[§]	Y[§]	

Table 4.4: Enrichments screened for *Geobacteraceae*. Data represent presence (Y) or absence (N) of *Geobacteraceae* strains, deduced from *Geobacter*-specific PCR ([§]) or 16S rRNA sequencing (*). Enrichments not assessed for the presence of *Geobacter* are left blank in the table. '?' represents sequences that were identified as equally similar to *Geobacter* and *Clostridium* genera.

4.4.3 Phylogenetic diversity

4.4.3.1 Bacterial 16S rRNA sequencing

DNA extracted from sediments collected at K, G, H and B was sequenced for the Bacterial 16S rRNA gene, and the ten most abundant genera in each are listed in Table 4.5. Genera that are known to include species capable of growth through MIR are denoted with an asterisk, and correspond to genera listed in Table 2.2 (see Chapter 2).

In the case of G, *Thermotoga* is the only genus in containing species capable of MIR, however a number of other such genera were identified in this sample that constitute <0.61%. Specifically, *Thermincola* (0.56%), *Bacillus* (0.28%), *Geothermobacterium* (0.22%), and *Thermolithobacter* (0.11%) were all identified in the sequence data to genus level, based on a 95-97% match with known sequences of microorganisms in the Blastn+ database. The iron-reducing microorganism *Geothermobacterium ferrireducens* was

identified to species level (greater than 97% match with sequences in the database) with a relative abundance of 0.22%. Thus, although changes in Fe^{2+} concentration were insufficient to represent MIR, a number of potential iron-reducing microorganisms were detected in the sediment with which enrichments were initiated. The same is true for H, in which a number of iron-reducing genera were detected but MIR was not evident in enrichments. Although no such genera are listed in Table 4.5, *Rhodoferax* (1.82%), *Geobacter* (0.97%), *Aeromonas* (0.60), *Geothermobacter* (0.26%), *Ferribacterium* (0.07%), *Geothrix* (0.07%) and *Bacillus* (0.02%) were all detected. A small number of sequences matched >97% to known iron-reducing microorganisms in the database, and include *Rhodoferax ferrireducens* (0.82%), *Geobacter psychrophilus* (0.15%), *Geothrix fermentans* (0.07%) and *Geobacter sulfurreducens* (0.04%).

In the case of the K, *Acidiphilium* and *Acidithiobacillus* are the two most abundant genera detected, and both include at species known to grow from MIR (see Table 2.1). A small proportion of sequences were assigned to *Acidithiobacillus ferroxidans* (0.69%). The one enrichment that was initiated with K tested positive for MIR, hence it is likely that species of *Acidiphilium*, and *Acidithiobacillus*, especially *A. ferrooxidans*, were responsible.

The largest number of genera known to contain iron-reducing microorganisms was identified in B, including *Geobacter* and *Rhodoferax*, which are fifth- and tenth-most abundant and represent 2.77% and 2.20% of identified sequences at genus level, respectively. Other genera include *Deferribacter* (0.86%), *Ferribacterium* (0.51%), *Geothrix* (0.40%), *Geothermobacterium* (0.23%), *Thermolithobacter* (0.17%), *Bacillus* (0.11%), *Geospychrobacter* (0.09%), *Acidiphilium* (0.06%), *Aeromonas* (0.06%) and *Desulfotalea* (0.03%). A number of sequences were matched (>97%) to *Rhodoferax ferriducens* (1.30%), *Geobacter psychrophilus* (0.13%), *Geobacter bemidjiensis* (0.08%), *Geobacter pickeringii* (0.08%) and *Desulfotaea psychrophilia* (0.03%).

G	K	H	B
Clostridium (40.52%)	*Acidiphilium** (24.89%)	*Nitrospira* (9.57%)	*Gallionella* (11.86%)
Fervidobacterium (34.17%)	*Acidithiobacillus** (22.92%)	*Acidobacterium* (8.00%)	*Acidobacterium* (6.20%)
Acetivibrio (6.97%)	*Desulfurella* (22.62%)	*Flavobacterium* (3.69%)	*Nitrosovibrio* (4.29%)
Caldicellulosiruptor (4.12%)	*Hydrogenobaculum* (20.35%)	*Dechloromonas* (2.87%)	*Azoarcus* (3.26%)
*Thermotoga** (2.51%)	*Acidimicrobium* (6.51%)	*Methylibium* (2.27%)	*Geobacter** (2.77%)
Dictyoglomus (1.90%)	*Alicyclobacillus* (0.89%)	*Aquiflexum* (2.23%)	*Ferrovum* (2.69%)
Lutispora (1.23%)	*Leptospirillum* (0.49%)	*Hyphomicrobium* (2.23%)	*Bradyrhizobium* (2.34%)
Thermus (0.89%)	*Prochloron* (0.34%)	*Verrucomicrobium* (2.23%)	*Verrucomicrobium* (2.34%)
Bacteroides (0.67%)	*Desulfotomaculum** (0.30%)	*Herbaspirillum* (2.12%)	*Denitratisoma* (2.32%)
Caloramator (0.61%)	*Acinetobacter* (0.25%)	*Denitratisoma* (2.05%)	*Rhodoferax** (2.20%)

Table 4.5: Most abundant genera in Geysir (G), Krýsuvik (K), Hvalfjördur (H) and Borgarnes (B) starting sediments from Iceland. Ten most numerous genera-assigned sequences are listed for each, as deduced from 16S rRNA sequencing of DNA extracted from bulk sediments. *Genera that are known to include species capable of MIR.

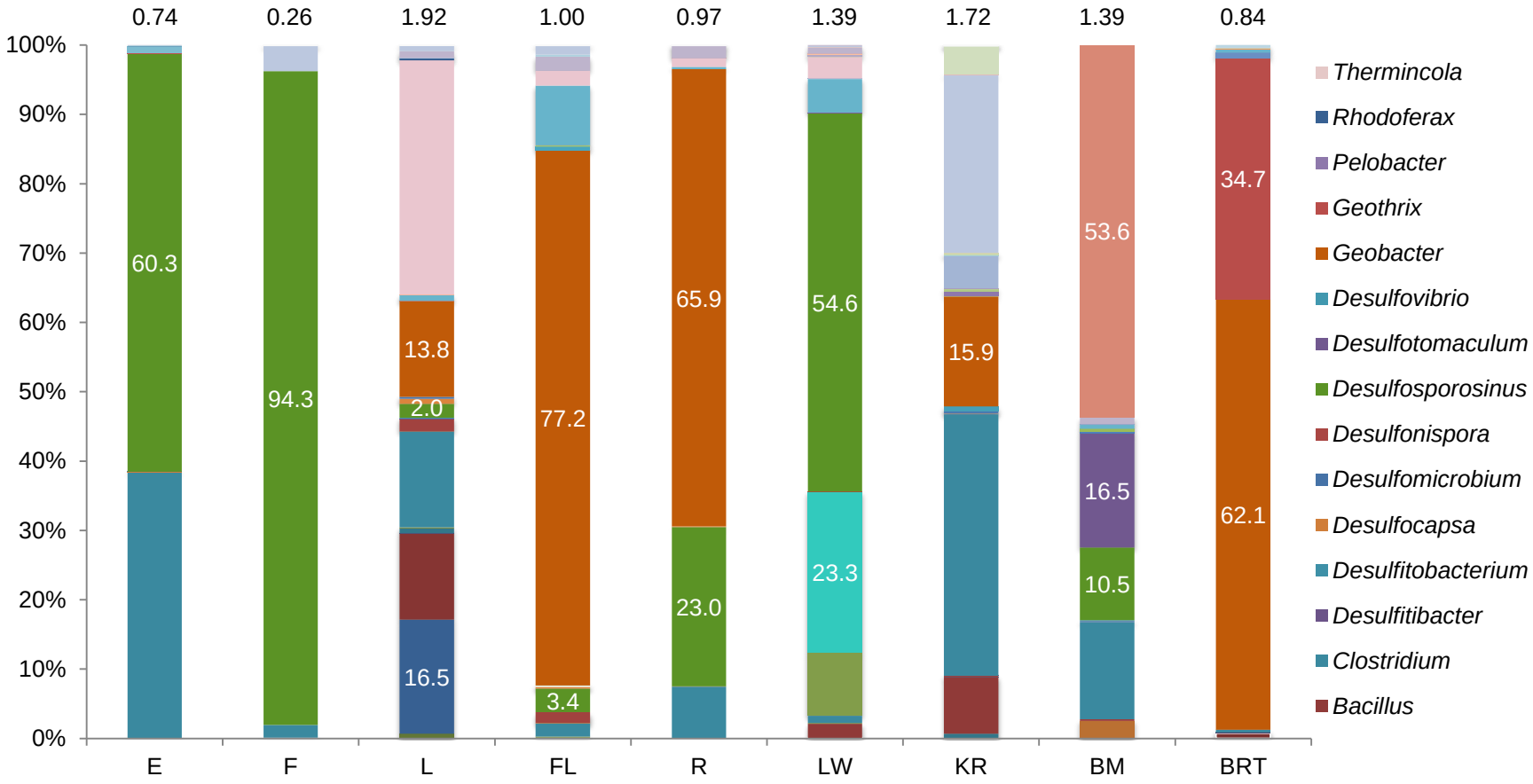

Figure 4.3: Genus-level taxonomic identification of DNA extracted from enrichments. Data are based on 16S rRNA sequence data. Shannon's index of diversity (H) is displayed above each sample. All subglacial enrichments displayed represent 4°C microcosms. Only genera know to include species capable of MIR are included in the legend, and the relative % abundance labeled accordingly. All enrichments are second-generation.

As expected, the highest values of H are evident in enrichments exhibiting the greatest diversity and the most even spread of taxa (see Figure 4.3). The highest H corresponds to L (1.92), and the lowest to F. In more than half of the sequenced enrichments (E, F, FL, R and BRT) the value of H is equal to or lower than 1.00, reflecting the relative dominance of one or more genera.

E is dominated (60.3%) with the genus *Desulforospinus*. The same is also true for F, in which *Desulfosporosinus* represents 94.3% of all sequences identified at genus-level. This genus is also dominant (54.6%) in LW 4°C, and present but not dominant in R (23.0%), RT (10.5%) and FL (3.4%). The enrichments in which *Desulfosporosinus* dominate are all devoid of *Geobacter*, suggesting this genus is responsible for the MIR measured in these enrichments.

In most of the enrichments, the heterotrophic genus *Clostridium* is prominent. In E *Clostridium* represents the majority of sequences not assigned to the dominating genus *Desulfosporosinus*. Similarly, in KR nearly 40% of the sequences were assigned *Clostridium*. This genus represents 14% of assigned genera in BM and L, and 7.4% in R. *Clostridium* is idetified in F, FL and LW, but only accounts for 1-2% of assigned sequences.

Three enrichments are dominated by species of the *Geobacter* genus. The most *Geobacter*-dominant enrichment is FL with a relative abundance of 77.0%. R and BRT follow with the relative abundance of *Geobacter* detected as 65.9% and 62.1%, respectively. *Geobacter* represented 15.9% of genus-assigned sequences in K, and 13.6% in L. R and FL harbour both *Desulfosporosinus* and *Geobacter* genera.

In BRT 96.8% of the identified genera are known to include iron-reducing microorganisms *Geobacter* and *Geothrix*. Sequences of these genera with more than a 97% match to sequences in the database were assigned to the species *Geobacter pelophilus* (41.25%), *Geobacter psychrophilus* and *Geothrix fermentans*. In BM, 70.1% of identified genera are known to include species of iron-reducing microorganisms, including *Thermincola* (53.6%) and *Pelobacter* (16.5%). A further 10.5% are *Desulfosporosinus*, the aforementioned genus that dominates in E, F and LW. Sequences that were assigned to species of known iron-reducing microorganisms include *Desulfitobacterium metallireducens*, *Desulfitobacterium aromaticivorans* and *Thermincola ferriacetica*, though the number of sequences matched for each represents less than 0.01% relative abundances of each. In KR only 15.9% of sequences assigned to genera represent potential iron-reducing microorganisms, in this case *Geobacter*. A number of the relatively few species-assigned sequences represent known iron-reducing microorganisms; *Geobacter sulfurreducens* (1.60%), *Geobacter grbiciae* (0.06%) and *Pelobacter acetylenicus* (0.04%). Almost a quarter

of all genus-assigned sequences in the LW enrichment were assigned to *Desulfitobacterium* (23.3%), and a small proportion of sequences were identified as iron-reducing species *Desulfitobacterium hafniense* (0.53%) and *Desulfitobacterium metallireducens* (0.03%). Of the 65.9% sequences assigned to *Geobacter* in R, none were sufficiently matched to assign to known species. In contrast, 46.29% of species-assigned sequences in FL were associated with *Geobacter psychrophilus*, and a much smaller number were assigned to the iron-reducer *Rhodoferax ferrireducens* (0.08%). *Rhodoferax* is assigned to 16.5% of sequences in L, and 16.17% of sequences are related to the known iron-reducing species *Rhodoferax ferrireducens*. None of the 13.8% of sequences that were assigned to the genus *Geobacter* were sufficiently matched to species level. Similarly, the 94.3% of sequences in F that are assigned to *Desulfosporosinus* have not been resolved to species level. In E, although 60.3% of sequences were assigned to the genus *Desulfosporosinus*, only 0.08% of all sequences were assigned to a species, namely *Desulfosporosinus lacus*.

4.4.3.2 DGGE

The gel image and resulting bands from DGGE analysis are shown in Figure 4.4, and demonstrate a range of phylogenetic diversity across the samples. The image is a processed version of the original, in which lane positions have been normalised for gel distortion using the marker (L 4°C; outermost lanes). This marker enrichment gave rise to 12 and 13 bands in the left-hand and right-hand lanes, respectively. In contrast, only 3 bands are evident for L 15°C, two of which appear to overlap with those of the 4°C enrichment. The F 4°C enrichment similarly manifested in 3 bands, whereas P 4°C displays 12. One more band is evident for the LW 15°C compared to the LW 4°C enrichment, and three appear to overlap. The 4°C proglacial enrichments ALp and ABp both exhibit 5 bands each, with the bands in each lane not appearing to overlap. From Figure 4.4 the most phylogenetically diverse enrichments in this subset are L 4°C and P 4°C, whereas the least diverse are L 15°C and F 4°C.

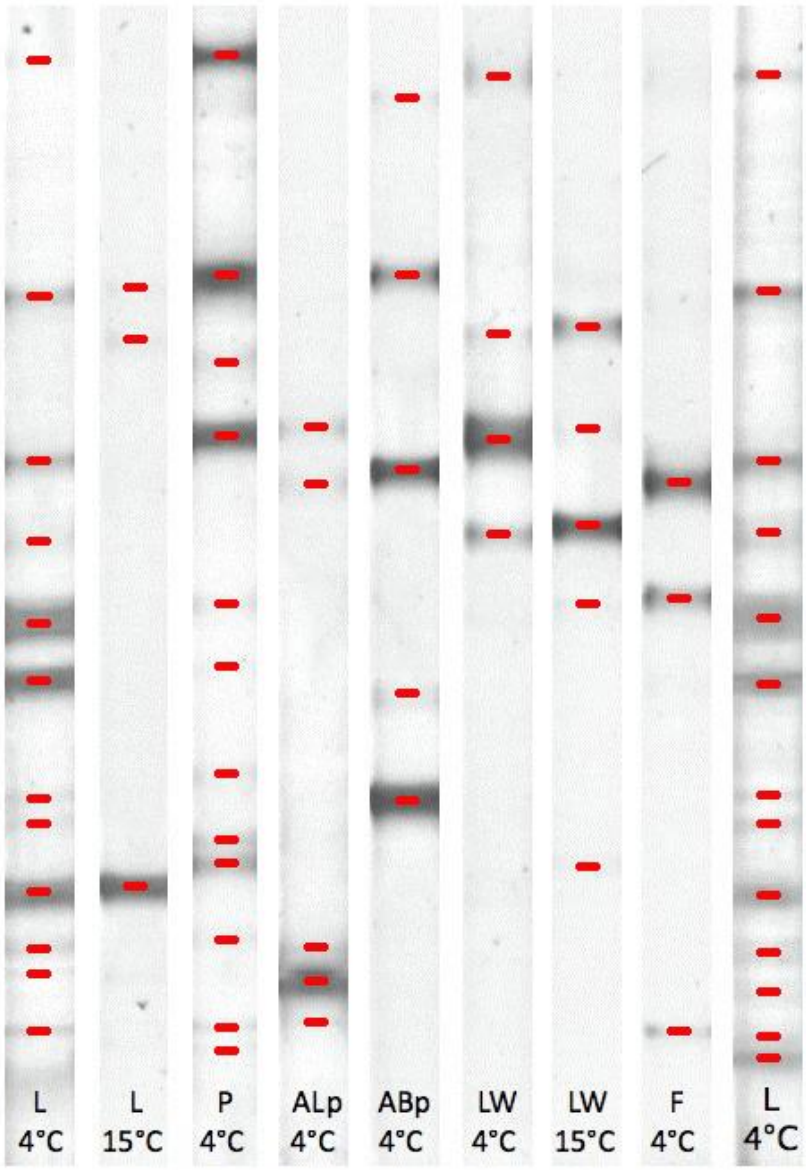

Figure 4.4: DGGE gel of a subset of subglacial and proglacial positive MIR enrichments. The image was processed using Gel Compar II, where outermost lanes represent the marker (L 4°C) used to normalise for gel distortions after a 10% background subtraction to remove noise. Red dashes denote bands subsequently recognised by the Gel Compar II software.

4.5 Discussion

The overall goal of this biogeographical study was to assess Mars-relevant environments for the presence of active iron-reducing microorganisms. This was achieved by initiating MIR enrichment cultures using sediment samples collected from each location, with the view to assess 1) whether MIR occurs, 2) whether growth temperature ranges reflect those of the original environment, 3) whether members of the *Geobacteraceae* family are present, and/or 4) which microorganisms are likely to be repsonsible for the observed MIR. These questions will be discussed below in light of the findings of this study.

4.5.1 Occurrence of MIR

Results from this study indicate that MIR is widespread in enrichments initiated with subglacial and river/stream sediments, but not in recent volcanic (Iceland) or arid (Namib) enrichments.

The positive enrichments from both Río Tinto samples suggests that the widely-available ferric iron in the river's waters is in fact being used for MIR, as previously suggested (Druschel *et al*., 2004; Coupland and Johnson, 2008). However it is somewhat surprising that MIR was detected, given the circumneutral nature of the enrichments and the low pH of the starting material. It is likely that adding the Río Tinto material to the neutral basal medium resulted in a pH somewhere between the two for the initial enrichment, though this effect would be greatly reduced after subculturing to a second-generation. That sediment from Krýsuvík gave rise to measurable MIR at 30°C is surprising, since the *in situ* pH and temperature measurements were 2.2 and 50.5°C, respectively.

4.5.2 Rates of MIR

Rates of MIR reported in Figure 4.2 represent first-order approximations, since the exact quantity of starting material used to establish enrichments varied from sample to sample. However it is interesting to compare the approximate rates between samples and over both generations.

Owing to the often-negative signal for MIR in initial enrichments, rates of MIR in subglacial sediments are generally lower in first- compared with second-generation enrichments. However, although the opposite is true for some enrichments, there does not appear to be a general pattern for other enrichments with respect to change in rates between first- and second-generations. This is surprising, since one would expect MIR to increase as iron-reducing microorganisms become more dominant over time. This may indicate that the role of non-iron-reducing microorganisms is important to the activity of iron-reducing microorganisms.

Rates of MIR have not previously been reported for the environments assessed in this study. The rates stated here therefore represent the first such measurements. However, they are not comparable to rates documented for other environments since they have not been normalised for the quantity of starting sediment. A number of studies report rates of MIR in incubations and enrichments normalised for dry weight of sediment. For instance, Achtnich and others (1995) found the total amount of ferric iron reduced in anoxic paddy soils reached 70 μmol over a period of 8 days, representing an average reduction rate of 8.75 μmol g^{-1} dry weight soil per day. Jensen *et al*. (2003) calculated MIR rates in Baltic Sea sediment cores from observed changes in concentration of poorly crystalline ferric oxide. The calculated rates were reported as change in concentration per unit area (cm^{-2}) per day, and throughout the depth of a sediment core were reported to reach 73 mM cm^{-2} d^{-1}.

The lack of reported MIR rates for environments typical of those studies here, coupled to the differing metrics used to report those from elsewhere, highlights the critical need to better incorporate these measurements into future enrichment studies. In particular, the sediment used to initiate enrichments should be measured at the start, and in dry form at the end, allowing for the most precise calculation of rates to be expressed in a range of ways.

4.5.3 MIR at different temperatures

Results of some MIR enrichments indicate that iron-reducing microorganisms are adapted to grow within a temperature range typical of the original environment. For example, based on a diagnostic positive or negative criterion, iron-reducing microorganisms in the US Río Tinto enrichment appear to be adapted to temperatures closer to 30°C than to 4°C or 15°C.

Temperature adaptations are most evident in enrichments initiated with subglacial sediments, in which MIR is more prevalent at 4°C than 15°C, and absent at 30°C; hence iron-reducing microorganisms in these sediments appear to be at least tolerant of cold temperatures (‘psychrotolerant’). The temperature adaptation between duplicated 4°C and 15°C second-generation enrichments for E, R, L, FL and F is clearly demonstrated in Figure 4.1. These graphs illustrate more productive MIR at 4°C (circles, dot-dashed line) compared to 15°C (squares, short-dashed line) in all cases except L, indicating that iron-reducing microorganisms in these environments are preferentially adapted to cold temperatures (‘psychrophilic’) rather than just tolerant of them.

MIR enrichment cultures serve to preferentially select for viable iron-reducing microorganisms, yet their proliferation in such cultures does not necessarily indicate they are active in their host environment. As such, it is not possible to extrapolate the apparent psychrophily of some iron-reducing microorganisms in subglacial sediments to higher rates of *in situ* MIR; this can only be addressed through further experiments aimed to mimic conditions of the host environment.

4.5.4 Phylogenetic diversity of MIR

The phylogenetic diversity of positive enrichments varies greatly between temperatures and sediments, as illustrated in Figure 4.3 and 4.4. The 4°C enrichments of P and L appear to be the most phylogenetically diverse in the DGGE data, with F 4°C and L 15°C representing the least diverse of the subset tested (Figure 4.4). Where enrichments overlap with phylogenetic methods, the DGGE results broadly corroborate with the 16S rRNA sequencing data. That is, sequences from samples with a larger number of DGGE bands have been assigned to a larger number of taxa, and those with fewer bands have been assigned to fewer. For instance, 3 bands are evident in DGGE analysis of F 4°C (Figure 4.4), in general agreement with the 6 taxa assigned at genus level, of which only three are apparent in Figure 4.3. Similarly, the 13 recognised bands of L 4°C correspond to 34 assigned genera, with only 12 apparent in Figure 4.3.

Members of the MIR-family *Geobacteraceae* are present in most MIR-positive enrichments, and dominate in a small number. However, the sulphate-reducing genus *Desulfosporosinus* is also present in most sequenced positive enrichments and similarly dominates a number of enrichments. Furthermore, *Desulfosporosinus* is more abundant in

the absence of *Geobacter*. Collectively these genera appear to be responsible for most of the MIR observed, though species of *Thermincola*, *Geothrix*, *Desulfitobacterium*, *Desulfotomaculum* and *Rhodoferax* also proliferated in some positive MIR enrichments.

Given that the Krýsuvík enrichment was not subject to 16S rRNA gene analysis, the microorganisms responsible for the observed MIR remain unknown. Yet the two most abundant genera identified in the bulk sediment 16S rRNA analysis are *Acidiphilum* and *Acidithiobacillus*, both known to include species capable of growth by MIR. However, the former is unable to use either lactate or acetate as electron donors (Kusel *et al*., 1999), and the latter is known to reduce ferric iron with elemental sulphur only (Pronk *et al*., 1992). The only other genus detected in this sediment that represents a potentially active iron-reducing microorganism in the subsequent enrichment is *Desulfotomaculum*. Although the relative abundance of this genus in the starting sediments is lower (Table 4.5), it is the most likely genus responsible for the observed MIR.

Of the numerous genera capable of MIR identified in 16S rRNA analysis of the Borgarnes stream sediment, only *Geothrix* and *Geobacter* proliferated over the two generations of enrichment, the latter being the most abundant. This is perhaps not surprising, since all known species of *Geobacter* are able to couple iron reduction to the oxidation of acetate, and the vast majority can use lactate. Furthermore, all strains of *Geobacter* tested for use of ferrihydrite as an electron acceptor could do so. The only species of *Geothrix* known to conduct MIR for growth (*G. fermentans*; Coates *et al*., 1999) is also able to use both lactate and acetate as electron donors, coupled to the reduction of ferrihydrite. Hence, these results indicate that the enrichment setup adopted in this study preferentially selects for these genera over others present. Alternatively, these results may indicate that all other genera containing iron-reducing microorganisms detected in the bulk sediment are in fact relatively inactive, or indeed were absent or significantly reduced in number in the inoculum used to initiate enrichments.

The three genera that are likely to be responsible for the MIR detected in the BM sample from Río Tinto are *Thermincola*, *Desulfotomaculum* and *Desulfosporosinus*. Sánchez-Andrea *et al* (2011) detected *Desulfosporosinus* in anaerobic sediments using 16S rRNA gene analysis, and a subsequent study (Sánchez-Andrea *et al*., 2012) identified *Desulfotomaculum* species as the dominant genus of microorganisms in sulphate-reducing enrichment cultures. Since *Desulfosporosinus* is known to include at least one species capable of iron reduction (*D.* lacus; Ramamoorthy *et al*., 2006), it is perhaps unsurprising that this genus is abundant in the positive enrichment. Similarly, *Desulfotomaculum reducens* is known to grow with ferric iron as an electron acceptor (Tebo and Obraztsova,

1998), thus a relatively high abundance of this genus is also unsurprising. In contrast, *Thermincola* has not been reported in studies of this type before, and thus its detection in this study represents a novel finding. This genus is the most abundant of the three, and the known iron-reducer *Thermincola ferriacetica* was identified at species level; hence these results strongly suggest that, along with *Desulfosporosinus* and *Desulfotomaculum*, this genus is implicated in MIR in Río Tinto sediments at moderately-low to circumneutral pH. Although known to proliferate in the Río Tinto system, the absence of *Acidithiobacillus ferrooxidans* in the 16S rRNA data (Figure 4.3) can perhaps be explained by the circumneutral pH selection pressure of these enrichments, in addition to a lack of elemental sulphur, the only known electron donor employed by this microorganism for MIR (Pronk *et al*., 1992).

Microorganisms implicated in subglacial MIR have been identified in a number of studies, but only one study is concerned with sediments proximal to those used here. Yde and others (2010) investigated the microbiology of basal ice at the margin of the Russell Glacier on the western margin of the Greenland ice sheet. Through the construction of a clone library, sequences associated with the genera *Rhodoferax* and *Geobacter* were identified. Results from the 16S rRNA gene analysis of this study illustrate that the latter genus dominates the second-generation enrichment, and *Rhodoferax* was not identified. Only two studies to date have enriched for MIR in subglacial sediments. The first, by Foght and others (2004), enriched for iron-reducing microorganisms in sediments from beneath two temperate glaciers in New Zealand using ferric-citrate and yeast extract. The enrichments were positive for MIR, but found the microorganisms responsible to be few in number. Similarly to the study by Yde *et al* (2010), clone library analysis of the sediments identified sequences closely related to the *Rhodoferax* genus. The second study to enrich for MIR was conducted by Mikucki and Priscu (2007), using outflow sediments from Blood Falls in Antarctica as their inocula. The 16S rRNA analysis of the sediment identified sequences closely related to the iron-reducer *Geopsychrobacter electrodiphilus*, although an isolate from a positive MIR enrichment was a close relative of *Shewanella frigidimarina*. In all other studies *Rhodoferax* has been reported and implicated in subglacial MIR (Skidmore *et al*., 2005; Lanoil *et al*., 2009; Mitchell *et al*., 2013). In contrast, *Rhodoferax* was identified only in the positive enrichment from Leverett glacier in this study. Given that *Rhodoferax ferrireducens* is capable of MIR with acetate or lactate as electron donors and ferrihydrite as an electron acceptor (Finneran *et al*., 2003), it seems unlikely that this strain would not proliferate in MIR enrichments if present and viable. Hence these results serve to demonstrate that enrichments established with sediments from five geographically-distinct

subglacial settings, *Rhodoferax* does not appear to carry out MIR, which can instead be attributed to *Geobacter* or *Desulfosporosinus* species, and in some cases both.

From this study it appears that MIR is carried out by a more diverse array of microorganisms than previously thought. Moreover, members of the family *Geobacteraceae* are not the only prolific iron-reducing microorganisms identified; microorganisms conventionally thought of as sulphate-reducers appear to be the dominant iron-reducing microorganisms in many sediments.

4.5.5 Factors controlling MIR

From the results it appears that MIR requires long-term stability of anoxic conditions and the presence of an active hydrological regime. The lack of MIR in sediments retrieved from the Namib Desert serve as evidence of both. As predicted, sediment collected from the mobile sand dunes of the Sand Sea was not conducive to MIR. In contrast, the waterlogged and likely organic-rich sediments of the Kuiseb River best resembled conditions of other sediments in which MIR has been reported, and did indeed harbour active iron-reducing microorganisms. With the exception of the sulphate spring, none of the gravel plain sediments were conducive to active MIR. Not only does the extreme aridity of this environment place desiccation stress on all organisms, regardless of metabolic pathway employed, but also anoxic conditions are likely to be restricted to infrequent heavy rain events when the plains become temporarily waterlogged. Furthermore, transfer of iron-reducing microorganisms from stable habitable sediments (such as those of the bed of the Kuiseb River) to newly habitable sediments (such as waterlogged soils of the gravel plains following heavy rain) involves subjecting microorganisms to aerobic stress that is deleterious to their survival. Therefore, the dominant factor affecting habitability for MIR in the Namib Desert is likely not the just the extreme aridity that renders it analogous to Mars, but also the lack of stable anoxia.

No MIR was evident in enrichments initiated with Fimmvörðuháls sediments. This can perhaps be attributed to a lack of available ferric iron, since the dominant iron phase in this environment is ferrous iron locked away in primary volcanic minerals, such as olivine, which is common in basalts. In older volcanic regions in Iceland, these basalts have weathered over time to form alteration products such as the ferric-iron bearing phyllosilicate, palagonite (Bishop *et al*., 2002). In addition, although the abundance of organic compounds

was not assessed at this site, it is likely that the environment is also lacking in organic electron donors for MIR due to the young age of the basalts. Furthermore, even if this region was conducive to MIR, transfer from established MIR habitats (such as nearby iron-rich streams) would similarly subject microorganisms to aerobic and desiccation stresses. However it is important to note that three first-generation enrichments from this site were positive for MIR but not carried forward to a second generation (FimmBB, FimmAH and FimmIF). Although two of these enrichments gave rise to ferrous iron production little more than the 5 mM threshold after 285 days, these enrichments nonetheless place uncertainty on the assertion that there was indeed a lack of available ferric iron in this environment.

Similar to the Namib samples, MIR was only detected in Icelandic enrichments derived from stream/river sediments. That is not to say all stream sediments gave rise to positive MIR enrichments; no enrichment initiated from Hvalfjörder sediments gave rise to MIR. However this is likely attributable to the lack of soil in the streambed to sample, and the enrichment inoculum was largely comprising small pebbles, plant roots and stream water. The close packing of finer-grained soils is likely to contribute to stable anoxia in a streambed, hence the soil-lacking nature of the material at Hvalfjörder may not be sufficiently anoxic for MIR to occur.

The lack of MIR in subglacial and proglacial sediments retrieved from Solheimajökull in Iceland is likely related to the reasons that Fimmvörðuháls sediments were not conducive to MIR. Not too distant from this recent volcanic field, the Solheimajökull system is subject to the in-fall of large quantities of ash liberated from volcanic eruptions. At the time of collection, much of the glacier was covered in the dark ash from the 2011 Grimsvötn eruption, the presence of which further speeds up the melting of the glacier. Given that the origin of this material is the same as that of the Fimmvörðuháls pass, there is similarly a lack of abundant and available ferric iron to serve as an electron acceptor. However, it is also possible that the environment lacks appreciable organic electron donors for MIR. Martteinsson and others (2013) conducted a clone library on DNA extracted from subglacial waters of the Vatnajökull ice cap, and detected high abundances of *Geobacter*, *Sulfospirillum* and *Desulfosporosinus* taxa. The authors suggest that species of *Geobacter* and *Desulfosporosinus* use molecular hydrogen as an electron donor for iron- and sulphate-reduction, respectively, though all three genera include iron-reducing microorganisms. It is therefore feasible that these microorganisms may have been present in sediment collected from the Solheimajökull system but were adapted to autotrophic MIR using hydrogen, an electron donor denied in the enrichments established in this study.

The large negative change in ferrous iron concentration in a number of first-generation Fimmvörðuháls enrichments may indicate the presence of anaerobic iron-oxidising microorganisms. These microorganisms are known to draw upon nitrate as a terminal electron acceptor (Weber *et al*., 2006b), and perchlorate has been implicated as an alternative electron acceptor for anaerobic iron oxidation in the literature (Coates and Achenbach, 2004; Bruce *et al*., 1999). However it is unclear whether these samples contained nitrate or perchlorate, and the cause of the ferrous iron depletions in these enrichments remains unknown. Where this effect is measured in 4°C and 15°C enrichments established with Fimmvörðuháls and Solheimajökull sediments, no such effect is measured in the corresponding 30°C enrichments. This is likely due to the substantially longer incubation times of the 30°C enrichments, and may indicate that iron-reducing microorganisms may be present in these sediments but take significantly longer to proliferate. Alternatively these effects could be abiotic.

Despite the lack of attention to MIR in subglacial environments, active iron-reducing microorganisms appear to be relatively widespread. The focus of most studies addressing the microbial ecology of subglacial systems has been on the inventory of microorganisms, with relatively few culture-based studies in which MIR is neglected. The results of this survey demonstrate that not only do iron-reducing microorganisms appear to be tolerant of the perennially-cold conditions, but psychrophilic MIR is widespread. Not all enrichments were positive, and results cluster according to the means by which sediments were retrieved. Specifically, all six of the basal sediment-laden ice that were chain sawed from the source material (E, F, L, R and LW) gave rise to positive 4°C. Comparatively few of the enrichments initiated with other sediment samples were positive for MIR. This may suggest that the nature of sample collection and long-term storage plays an important role in the likelihood of iron-reducing microorganisms remaining viable, where the retrieval of large blocks of debris-rich ice that are kept frozen better maintain the microbial consortia within, compared with scooping up relatively smaller quantities of debris with sterilised spatulas, and storing them at the much colder temperature of –80°C. Interestingly, sediment collected from E, R, L, LW and F had been stored at –30°C since their arrival to the laboratory. This indicates that not only are active iron-reducing microorganisms capable of MIR at low temperatures, but they may also be primed to withstand significantly lower temperatures, in some cases for several years.

4.5.6 Implications for the biogeography of terrestrial MIR

There are several implications of this research that relate to the understanding of the role and prevalence of MIR on Earth. Firstly, this research highlights that MIR is not ubiquitous to environments on Earth, and appears to be controlled by the longevity of anoxia in addition to the availability of redox couples. Unsurprisingly, MIR is widespread in waterlogged soils of stream- and river-beds, where the sediments remain anoxic and serve as a reliable source of redox substrates.

Secondly, the detection of MIR in Río Tinto samples suggests that this metabolism operates in this system, which is of much lower pH than most other reports of MIR on Earth. Furthermore, the contrasting microbial ecology of the biomat compared to other positive enrichments demonstrates that a wide variety of microorganisms are responsible for MIR, many of which would not traditionally be described as iron-reducing microorganisms. Additionally, the widespread occurrence of MIR at low temperatures in subglacial sediments reveals that MIR is likely to be an important process in these settings, and represents the most comprehensive demonstration of psychrophilic MIR to date. Given the geographical extent of ice cover on Earth, the contribution of MIR to the cycling of iron and carbon in subglacial environments is likely to be grossly underestimated.

This study demonstrates that MIR occurs in environments characterised by lower pH and temperatures than previously thought, and a wider variety of microorganisms are responsible. Indeed, the phylogenetic diversity revealed in molecular techniques employed here illustrates that defining an organism by the metabolism it was first characterised by (e.g. sulphate-reducers, iron-reducers) is detrimental to the way their role in microbial communities is understood. In this case, microorganisms traditionally thought of as sulphate-reducers appear to be responsible for widespread iron-reduction. Perhaps a naming system independent of metabolisms would avoid potential discrimination in this way, and represent a more useful device in microbial ecology.

4.5.7 Implications for MIR on Mars

These results indicate that the relatively colder and previously more acidic environments on Mars are not necessarily uninhabitable for iron-reducing microorganisms, provided that a supply of appropriate redox constituents and other requirements are met. The

lack of MIR in Namib Desert samples is difficult to attribute to aridity alone, since the lack of anoxia in sediments seems also to have played a part. Transfer of microorganisms from established MIR habitats to newly available MIR habitats further exposes microorganisms to exposure to aerobic conditions. By comparison, the lack of appreciable quantities of oxygen in the Martian atmosphere renders the selective pressure of anoxia redundant. The lack of MIR in recent volcanic environments of Iceland points to a deficiency in the appropriate electron donors and acceptors required for MIR. On Mars, the signature of ferric iron is ubiquitous at the surface, and present in a wide variety of minerals (Table 2.3). Hence, on Mars the abundance and accessibility of potential electron donors is the most probable limiting factor for MIR.

4.5.8 Limitations

A number of limitations exist in this study. Firstly, the quantity of sediment used to initiate enrichments was not equal or measured, thus the direct comparison of rates and magnitude of MIR is problematic. Furthermore, different enrichments were allowed to run for different lengths of time, most notably the first generation 30°C enrichments initiated with sediments from Río Tinto, Iceland and the Namib Desert which were incubated for several months longer than all other enrichments. The criterion of a positive enrichment after 40 days is likely to have selected against microorganisms that are slow-growing or take longer to proliferate, and may explain why 30°C enrichments of some sediments are positive for MIR whilst the 4°C and 15°C counterparts are negative.

An additional limitation lies in the small number of generations cultured before assessing for MIR. In cases where the starting material was rich in sulphate (such as SPS02 and most likely K) it is possible that microbial sulphate-reduction was responsible for the increased ferrous iron concentrations measured. In most cases microbial sulphate reduction produces sulphide, which can abiotically reduce ferric iron. Although this effect should have been greatly reduced upon subculturing to a second generation, its occurrence in enrichments thought to be positive cannot be ruled out. Neither the 15°C SPS02 nor the 30°C K positive enrichments were subject to phylogenetic analysis; hence the presence of sulphate-reducing taxa cannot be assessed. Abiotic sulphide iron reduction could be better evaluated through phylogenetic anaylsis, and avoided through the initiation of more generations.

A number of results regarding the presence or absence of *Geobacteraceae* species were conflicting. In all cases where both *Geobacter*-specific PCR and 16S rRNA gene sequencing were conducted, *Geobacter* were detected in the former but not the latter. The PCR primers used in this study had been assessed for their specificity to target regions of the 16S rRNA gene in all genera belonging to the *Geobacteraceae* family, and proved to be reliable (Holmes *et al*., 2002). Therefore this discrepancy likely reflects that the number of *Geobacteraceae* 16S rRNA gene copies was inherently low, such that they were only detected using the *Geobacteraceae*-specific PCR primer set and not in the 16S rRNA sequencing data.

4.6 Conclusions

The aim of this study was to assess the prevalence of active iron-reducing microorganisms in environments considered analogous to those on Mars. This was achieved through the establishment of MIR enrichment cultures, and the conclusions drawn from this study are as follows:

- MIR is widespread in river and stream sediments and beneath glaciers, but not in recent volcanic environments or extremely arid environments
- Iron-reducing microorganisms in subglacial environments appear to be at least psychrotolerant, and in some cases psychrophilic
- The controlling factors affecting MIR appear to be the long-term stability of anoxic conditions and the availability of appropriate redox constituents
- The contribution of MIR to the global cycling of iron and carbon is likely to be underestimated
- Colder and more acidic environments thought to prevail on Mars, both now and in the past, fall within habitable limits of MIR as we know it on Earth

Based on this study, the hypothesis that MIR is carried out by a wide diversity of taxa extending beyond the supposedly dominant *Geobacteraceae* family can be accepted. This outcome calls for a paradigm shift in the way in which microorganisms classified by their principal mode of metabolism should be considered. Specifically, microorganisms should

not be defined by the metabolism they were first characterised by. That a microorganism should be considered a sulphate-reducer because of how it is named halts progress in studying the role of such microorganisms within microbial communities. This study demonstrates that certain microorganisms are in fact versatile in their ability to harness energy from a much wider range of redox-driven metabolisms than previously thought. This bias could be avoided by moving towards a taxonomic nomenclature that is independent of the metabolic pathways adopted by microorganisms.

In order to further the outcomes of this work, future research should be directed towards two specific goals. Firstly, MIR in enrichments should be established in which molecular hydrogen is supplied as an electron donor. This may reveal a greater number of viable iron-reducing microorganisms in organic-deficient environments. Secondly, although this study has identified sediments containing viable iron-reducing microorganisms, it has not addressed to what degree these microorganisms are active in their natural settings. Future enrichment studies should therefore include parallel incubation studies in which the natural environment is closely replicated. From such studies it will be apparent whether MIR does indeed occur without artificially selecting for it, in addition to the added benefit of characterising realistic rates.

Chapter 5: Does recalcitrant carbonaceous material in the terrestrial subsurface serve as a source of electron donors for MIR?

5.1 Introduction

A large number of organic compounds have been identified as electron donors for MIR (see Table 2.2). However, these compounds are typically tested in isolation, usually as part of the characterisation of newly isolated iron-reducing microorganisms. As a result, whether bulk carbonaceous material in the terrestrial subsurface represents a source of cellular carbon and electron donors for MIR is unknown.

More than 90% of the organic matter on Earth exists in sedimentary rocks (including shales) in the form of kerogen, macromolecular organic matter that is typically insoluble in organic solvents (Vandenbroucke and Largeau, 2007). Kerogen is fossilized organic matter, and was formed through the selective preservation of high molecular weight biopolymers under oxygen-deficient conditions (Killops and Killops, 2005). These components of life were more resistant to decay during diagenesis, and their preserved remains often represent less than 1% of the starting organisms (Sephton and Hazen, 2013). The organic material contained within kerogen is dominated by hydrocarbons, and as such these materials often represent an economically viable source of fossil fuels. Although few hydrocarbons are known to support MIR, some functionalised components (such as fatty acids, carboxylates and phenols) survive diagenesis through their association with the recalcitrant biopolymers (Killops and Killops, 2005). It is the presence of these solvent-soluble compound classes that raise the question of whether kerogens serve as a source of electron donors for MIR and similar chemoorganotrophic redox-based metabolisms.

The organic chemistry of kerogens strongly correlates with the starting material, leading to the distinction of several different types. Four types are recognised, and the degree to which the organic matter in each is functionalised and oxidised increases from Type I to Type IV. Type I kerogens are relatively rare, and are formed predominantly from algae in anoxic mud and shallow water environments, such as lakes and lagoons. These kerogens are characterised by high H/C and low O/C ratios, and mainly contain long-chained aliphatic compounds, owing to a significant contribution from lipid material (Killops and Killops, 2005). The more common Type II kerogens can potentially form in any environment, though

phytoplankton constitutes a major starting material. These kerogens similarly have a high H/C and low O/C ratio; yet carboxylic acid and ketone compound classes are more prominent in Type II compared with Type I kerogens (Killops and Killops, 2005). Type III kerogens are common and derive solely from vascular land plants (Sephton and Hazen, 2013). These kerogens are characterised by relatively low H/C and high O/C ratios, and ketone and carboxylic acid groups are more prominent still (Killops and Killops, 2005). A more recent addition to kerogen classification is Type IV kerogens. Sometimes not considered a true kerogen due to their lack of hydrocarbon potential, these kerogens comprise of reworked and oxidised organic matter, leading to their characteristically high O/C ratios (Killops and Killops, 2005).

A number of studies identify microbial communities in association with macromolecular shale organic matter, and implicate kerogens as a viable source of organic carbon and electron donors for microbial metabolism. For example, Colwell and colleagues (1997) successfully enriched iron-reducing and heterotrophic bacteria from cores of sandstone and shale that make up the Late Cretaceous and Early Tertiary rocks in the Piceance Basin of western Colorado, USA. Additionally, the presence of microbial communities at sand-shale interfaces within Cretaceous rocks was interpreted as evidence that these communities use organic material in shales as the main energy source for fermentation and sulphate-reduction (Krumholz *et al*., 1997). More recently, Shen and Buick (2004) concluded that organic carbon in the form of kerogens is the principle electron donor for microbial sulphate-reduction at the ancient North Pole barite deposit in northwestern Australia. The majority of studies implicating the use of organic matter in kerogens for microbial metabolism, however, are concerned with subglacial environments. Grasby and others (2003) identify that the organic carbon in underlying organic-rich shales at sulfur-springs on Ellesmere Island, Canada, is sufficient to facilitate microbial metabolisms. Similarly, bedrock kerogens were cited as the probable source of electron donors for microbially-mediated sulphate-reduction beneath Finsterwalderbreen, Svalbard (Wadham *et al*., 2004).

Despite the proximity of anaerobic microbial communities to ancient organic matter in the terrestrial subsurface, utilisation of kerogen-derived organics is yet to be demonstrated. Indeed, determination of the exact carbon substrates used my microbial communities in subglacial environments has been recognised as a vital research question to be addressed (Hodson *et al*., 2008).

The overall goal of this study is to assess whether bulk carbonaceous material represents a source of electron donors for MIR. The carbonaceous material evaluated

includes well-characterised kerogens, and less well defined shale rocks that bear kerogens. The hypothesis being tested in this study is that recalcitrant carbonaceous material common to the terrestrial subsurface represents a source of available electron donors for MIR. This hypothesis is tested through microbial growth experiments using a MIR enrichment culture established from Icelandic stream sediments. Specific research questions addressed are:

i. Does MIR occur when shale or kerogen is supplied as the sole source of electron donors?
ii. Does the presence of shale or kerogen inhibit MIR when a full redox couple is supplied?
iii. Does kerogen type affect the outcome to the above questions?

5.2 Materials and methods

5.2.1 Shale samples

Two shale samples were used in growth experiments, both of which originated from the Early Jurassic formation on the north Yorkshire coast, UK. The first was collected at an exposure at Port Mulgrave, and the second from the side of a cliff near the village of Staithes on the Boulby coastline (herein referred to as 'Boulby'). Both shale samples were collected and donated by other researchers acquiring the specimens for independent study.

The formation from which these samples originate was deposited during the Torcian oceanic anoxic event approximately 181 million years ago. Owing to its marine origin, this shale contains widespread ammonite fossils, and contains as much as 15 wt % organic carbon (Cohen *et al*., 2004). Through collaboration with researchers at Imperial College London, both shale samples were analysed for extractable organic matter (EOM). Prior to quantification, organic matter was solvent-extracted by sonicating shale samples in a mixture of dichloromethane and methanol (at a ratio of 93:7), before separating via centrifugation. This process was repeated three times. Results of this procedure found EOM to be less than 1 wt % in both shale samples. Total organic carbon content was not measured. Through the same collaboration, 'whole rock' pyrolysis gas chromatography-mass spectrometry (py-

GCMS) was additionally conducted on bulk shale samples. Briefly, samples were loaded into quartz sample tubes and transferred to an autosampler pyrolysis unit (Model 2500, CDS Analytical Inc., Oxford, UK) in which they were subjected to pyrolysis at 610°C (heating rate of 20°C per minute). Pyrolysis products were then inserted into a gas chromatograph, and the organic compounds detected after being passed into the mass spectrometer (Montgomery, 2014; personal communication).

The pyrolysis step in this technique breaks down larger compounds to smaller ones, making it difficult to identify the precise compounds present. However, it is nonetheless useful for identifying functional groups present. Port Mulgrave contained mostly hydrocarbons, indicated by the presence of such breakdown products as benzene, toluene, xylene and naphthalene. Similar hydrocarbon products were detected during py-GCMS analysis of Boulby, with the addition of sulfur-containing compounds such as thiopthenes (Montgomery, 2014; personal communication). Given their common geological origin, it is unsurprising that the array of compound classes is similar between Boulby and Port Mulgrave shales.

5.2.2 Kerogen samples

Although the carbonaceous material within shales is kerogen, the above samples are relatively uncharacterised with respect to their kerogen Type. Therefore, four well-classified kerogen samples were also used in this study, each of which is thought to be representative of the four major types outlined above. These samples were supplied and analysed through the aforementioned collaboration with researchers at Imperial College London. Each sample is briefly described below, along with corresponding total organic carbon (TOC). Briefly, TOC was measured by twice treating a 100 mg subsample with a solution of 0.5 M HCl, before washing in high purity water. The sample was dried and subsequently inserted into a carbon analyser (LECO Corporation, St. Joseph, MI, USA) in which it was subject to 1000°C in the presence of oxygen. This serves to completely oxidise the organic carbon to carbon dioxide, which was quantified using an infra-red cell previously calibrated using samples of known carbon content (Montgomery *et al*., 2013).

The Type I kerogen is a sample of Late Carboniferous black shale, retrieved from Port Edgar, west Lothian, Scotland, UK. The TOC content of this specimen is 13.43 wt. %.

The Type II kerogen used is a sample of the lower Jurassic Blue Lias black shale, collected from Monmouth Beach, Dorset, UK. This sample has a TOC content of 8.14 wt %. The Type III kerogen is a highly volatile bituminous coal, originating from the Ensdorf colliery of Saarland, Germany. TOC content of this kerogen is 56.4 wt %. The Type IV kerogen is a sample of charcoal obtained from the Upper Greensand formation at Durdle Door, Dorset, UK. By definition the TOC content of this sample is 100%. Irrespective of the individual TOC values, the fraction of extractable organic matter (EOM) of each kerogen sample was found to be less than 1 wt % in all cases.

Compared with the shale samples, the organic material in kerogens were more extensively characterised with py-GCMS by researchers at Imperial College London (details of procedure as above), and the data are summarised in Figure 5.1. The carbonaceous material in the Type I kerogen sample appears to be entirely made up of aliphatic hydrocarbons. The Type II kerogen similarly contains aliphatic hydrocarbons, in addition to more aromatic derivatives and alkyl benzenes. The Type III kerogen contains these same compound classes in addition to a number of diverse phenol compounds. The Type IV kerogen contains the most aromatic compounds and phenols, and appears not to contain any aliphatic hydrocarbons. These data compliment the traditional description of the kerogen type classification described in the introduction, whereby the ratio of H/C is highest for Type I kerogens and decreases to Type IV, and O/C ratio is low for Type I and increases to Type IV kerogens.

It is important to note that py-GCMS is not an appropriate tool to assess whether known electron donor compounds exist within these samples. Different analytical tools are required to measure the presence and abundance of smaller compounds, such as amino acids and small organic acids that are widely used in MIR. Nonetheless, the py-GCMS data indicate the presence of compound classes that are known to include electron donors (phenols, aliphatic and aromatic hydrocarbons). For the purpose of this study, it is assumed that compounds that can support MIR, whether previously identified to do so, are contained within all shale and kerogen samples.

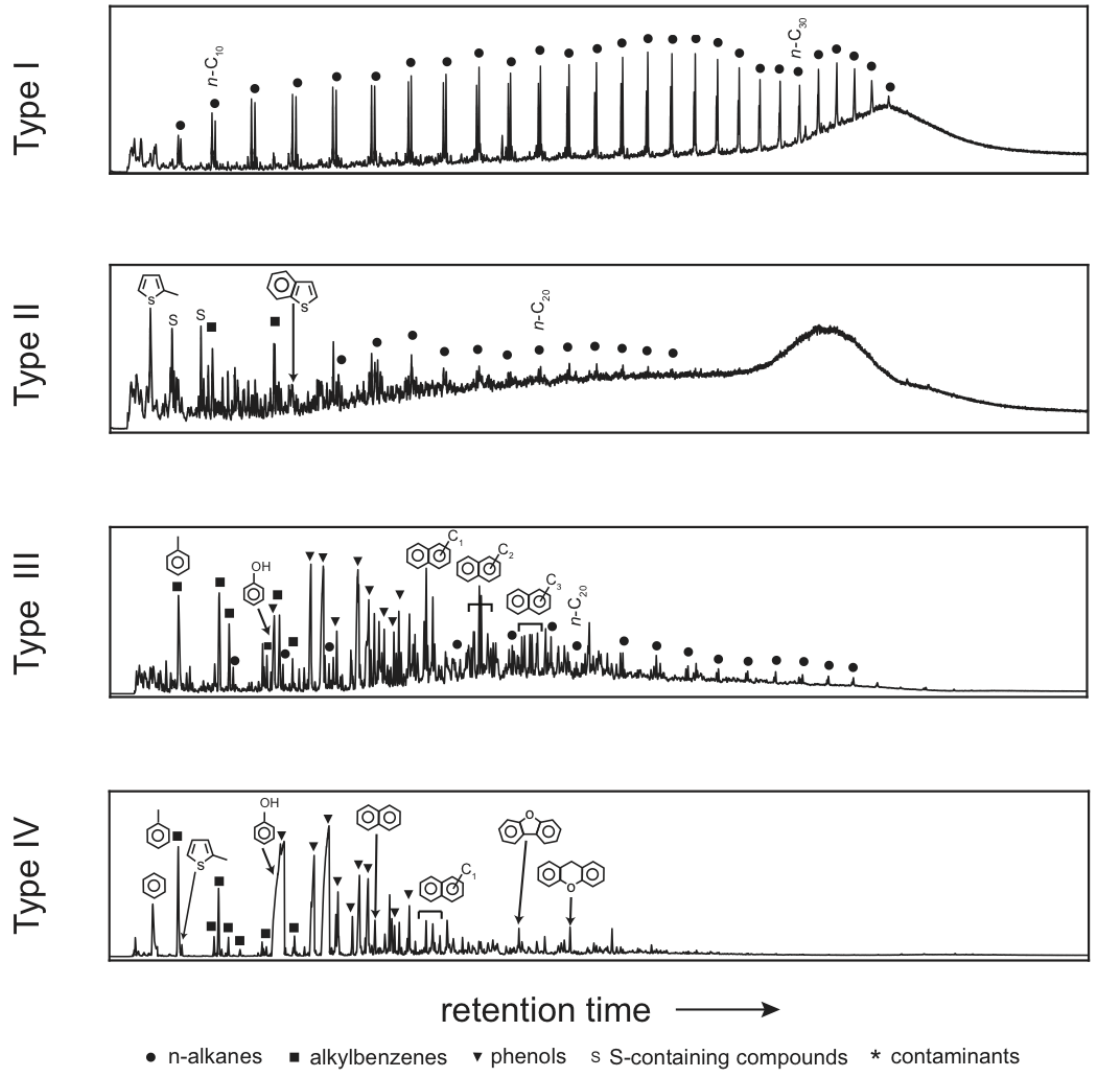

Figure 5.1: Major organic components in kerogen samples. Chromatograms show results from pyrolysis gas chromatograph-mass spectroscopy analysis for all kerogen samples, labeled for major components. Analysis was conducted through collaboration with researchers at Imperial College London.

5.2.3 Microorganisms and growth conditions

The Borgarnes enrichment culture, established from sediments from the bed of an iron-rich Icelandic stream, was used as the inoculum for these experiments (see Chapter 4 for details). The culture was grown in electron donor-limiting medium at room temperature in the dark prior to inoculation. The MIR redox couple supplied to this culture was kept the same as that used to initiate the culture, namely using acetate and lactate in equal concentration as electron donors and ferrihydrite as the electron acceptor. MIR in this experimental culture was monitored to ensure all the ferric iron had been reduced before being used in shale and kerogen experiments.

The growth medium used in these experiments is the same freshwater bicarbonate-buffered basal medium used to enrich for iron-reducing microorganisms as outlined in Chapter 4. This medium contained (in grams per litre of deionised water): $NaHCO_3$ (2.5), NH_4Cl (0.25), $NaH_2PO_4.H_2O$ (0.06), KCl (0.2), and vitamins (10 ml) and trace elements (10 ml). The vitamin mixture contained (in mg per litre of deionised water): biotin (2.0), folic acid (2.0), pyridoxine-hydrochloride (10.0), riboflavin (5.0), thiamine (5.0), nicotinic acid (5.0), pantothenic acid (5.0), vitamin B-12 (0.1), *p*-aminobenzoic acid (5.0) and thioctic acid (5.0). The trace elements mixture contained (in grams per litre of deionised water): nitrilotriacetic acid (1.5), $MgSO_4$ (3.0), $MnSO_4.H_2O$ (0.5), NaCl (1.0), $FeSO_4.7H_2O$ (0.1), $CaCl_2.2H_2O$ (0.1), $CoCl_2.6H_2O$ (0.1), $ZnCl_2$ (0.13), $CuSO_4.5H_2O$ (0.1), $AlK(SO_4)_2.12H_2O$ (0.01), H_3BO_3 (0.01), $NaMoO_4$ (0.025), $NiCl_2.6H_2O$ (0.024) and $NaWO_4.2H_2O$). Vitamin and trace element solutions were stored at 4°C in the dark.

5.2.4 Experimental setup

All shale and kerogen samples were powdered as finely as possible by hand, using a furnaced (400°C, 6 hours) porcelain pestle and mortar. It is worth nothing that the shale and Type II kerogen samples appeared to be finer grained than other samples. For the shales experiment, 1g aliquots of powdered sample were added to furnaced 10 ml serum vials in a laminar flow hood, and 8 ml autoclaved basal medium added. For each shale, two triplicates were setup; one to test whether the material serves as a source of electron donors for MIR, the other to test for inhibition.

Serum vials were then sealed with autoclaved butyl rubber stoppers and crimped with aluminium caps. For the kerogens experiment, exactly the same setup was adopted. However, the experiment was repeated due to unsatisfactory results in the controls, leaving insufficient quantities of kerogens to repeat with 1 g aliquots. Consequently, only 0.5 g aliquots were used in the repeated experiment, and 4 ml of sterile basal medium. Thus the ratio of carbonaceous material to medium was the same in both experiments. It is worth noting that the addition of basal medium to the Type III kerogen resulted in hydrophobic behaviour of the material, with the kerogen floating on top of the medium. After serum vials containing this material were sealed, the kerogen appeared to expand to completely fill the available head space.

After sealing, serum bottles were purged with N_2/CO_2 (80:20) through 0.2 µm syringe filters (10 minutes for 8 ml media, 7 mins for 4 ml). All replicates were amended with ferrihydrite (~50 mM for shales experiment, ~20 mM for kerogens) from sterile anoxic stock. In addition to these shale and kerogen microcosms, the following triplicate controls were set up:

- Positive control: ferrihydrite plus acetate and lactate. The purpose of this control is to show that the inoculated iron-reducing microorganisms are capable of MIR when supplied with a full redox couple.
- Negative control: ferrihydrite but no electron donors. This control serves to establish whether MIR will occur in the absence of electron donors.
- Blank: ferrihydrite plus acetate and lactate (but not inoculated). This control is included to indicate whether ferric iron is abiotically reduced in the absence of iron-reducing microorganisms, when a full redox couple is present.

All replicates were then amended with $FeCl_2$ (~1.3 mM) to serve as a mild reducing agent, before correcting the pH to 6.9-7.0. A noteworthy observation is that the initial pH of Type IV kerogen replicates was very low (circa pH 4.0 and 2.7 for inhibition and electron donor tests, respectively). Consequently, whilst the pH of all other replicates could be corrected using anaerobic gases alone, addition of sterile anoxic NaOH was required to achieve pH 6.9-7.0 in Type IV kerogen replicates. This was observed in both the initial and repeated experiments.

For the shales experiment, all replicates (except blanks) were inoculated with 1 ml of the second-generation Borgarnes enrichment culture. The same inoculum was added to the

initial kerogens experiment. However, the positive controls of this first kerogen experiment demonstrated low levels of MIR, indicating that the microorganisms in the inoculum were in some way stressed or not able to metabolise as anticipated. As a result it was decided that the replicates of the repeated experiment should instead be inoculated with 200 µm of the pooled positive controls from the shales experiment. This approach was chosen since the positive controls in the shales experiments had already demonstrated the expected levels of MIR at the time the second kerogens experiment was initiated. It should be noted that the setup of these positive controls is identical to those used to establish successive generations of MIR enrichment cultures in Chapter 4. Therefore, this pooled positive control used as the inoculate in the repeated kerogens experiment is actually a third-generation culture of the Borgarnes enrichment, compared with the second-generation culture used in the shales experiment. All replicates of both experiments were incubated at room temperature in the dark.

5.2.5 Analytical methods

Microbial iron reduction was determined by measuring the concentration of HCl-extractable ferrous iron over time using the ferrozine assay. To assess whether results of the ferrozine assay correlate to cell numbers, growth was determined by microbial enumerations in the positive and negative controls of the kerogens experiment. Details of these procedures are outlined in Chapter 3. Cell enumerations for shale and kerogen tests were not possible owing to intense auto-fluorescence given off by the carbonaceous materials. Mean changes in ferrous iron reduction and cell numbers between different tests compared with controls were assessed for statistical significance using a student’s t-test (2-tailed, type 2, critical vale of 0.05), and corresponding p-values presented below.

5.3 Results

5.3.1 Shales

The mean change in Fe^{2+} concentration measured in the positive control reached a maximum of 43.51 ± 7.23 mM after 8 weeks of incubation, with a lower final concentration of 29.07 mM after 32 weeks. In contrast, the Fe^{2+} concentration in the negative control peaked at 6.17 ± 1.05 mM after 13 weeks, with a final concentration of 2.69 ± 0.60 mM after 32 weeks. The change in Fe^{2+} concentration in the triplicated blanks, which contained the full redox couple but no iron-reducing microorganisms, fluctuated from –0.62 ± 0.17 mM at weeks 6 and 8 to 1.41 ± 0.05 mM at 3 weeks. From these results it is clear that significantly less MIR had occurred by the end of the experiment in the absence of an electron donor (negative control) compared to when a full redox couple was supplied (positive control; p-value < 0.05). Furthermore, an insignificant change in Fe^{2+} occurred between the start and end of the experiment in the absence of the enrichment culture (blanks; p-value > 0.05), indicating that abiotic changes in Fe^{2+} concentration were negligible.

5.3.1.1 Use of shales as a source of electron donors for MIR

Results from growth experiments in which shales were supplied as the sole source of electron donors for MIR are summarised in Figure 5.2. In general, neither Port Mulgrave nor Boulby shales gave rise to levels of MIR significantly different to those measured in the negative controls. However, there are points in both experiments at which the change in ferrous iron concentration exceeded that in the negative control. Where Port Mulgrave shale was supplied as the source of electron donors, the concentration of Fe^{2+} at week 13 was 10.91 ± 2.25 mM compared to 6.17 ± 1.05 mM in the negative control. The difference between the two means is statistically significant (p-value < 0.05). Similarly, when the Boulby shale was added as the sole source of electron donors, the concentration of Fe^{2+} at week 21 was significantly higher than that of the negative control (6.77 ± 0.27 mM with shale compared to 4.30 ± 0.86 mM without an electron donor; p-value < 0.05). However at no other time points was there a significant difference when either shales were added compared with the positive control.

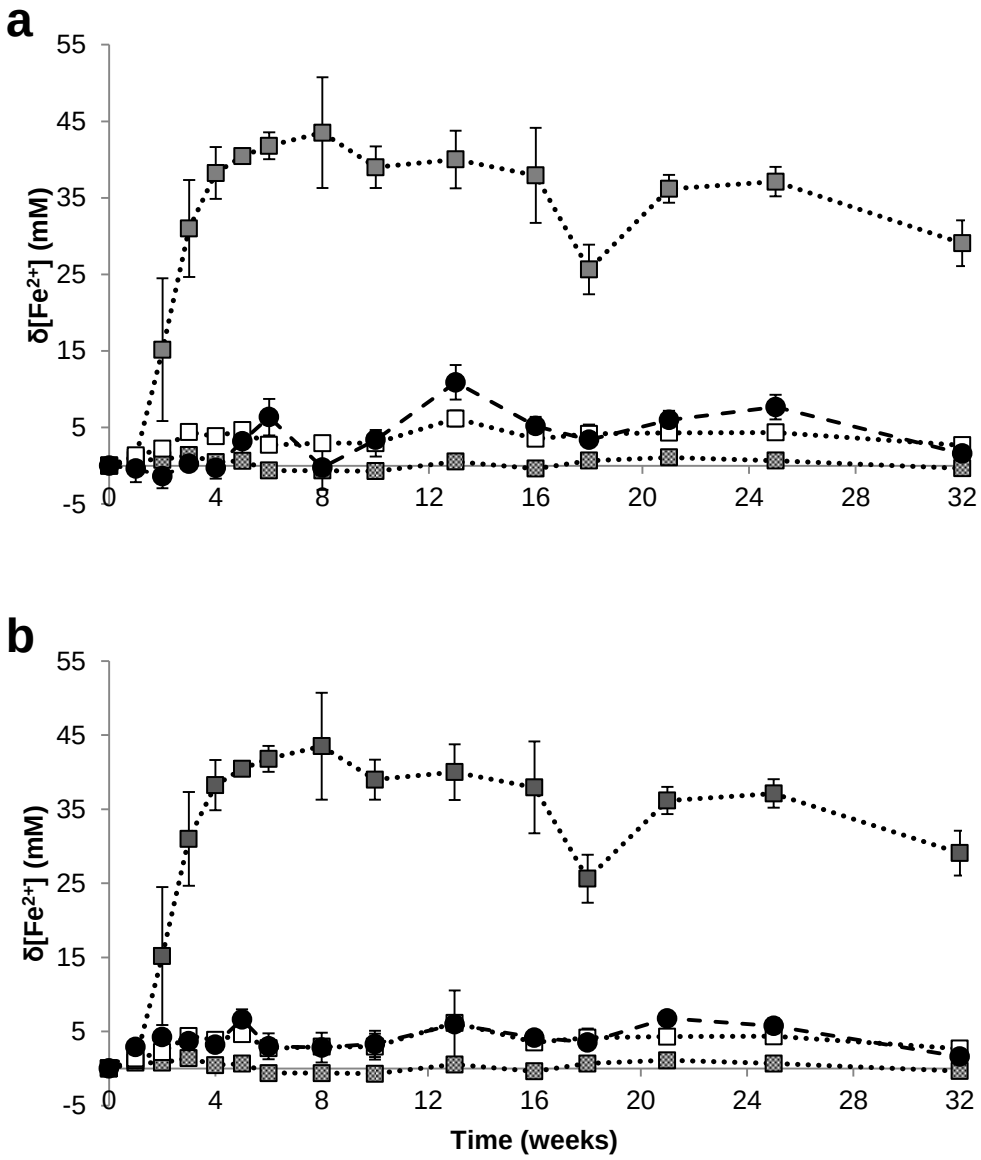

Figure 5.2: MIR growth experiments with shales. Results, expressed as change in Fe^{2+} concentration (mM) with time (weeks) are shown for tests to assess whether Port Mulgrave (a) and Boulby (b) shales serve as the sole source of electron donors for MIR by the Borgarnes enrichment culture (circles, dashed lines). Ferrihydrite was supplied as the terminal electron acceptor. Controls are represented by dotted lines, where the positive control (full redox couple added) is depicted with solid grey squares, the negative control (no donor supplied) with empty squares, and the blank (full redox couple, no culture added) with crosshatch-filled squares. Error bars represent standard deviation of triplicate measurements.

5.3.1.2 Inhibition of MIR by shales

When Port Mulgrave shale was added to medium containing a full redox couple, the final concentration of Fe^{2+} measured after 32 weeks was 22.03 ± 4.71 mM compared with 29.07 ± 3.01 mM in the positive control (see Figure 5.3a). Although this difference is statistically significant (p-value < 0.05), the mean difference between Fe^{2+} concentration between the negative contol and Port Mulgrave inhibition tests at the same time point is also significant (p-value < 0.05). The results plotted in Figure 5.3a demonstrate that, although MIR in the presence of Port Mulgrave shale is of a similar magnitude to that of the positive control by the end time point, it is otherwise lower for much of the duration of the experiment. Therefore, the presence of this shale appears to inhibit MIR by the Borgarnes enrichment culture, but not completely.

The presence of Boulby shale in media containing a full MIR redox couple does not give rise to significantly different mean concentrations of Fe^{2+} by the end of the experiment (p-value > 0.05). The final concentration in the Boulby inhibition test was 30.14 ± 4.23 mM compared with 29.07 ± 3.01 mM in the positive control. Results plotted in Figure 5.3b indicate that the rate of MIR by the Borgarnes enrichment culture was initially slower in the presence of Boulby shale, but after 13 weeks the concentration of Fe^{2+} was in line with, or higher than, that of the positive control. The presence of this shale does not appear to ultimately inhibit MIR by this enrichment culture.

5.3.2 Kerogens

The mean change in Fe^{2+} concentration of the negative control increased to 3.08 ± 0.21 mM after 19 weeks of incubation. This is significantly lower than the final concentration of 6.10 ± 1.28 mM measured in the positive control (p-value < 0.05), though Fe^{2+} concentration peaked at 20.10 ± 2.67 mM at week 8 and did not significantly change by week 12 (p-value > 0.05). The Fe^{2+} concentration of the blank (full redox couple but no cells) decreased to –1.69 ± 0.60 mM by the end of the experiment, though changes in concentration at all other measurement times were smaller in magnitude. The results from these controls demonstrate that, despite the final decrease in Fe^{2+} concentration, the enrichment culture was able to reduce all of the available ferric iron in the presence of

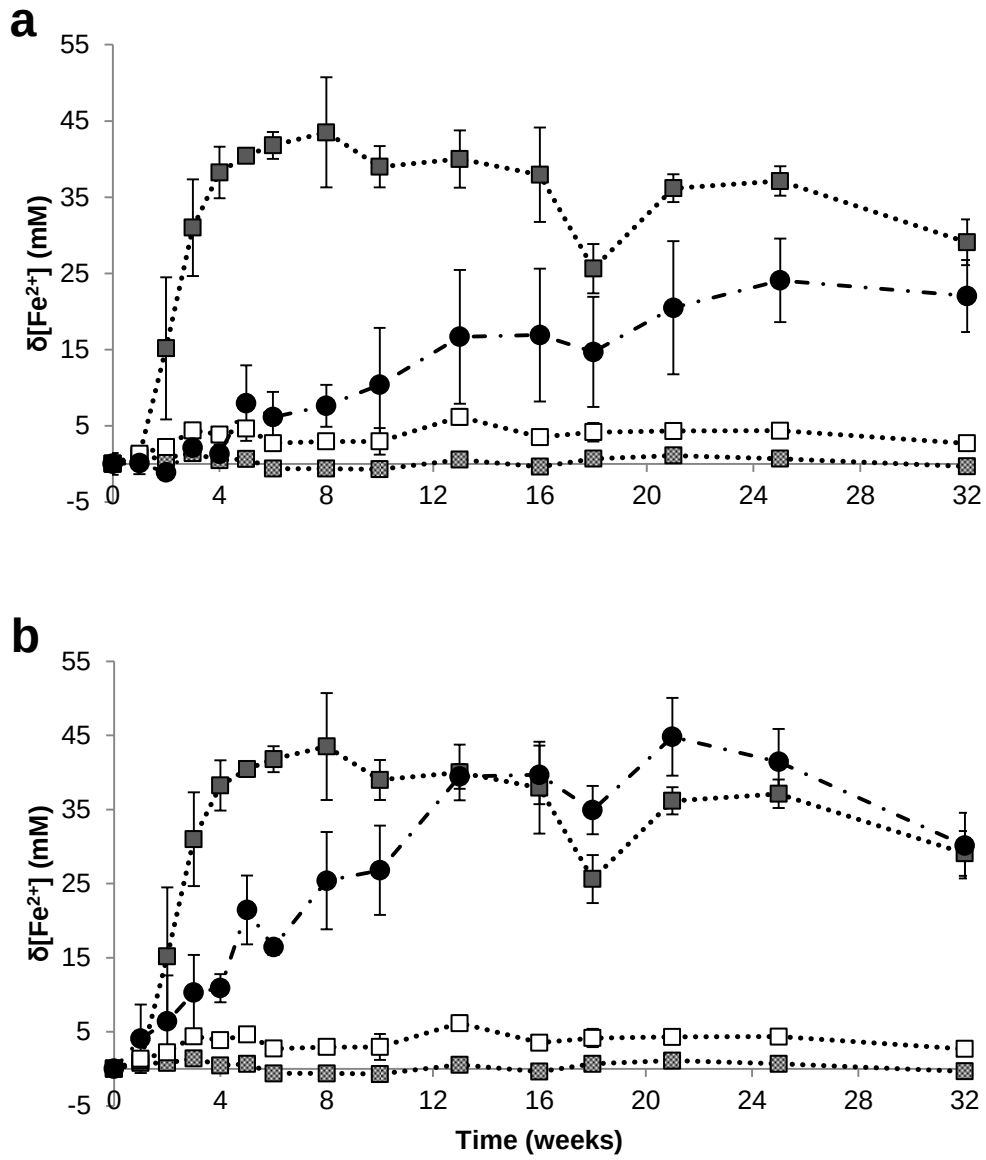

Figure 5.3: MIR inhibition tests with shales. Results, expressed as change in Fe^{2+} concentration (mM) with time (weeks), are shown for tests to assess whether the presence of Port Mulgrave (a) and Boulby (b) shales inhibits MIR by the Borgarnes enrichment culture when acetate and lactate are supplied as electron donors (circles, dot-dashed line). Ferrihydrite was supplied as the terminal electron acceptor. Controls are represented by dotted lines and squares, where the positive control (full redox couple added) is depicted with solid grey squares, the negative control (no donor supplied) with empty squares, and the blank (full redox couple, no culture added) with crosshatch-filled squares. Error bars represent standard deviation of triplicate measurements.

electron donors. The change in Fe^{2+} was significantly lower when no electron donors were supplied, and negligible in the absence of iron-reducing microorganisms.

The mean number of cells in the negative control (no electron donor) of the kerogens experiment increased from $1.43 \times 10^6 \pm 3.02 \times 10^5$ cells ml^{-1} to $2.18 \times 10^6 \pm 1.42 \times 10^6$ cells ml^{-1} after 19 weeks of incubation. This represents a change of $7.35 \times 10^5 \pm 1.46 \times 10^6$ cells ml^{-1}, which is not statistically significant (p-value > 0.05). The mean number of cells in the positive control (full redox couple) increased by $5.84 \times 10^6 \pm 3.90 \times 10^6$ cells ml^{-1}, from $2.83 \times 10^6 \pm 1.30 \times 10^6$ cells ml^{-1} at the start to $8.67 \times 10^6 \pm 3.68 \times 10^6$ cells ml^{-1} after 19 weeks. This change over the course of the experiment is statistically significant (p-value < 0.05). However, the difference between the overall changes of the negative compared with the positive controls over time is not statistically significant (p-value > 0.05). When coupled to the large error associated with the cell counts, whether MIR correlates with cell growth in these experiments remains inconclusive.

5.3.2.1 Use of kerogens as a source of electron donors for MIR

Figure 5.4 summarises results from tests to assess the use of kerogens as a source of electron donors for MIR by the Borgarnes enrichment culture. For the Type I kerogen, there is a reduction in Fe^{2+} concentration for the duration of the experiment, which falls below that of the negative control and the blanks (Figure 5.4a). Addition of this kerogen did not give rise to MIR. Similarly, although the changes in Fe^{2+} concentration over time were not negative when the Type II kerogen was added as an electron donor source, the mean concentration generally overlapped with that of the negative control (Figure 5.4b), indicating MIR was not supported. However, at week 3 the mean concentration of Fe^{2+} in the tests with Type II kerogen was higher than that in the negative control, measuring 4.49 ± 0.75 mM and 1.61 ± 1.40 mM respectively. This is the only time point at which the difference between these triplicates is statistically significant (p-value < 0.05). When the Type III kerogen was added as a sole source of electron donors, the concentration of Fe^{2+} was equal to or less than that in the negative control, and aligned more with that of the blank, which contained the full redox couple but no cells (Figure 5.4c). Similar to the Type I kerogen, at no point during this experiment did the presence of Type III kerogen give rise to Fe^{2+} concentrations indicative of MIR. When the Type IV kerogen was supplied as the sole electron donor source, the concentration of Fe^{2+} exceeded that of all other tests at week 3 (Figure 5.4d), in line with the

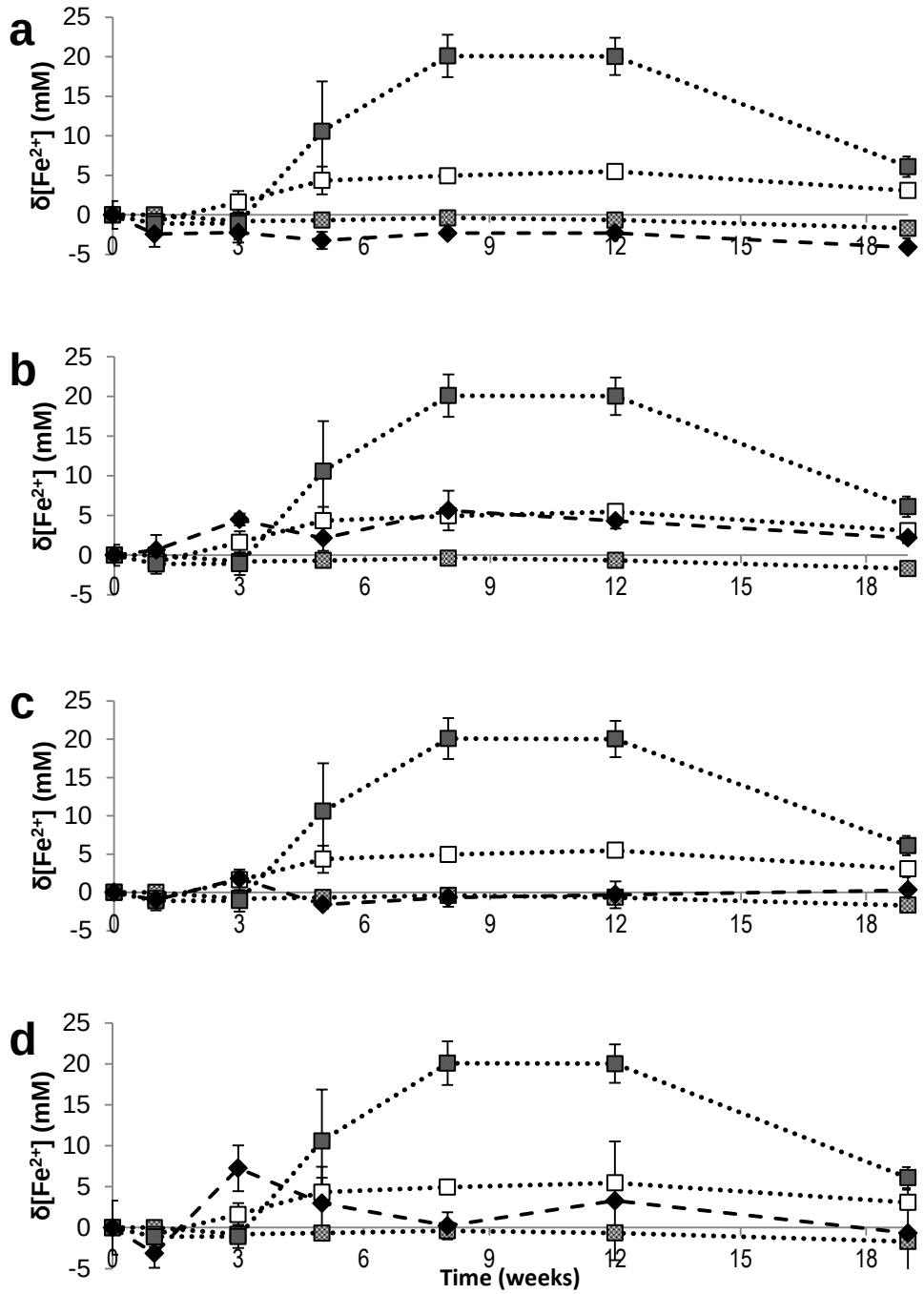

Figure 5.4: MIR growth experiments with kerogens. Results, expressed as change in ferrous iron concentration with time, are shown for tests in which Type I (a), Type II (b), Type III (c) and Type IV (d) kerogens are supplied as the sole source of electron donors for MIR by the Borgarnes enrichment culture (diamonds, dashed lines). Ferrihydrite was supplied as the terminal electron acceptor. Controls are represented by dotted lines, where the positive control (full redox couple added) is depicted with solid grey squares, the negative control (no donor supplied) with empty squares, and the blank (full redox couple, no culture added) with cross-hatch filled squares. Error bars represent standard deviation of triplicate measurements.

concentration measured when the Type II kerogen was added. In this case, addition of Type IV kerogen gave rise to a mean increase in Fe^{2+} concentration of 7.26 ± 2.80 mM compared with 1.61 ± 1.40 mM in the negative, a statistically significant difference (p-value < 0.05). However, as with the Type II kerogen, at no other point was Fe^{2+} concentration greater than that of the negative control. This indicates that the addition of Type IV kerogen as the sole electron donor source did not support MIR.

5.3.2.2 Inhibition of MIR caused by kerogens

Figure 5.5 summarises the results of tests to assess the inhibitory effect of kerogens. The results show that the presence of Type II kerogen does not inhibit MIR. Here, the final mean concentration of Fe^{2+} is 14.23 ± 1.24 mM, which is significantly higher than that of the positive control measured at the same time point (p-value < 0.05), but significantly lower than the mean Fe^{2+} concentration for the positive control of 20.04 ± 2.36 mM measured at week 12 (p-value < 0.05). Therefore, although the presence of Type II kerogen did not give rise to full reduction of the available ferric iron during the course of the experiment, it did not prevent MIR by the Borgarnes enrichment culture.

In contrast, the presence of Type I and Type III kerogens gave rise to concentrations of Fe^{2+} in line with those measured in the blanks, indicating complete inhibition of MIR. The concentration of Fe^{2+} in the presence of the Type IV kerogen was insignificantly different from that of the negative control at all time points (p-values > 0.05), similarly indicating inhibition of MIR by the Borgarnes enrichment culture.

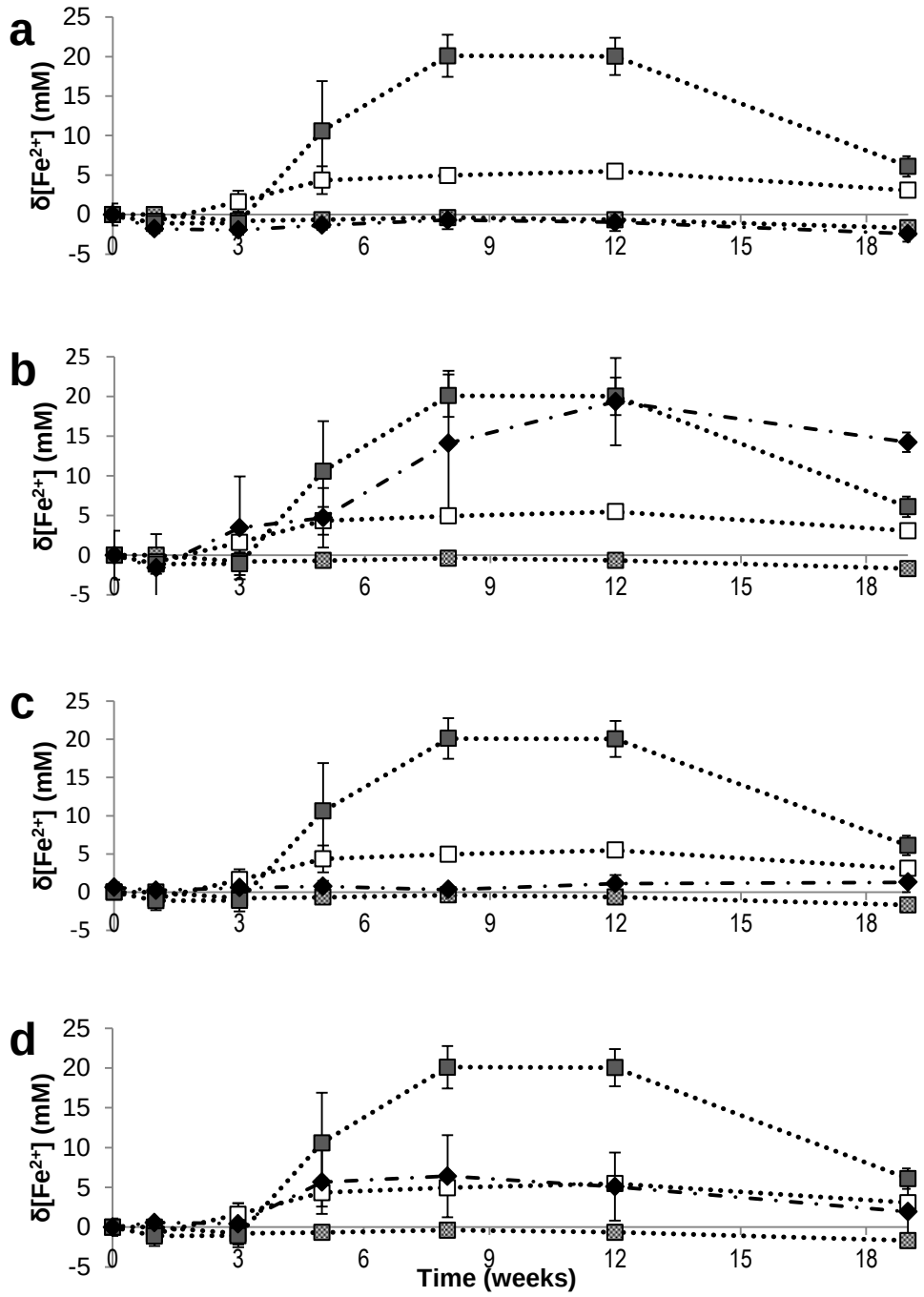

Figure 5.5: MIR inhibition tests with kerogens. Results, expressed as change in Fe^{2+} concentration (mM) with time (weeks), are shown for tests to assess whether Type I (a), Type II (b), Type III (c) and Type IV (d) kerogens inhibit MIR by the Borgarnes enrichment culture when acetate and lactate are supplied as electron donors (diamonds, dot-dashed lines). Ferrihydrite was supplied as the terminal electron acceptor. Controls are represented by dotted lines, where the positive control (full redox couple added) is depicted with solid grey squares, the negative control (no donor supplied) with empty squares, and the blank (full redox couple, no culture added) with crosshatch-filled squares. Error bars represent standard deviation of triplicate measurements.

5.4 Discussion

This study employed microbial growth experiments to assess whether ancient carbonaceous material, widespread in the terrestrial subsurface (in shales and kerogens), can serve as the sole source of electron donors for MIR by an iron-reducing enrichment culture. The above results demonstrate that none of the shale or kerogen samples supported MIR when no additional electron donors were supplied (Figures 5.2 and 5.4). As such, the overall hypothesis underpinning this study can be rejected. Furthermore, the presence of Type I, III and IV kerogens (Figure 5.5) and the Port Mulgrave shale (Figure 5.3a), inhibits MIR when the enrichment culture is supplied with a full redox couple. The effect of inhibition caused by kerogens does not appear to correlate with increasing type, since Type II is the only kerogen that does not inhibit MIR. In addition, the greatest degree of inhibition was induced by the presence of Types I and III kerogens (Figure 5.5), providing further evidence that kerogen inhibition does not correlate with increasing O/C ratios characteristic of increasing type number. The results are discussed in more detail below.

5.4.1 Use of bulk carbonaceous material as a source of electron donors for MIR

Given the recalcitrant nature of kerogen, it is perhaps unsurprising that they do not serve as a source of electron donors for MIR, either in the experiments using well-characterised kerogen samples or with shales. That so much of the terrestrial organic carbon remains locked away in these carbonaceous materials suggests that the constituent compounds are inaccessible to subsurface microbial communities. One possible explanation as to why the kerogens did not support MIR could be adsorption of usable organic compounds onto clay minerals. Shales are clay-rich sedimentary rocks. Martins and others (2011) observed that desert soils containing a high percentage of clay minerals prevented the detection of organic compounds, despite the direct addition of amino acids to the soil. This adsorption effect is likely to be operating in shales, and may account for the lack of MIR in experiments with added kerogens and shales. Namely, it is possible that any compounds present that may serve as electron donors for MIR were adsorbed onto the surface of clay minerals, rendering them unavailable for the iron-reducing microorganisms to use. However, this phenomenon cannot fully explain the results, since tests in which shales and

Type II kerogen were added to media containing acetate and lactate permitted MIR. Complete adsorption of these organics would not have given rise to this result.

Another explanation may be the enhanced recalcitrance of this material to oxidation under anoxic conditions. A number of studies have reported heightened rates of degradation of ancient sedimentary carbonaceous material upon exposure to aerobic conditions. Petsch *et al*. (2000) observed sharp oxidation fronts in shales following oxygen exposure. Similarly, Moodley and others (2005) used ^{13}C-labelled diatoms to demonstrate that organic matter in eastern Mediterranean sapropels, a kerogen precursor, was rapidly remineralized under oxic, compared with anoxic, conditions. LaRowe and van Cappellen (2011) argue that the oxidation of the complex high molecular weight organic matter is only thermodynamically favourable when a strong oxidising agent, such as oxygen or nitrate, is used as a terminal electron acceptor. They calculated that oxidation of Type I to IV kerogens was thermodynamically inhibited when sulphate or ferric iron were used as the terminal electron acceptor compared with stronger oxidants. Therefore it is possible that conducting these microbial growth experiments under anoxic conditions rendered the thermodynamics of organic matter oxidation for MIR unfavourable.

Although TOC content varied between the different kerogen types, the proportion of solvent-soluble (EOM) organic compounds was less than 1 wt % in all shale and kerogen samples. It is this fraction of the materials in which smaller compounds, most likely to serve as electron donors for MIR, are found. Therefore the lack of MIR may reflect an insufficient concentration of electron donors leached from the carbonaceous material to give rise to measurable MIR using the ferrozine assay. As previously mentioned, pyrolysis GCMS is not an appropriate technique to detected these compounds. Therefore it is unclear whether known electron donors were in fact present and sufficiently abundant to support MIR.

The 16S rRNA sequence data reported in Chapter 4 indicates that the Borgarnes enrichment culture is almost entirely dominated by microorganisms associated with *Geobacter* and *Geothrix* genera (see Figure 4.3). *Geothrix fermentans* was identified as the closest known strain for a number of sequences. This microorganism is known to adopt a heterotrophic metabolism in addition to its ability to acquire energy through MIR (Coates *et al*., 1999). It is therefore possible that these microorganisms were able to ferment organic compounds that did not support MIR in microbial growth experiments. However, if this is the case the lack of MIR indicates that any fermentation products were not used as electron donors for MIR, either because they were not suitable, or were present in insufficient concentrations. Petsch and others (2001) demonstrated that aerobic heterotrophic bacteria used macromolecular organic material in black shales as the sole source of carbon,

accounting for up to 94% of lipid carbon in these microorganisms. However, given the aforementioned thermodynamic inhibition of kerogen oxidation predicted in anoxic environments, it is likely that *G. fermentans* or any other anaerobic heterotrophs present in the enrichment culture were unable to metabolise in this way. Whether this carbonaceous material constitutes a source of energy and carbon for a predominantly anaerobic heterotrophic culture warrants further investigation.

From these results it seems that the observed lack of MIR when these bulk carbonaceous materials were supplied as the sole electron donor source can be attributed to a lack of utilisable electron donors, either because they were not present, or were present at insufficient concentrations. However it is also feasible that the use of organic material in kerogens by any microorganism is energetically unfavourable in anoxic environments.

5.4.2 Inhibition of MIR in the presence of bulk carbonaceous material

The polycyclic aromatic hydrocarbon (PAH) napthalene is known to inhibit microbial growth in some strains of aerobic bacteria (Shuttleworth and Cerniglia, 1995), therefore it is possible that the presence of this and similar PAH compounds inhibited the activity of iron-reducing microorganisms in this study. However, the presence of the Type I kerogen also inhibited MIR, despite the absence of PAHs in the pyrolysis GCMS data (Figure 5.1), suggesting other factors are responsible for the observed inhibition. It is possible that inorganic components in the materials are inhibitory, though an inorganic mechanism does not account for the inhibition observed in the presence of the Type IV kerogen, which is 100 % carbonaceous. That the shales and type II kerogen appeared generally not to inhibit MIR may suggests that inhibition is related to physical properties of the materials, such as particle size. As noted in section 5.2.4 above, these materials appeared to be finer grained than the other kerogen samples. It therefore appears that a combination of physical and chemical factors may contribute towards inhibition, and warrants further investigation.

5.4.3 Implications for MIR on Earth

From the findings of this study it appears that kerogens and the shales that contain them do not represent a source of electron donors for microbial iron reduction in subglacial environments. Furthermore, since numerous sulphate-reducing microorganisms are also capable of reducing iron for energy conservation using the same array of electron donors, it is unlikely that sulphate-reducing microorganisms are ultimately sustained by bedrock kerogen in these environments, as predicted by Wadham *et al* (2004).

In the absence of bioavailable organics, molecular hydrogen may serve as an electron donor for microbial iron and sulphate reduction in subglacial environments. Many microorganisms capable of these metabolisms are able to reduce iron autotrophically, using an inorganic source of carbon (see Table 2.2). Such chemoautotrophic microbial activity has been reported beneath Taylor Glacier in the Antarctic (Mikucki and Priscu, 2007) and in a subglacial volcanic crater in Iceland (Gaidos *et al*., 2004). Although the volcanic nature of the latter environment is responsible for the availability of hydrogen and carbon dioxide (Gaidos *et al*., 2004), hydrogen is liberated from freshly crushed rock in the presence of water (Kita *et al*., 1982; Freund *et al*., 2002). The crushing of rocks retrieved from subglacial environments has similarly been found to release hydrogen (Telling, 2013; personal communication). Therefore, in the event that kerogen-derived organic material is unavailable to complex subglacial microbial communities, it is feasible that MIR and other redox-driven microbial metabolisms operate using hydrogen as an electron donor.

As the fourth most abundant element on Earth, microbial metabolisms fuelled by iron are widespread. However, the observed inhibition in the presence of a number of kerogens raises the interesting question of whether these carbonaceous materials may exert a selection pressure on iron-reducing microorganisms in subsurface environments.

5.4.4 Implications for MIR on Mars

Although formed from different starting material, the chemical composition of kerogens is similar to that of the organic matter in carbonaceous chondrites (Bandursky and Nagy, 1976; Kerridge, 1987; Becker *et al*., 1999; Botta and Bada, 2002). Indeed, owing to their widespread abundance on Earth, kerogens are often used as an analogue of the more scientifically valuable and relatively rare carbonaceous chondrites (Matthewman *et al*.,

2013). The findings of this study suggest that iron-reducing microorganisms are unable to use organic material of this type as a source of electron donors, at least not in the absence of heterotrophic microorganisms. This brings into question the hypothesis that carbonaceous chondrites can be used as a source of electron donors for MIR in the subsurface of Mars.

5.4.5 Limitations

One limitation of this study is the lack of cell counts for the experiments involving shale and kerogen samples. Such data would not only indicate whether the *Geothrix* species present were able to metabolise the organic carbon through fermentation, but may also elucidate whether inhibitory effect of adding kerogen to cultures results in a decrease in cell numbers. It is possible that other DNA stains would prevent the issue of autofluorescence encountered in this study. Petsch and others (2001) used acridine orange to visualise cells associated with organic material in sediment cores, with no apparent autofluorescence. However, given that growth of *Geothrix* species by way of heterotrophic metabolism cannot be ruled out, additional experiments with only anaerobic heterotrophs would be required to draw conclusions from cell counts.

An additional limitation is the restricted data on the organic compounds present in these materials. Although the pyrolysis GCMS data indicates the presence of compound classes that align with compounds known to serve as electron donors (such as phenols), it is not clear that known electron donors were actually present. A robust organic inventory would thus significantly add to this study, and would highlight whether the lack of MIR was in fact due to a total lack of available electron donors or not. Such data that indicated the presence of sufficiently abundant electron donors to result in detectable MIR would render the findings of this study more significant.

5.5 Conclusions

This study is the first to demonstrate that kerogens do not serve as a source of electron donors for an iron-reducing enrichment culture. The initial hypothesis that ancient carbonaceous material common to the terrestrial subsurface can thus be rejected. Furthermore, MIR by this culture was inhibited by the presence of most kerogen types; a phenomenon that has not before been reported. The following can be concluded from the findings of this study:

- The lack of MIR in these experiments is likely due to a lack of usable electron donors in the carbonaceous material
- Inhibition of MIR in the presence of kerogens is likely to be complex, and caused by a combination of chemical and physical factors. Furthermore, inhibition does not correlate with increasing type number (and thus O/C ratio)
- These findings align with the theoretical prediction that the oxidation of organic matter in kerogens is not thermodynamically favourable in the absence of oxygen and/or stronger oxidants to serve as the terminal electron acceptor

This study would greatly benefit from further research that addresses two additional scientific questions. Firstly, are organic compounds that are known to serve as electron donors for MIR present in the organic inventory of these kerogens? Secondly, are anaerobic heterotrophic microorganisms capable of using the organic material in kerogens? If so, are the fermentation products of this metabolism known to support MIR? The first question can be addressed by applying more appropriate organic analysis tools to the shale and kerogen samples, such as ion chromatography. To answer the second question, additional microbial growth experiments using an anaerobic heterotroph enrichment culture would be required. Finally, although kerogens are a useful analogue to relatively rare and valuable extraterrestrial carbonaceous material, studies using carbonaceous meteorites are essential to constrain the available chemical energy on Mars for MIR, and serve as a logical next step to this research.

Chapter 6: Do extraterrestrial non-proteinogenic amino acids serve as electron donors for MIR?

6.1 Introduction

In order to address the feasibility of MIR on Mars, it is essential to constrain the range of chemical energy sources available to support MIR. As identified in Chapter 2, there are many more compounds that have been detected in carbonaceous meteorites than have been tested as electron donors for MIR. This study aims to address this knowledge gap by testing one group of organic compounds common to carbonaceous chondrites: amino acids. The presence of this compound class in carbonaceous meteorites is particularly interesting for two reasons. Firstly, a large number of the amino acids are non-proteinogenic, in contrast to the amino acids tested as electron donors for known iron-reducing microorganisms to date (see Table 2.2). Furthermore, most of the amino acids detected in carbonaceous chondrites over the last four decades are nonexistent, or extremely rare, in the terrestrial biosphere (Botta and Bada, 2002). The question of whether iron-reducing microorganisms can utilise these rare non-proteinogenic amino acids as electron donors for MIR therefore remains unanswered. Secondly, chiral amino acids in carbonaceous chondrites are present in racemic mixtures, unlike on Earth where life preferentially selects for L-amino acids only. Common terrestrial D-amino acids have been shown to partially or fully inhibit growth of a number of bacteria, including *Lactobacillus arabinosus* (Fox *et al*., 1944), *Bacillus subtilis* (Trippen *et al*., 1976), strains of *Erwinia* (Grula, 1960) and *Thiobacillus* (Lu *et al*., 1971), *Corvnebacterium collunge* (Trippen *et al*., 1976) and *Escherichia coli* (Davis and Maas, 1949; Rowley, 1953a, 1953b). The majority of amino acids tested in these studies are proteinogenic, and most of the strains subjected to these tests operate an aerobic and heterotrophic mode of metabolism. Therefore it remains to be seen whether D-forms of non-proteinogenic amino acids of extraterrestrial origin inhibit redox-driven anaerobic microbial metabolisms, such as MIR.

The hypothesis underpinning this study is that iron-reducing microorganisms can utilise non-proteinogenic amino acids common to carbonaceous chondrites as electron donors for MIR. To test this hypothesis, a number of pure strains of iron-reducing microorganisms and

a MIR enrichment culture were assessed for 1) their ability to conduct MIR using these compounds as the sole electron donor, and 2) inhibition caused by D-forms of chiral amino acids. This study will additionally serve to broaden the inventory of meteoritic organics tested for their use as electron donors, allowing to better constrain the available chemical energy for MIR on Mars.

6.2 Materials and methods

6.2.1 Microorganisms

Actively growing cultures of *Geobacter bemidjiensis* (Nevin *et al*., 2005; DSM-16622), *Geopsychrobacter electrodiphilus* (Holmes *et al*., 2004; DSM-16401) and *Geobacter metallireducens* (Lovley *et al*., 1993a; DSM No-7210), in addition to a freeze-dried culture of *G. metallireducens*, were obtained from the Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH (DSMZ; Braunschweig, Germany). The enrichment culture used in this study was the second-generation room temperature MIR enrichment established from Borgarnes river sediments discussed in Chapter 4.

6.2.2 Media and growth conditions

Strict anaerobic culturing techniques were used throughout, as outlined in Chapter 3. *G. bemidjiensis* and *G. metallireducens* were grown on freshwater bicarbonate-buffered medium, containing (per L) 2.5 g $NaHCO_3$, 0.25 g NH_4Cl, 0.06 g NaH_2PO_4, 0.1 g KCl, 10 ml vitamin solution and 10 ml trace elements solution. The vitamin solution contained (per L) 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyridoxine-HCl, 5.0 mg riboflavin, 5.0 mg thiamine, 5.0 mg *p*-aminobenzoic acid and 5.0 mg thioctic acid. The trace elements solution contained (per L) 1.5 g NTA, 3.0 g $MgSO_4$, 0.5 g $MnSO_4.H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.1 g $CoCl_2.6H_2O$, 0.13 g ZnCl, 0.01 g $CuSO_4.5H_2O$, 0.01 g $AlK(SO_4)_2.12H_2O$, 0.01 g H_3BO_3, 25.0 mg $NaMoO_4$, 25.0 mg $Na_2WO_4.2H_2O$ and 24.0 mg $NiCl_2.6H_2O$. In all experiments Fe(III)-citrate (50 mM) was added from sterile anoxic stock

as the electron acceptor, and in chiral inhibition experiments acetate (10 mM) was supplied as the electron donor. The final pH of the growth medium was adjusted to 6.8 - 7.0, and both strains were incubated in the dark at 30°C.

The enrichment culture was grown on the same basal medium as above, and supplemented with ferrihydrite (approximately 20 mM) as the electron acceptor, and acetate (10 mM) and lactate (10 mM) as electron donors for inhibition tests. $FeCl_2$ (1.7 mM) was added as a reducing agent. The final pH of the medium was 6.8-7.0, and the culture was incubated in the dark at room temperature.

G. electrodiphilus was grown on a basal medium containing (per L) 0.6 g KH_2PO_4, 0.3 g NH_4Cl, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$ and 20 g NaCl. After autoclaving, this medium was supplemented with (per 10 ml) 0.35 ml 10 % (wt/vol) $NaHCO_3$, 0.01 ml SL-10 trace element solution, 0.01 ml selenite-tungstate solution and 0.01 ml vitamin solution (as above). All supplements were made from sterile anoxic stocks using sterile needles and syringes flushed with N_2. The SL-10 trace element solution contained (per L) 10 ml 7.7 M HCl, 1.5 g $FeCl_2.4H_2O$, 0.07 g $ZnCl_2$, 0.1 g $MnCl_2.4H_2O$, 6.0 mg H_3BO_3, 0.19 g $CoCl_2.6H_2O$, 2.0 mg $CuCl_2.2H_2O$, 24.0 mg $NiCl_2.6H_2O$, 36.0 mg $Na_2MoO_4.2H_2O$ and 0.5g Na_2-EDTA. The solution was adjusted to pH 7.0. The selenite-tungsten solution contained (per L) 0.5 g NaOH, 3.0 mg $Na_2SeO_3.5H_2O$ and 4.0 mg $Na_2WO_4.2H_2O$. This strain was used in inhibition tests only, in which the electron donor and acceptor supplied were acetate (10 mM) and Fe(III)-citrate (50 mM), respectively. The strain was incubated in the dark at room temperature.

6.2.3 Experimental setup

The non-proteinogenic amino acids used in this study are summarised in Table 6.1, and include *N*-ethyl glycine (N-EtGly), norvaline (Nor), α-aminobutyric acid (AABA), α-aminoisobutyric acid (AIB), β-aminobutyric acid (BABA) and γ-aminobutyric acid (GABA). These amino acids were chosen for their known presence in a number of carbonaceous meteorites (see references in Table 6.1), and for their commercial availability. All compounds were obtained from Sigma-Aldrich (Dorset, UK). Only racemic (DL-) mixtures of the chiral amino acids (Nor and AABA) were tested as electron donors. Note that although BABA is also chiral, it was not possible to obtain individual D- and L- forms for the purpose of testing inhibition in this study.

Amino Acid	Abbrev.	MF	Meteorites Detected In
N-ethylglycine	*N*-EtGly	$C_4H_9NO_2$	Mch[1-4]
Norvaline*	Nor	$C_5H_{11}NO_2$	Mch[1-4] AlSit[5]
α-aminobutyric acid*	AABA	$C_4H_9NO_2$	Mch[1,2,6,8] My[6,8] Org[8] Iv[8]
α-aminoisobutyric acid	AIB	$C_4H_9NO_2$	Mch[4,6,8,10] My[6,8] AlSit[5] AH[7] Org[6,8] Iv[6,8]
β-aminobutyric acid*[¶]	BABA	$C_4H_9NO_2$	Mch[1-4,6] My[6] AlSit[5], Org[6] Iv[6]
γ-aminobutyric acid	GABA	$C_4H_9NO_2$	Mch[1-3,8] My[8] Org[8] Iv[8]

Table 6.1: Amino acids used in this study and the carbonaceous chondrites they have been detected in. *Denotes chiral amino acids, *[¶]indicates chirality not tested for inhibition. Mch = Murchison, AlSit = Almahata Sitta, My = Murray, Org = Orgueil, Iv = Ivuna, AH = Allan Hills C2. Refs: [1] Kvenvolden *et al*., 1971, [2] Ponnamperuma, 1972, [3] Lawless, 1973, [4] Cronin and Pizzarello, 1983, [5] Glavin *et al*., 2010, [6] Botta and Bada, 2002, [7] Cronin *et al*, 1979, [8] Ehrenfreund *et al*., 2001.

To assess their use as electron donors for MIR, amino acids were added to media containing an electron acceptor but no other electron donor, at a final concentration of 10 mM. This is a common concentration adopted when testing alternative electron donors in the characterisation of newly isolated strains of iron-reducing microorganisms, and was chosen for this study accordingly (e.g. Caccavo *et al*., 1996; Kashefi *et al*., 2003; Nevin *et al*., 2005). For inhibition tests, D-, L- and DL- Nor and AABA were added to media already containing an electron donor and acceptor, at a final concentration of 10 mM. Additional tests assessing concentration sensitivity were conducted at 1 mM and 0.1 mM with *G. bemidjiensis*. All amino acid supplements were carried out from sterile anoxic stocks. For pure strains, replicates were inoculated with 200-300 µl late log-phase concentrated cells that had been washed twice with 30 mM sterile anoxic bicarbonate buffer and concentrated tenfold. Each replicate of the enrichment experiment was inoculated with 1 ml unwashed active culture that had fully reduced the ferric iron in the medium. Pure strain cultures were not washed prior to inoculation into inhibition tests since the same electron donors and acceptors were present in experimental microcosms, hence carry-over was not a concern. All inhibition tests were inoculated with 250-900 µl of the appropriate culture in the late log-phase of growth.

All experiments were conducted in triplicate, and included positive controls (known electron donor and acceptor supplied), negative controls (electron acceptor but not donor supplied) and blanks (electron donor and acceptor, no culture).

6.2.4 Analytical methods

Microbial iron reduction was determined by measuring the concentration of HCl-extractable ferrous iron over time using the Ferrozine assay. Growth was determined by microbial enumerations. Both techniques are outlined in Chapter 3. The mean change in ferrous iron production between different tests compared to controls were assessed for statistical significance using a student's t-test (2-tailed, type 2, critical value of 0.05), and the corresponding p-values presented below.

6.3 Results

6.3.1 Use of amino acids as electron donors for MIR

Results of tests in which amino acids were assessed for use as sole electron donors in MIR are summarised in Figure 6. No MIR was observed when any of the amino acids were supplied as the sole electron donor in cultures inoculated with *G. bemidjiensis* (Figure 6.1a). Addition of GABA to media inoculated with the Borgarnes enrichment culture gave rise to an increase in 23.13 ± 14.84 mM Fe^{2+} over the course of the experiment, compared to 4.12 ± 1.87 mM in the negative control (ferric iron but no electron donor added) and 16.78 ± 3.05 mM in the positive control (ferric iron plus acetate and lactate; see Figure 6.1b). The difference between the means of the negative control and GABA is not statistically significant (p-value > 0.05), though two of the replicates in the GABA test gave rise to a higher overall increase in Fe^{2+} concentration than the other ([1] 8.03 mM, [2] 39.52 mM and [3] 21.83 mM). Omitting the lower value from the t-test renders this difference significant (p-value < 0.05).

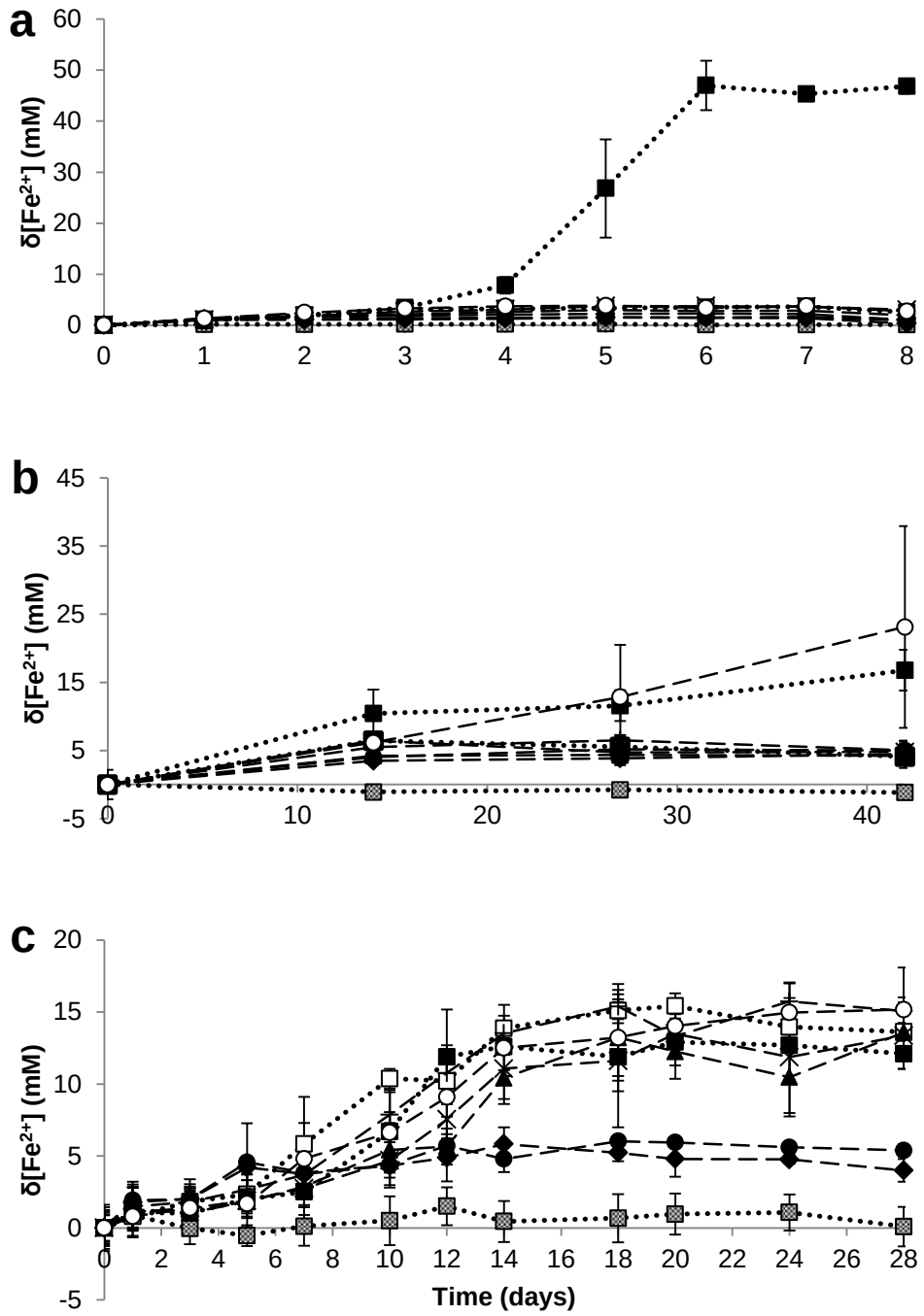

Figure 6.1: MIR with nonproteinogenic amino acids. Each compound (10 mM; dashed lines) was supplied to *Geobacter bemidjiensis* (a), Borgarnes enrichment (b) and *Geobacter metallireducens* (c) as the sole electron donors. Dotted lines represent positive control (10 mM acetate; filled squares), negative control (no electron donor; empty squares) and blank (full redox couple but no culture; crosshatch-filled squares). Amino acids tested are N-EtGly (X), DL-Norv (circles), DL-AABA (diamonds), AIB (triangles), BABA (+) and GABA (empty circles). Error bars represent standard deviation of triplicate measurements.

Tests to assess the ability of *G. metallireducens* to use the amino acids were unsuccessful due to growth in the negative control (Figure 6.1c). The positive control, in which acetate was supplied as the electron donor for the reduction of Fe(III)-citrate, gave rise to an overall increase of 12.10 ± 1.42 mM Fe^{2+}, compared to 13.62 ± 0.79 mM in the negative (in which no electron donor was supplied). This apparent growth in the negative control indicates the presence of an available electron donor, despite the omission of acetate from this and all amino acid tests. Consequently the test of whether the amino acids can serve as electron donors for *G. metallireducens* is invalid; the amino acids appear not to be the sole source of electron donors in every case (this phenomenon is discussed at length in Chapter 7 and is caused by contamination).

Regardless of these findings, the production of Fe^{2+} in tests for the use of chiral amino acids by *G. metallireducens* was observed to be lower than that of the positive and negative controls. Specifically, when DL-Nor was supplied an overall Fe^{2+} production of 5.39 ± 1.51 mM was measured, and 3.99 ± 1.42 mM Fe^{2+} was produced when DL-AABA was added; the difference between these and the positive control was significant in both cases (p-values < 0.05), and is evident in Figure 6.1c. Given that MIR by this strain appears to have access to a full redox couple in the absence of added acetate, and that full reduction of ferric iron is observed upon addition of non-chiral amino acids, these results indicate that the presence of the chiral amino acids inhibits MIR.

6.3.2 Inhibition of MIR by D-enantiomers

The effect of adding D- and L-forms of both chiral amino acids on MIR by *Geobacter bemidjiensis* is shown in Figure 6.2. Specifically, the addition of 10 mM D-Nor to acetate- and ferric iron-containing media led to the production of 1.77 ± 1.52 mM Fe^{2+} after 15 days, compared with 50.30 ± 3.85 mM production in the positive control. This difference is statistically significant (p-value < 0.05). Addition of 10 mM D-AABA lead to the production of 1.99 ± 1.13 mM Fe^{2+}, a similarly significant difference to the positive control (p-value < 0.05). The presence of 10 mM L-Nor resulted in a change of 14.68 ± 4.54 mM Fe^{2+}, significantly different to the mean change in Fe^{2+} concentration in the positive control (p-value < 0.05). Addition of 10 mM L-AABA did not result in a significantly different mean change in Fe^{2+} concentration (p-value > 0.05). The effect of adding the racemic form of Nor or AABA was not assessed for this strain.

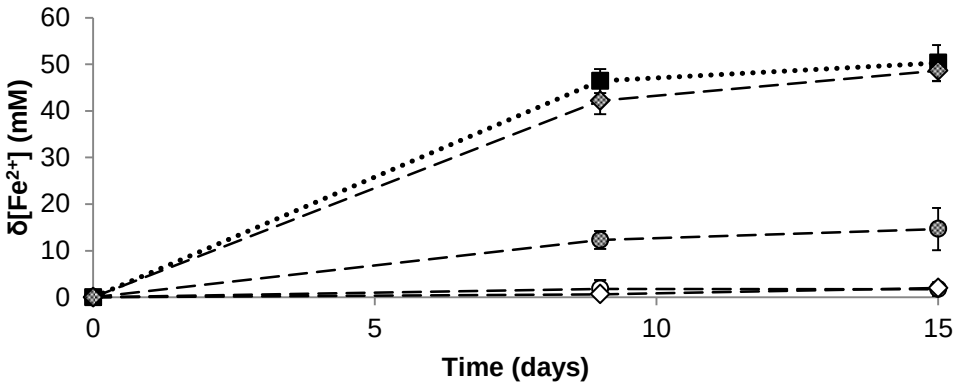

Figure 6.2: Inhibition of MIR by D-enantiomers. Data represent mean change in Fe^{2+} concentration (mM) with time (days) when *G. bemidjiensis* is grown on acetate (10 mM) and Fe(III)-citrate (50 mM) spiked with chiral amino acids (dashed lines). D-Norv (empty circles), L-Norv (crosshatch-filled circles), D-AABA (empty diamonds) and L-AABA (crosshatch-filled diamoonds) were added at a final concentration of 10 mM. Dotted black line and black squares represent positive control (no amino acid added). Error bars represent standard deviation of triplicate measurements.

The effect of adding 10 mM D- and L-forms of the chiral amino acids on MIR by *Geobacter metallireducens* is shown in Figure 6.3a. Addition of D-Nor and D-AABA gave rise to changes of 4.60 ± 1.49 mM Fe^{2+} and 1.12 ± 0.53 mM Fe^{2+}, respectively, compared to an increase of 36.14 ± 5.50 mM Fe^{2+} in the positive control over the course of 5 days. Both D-inhibited means are significant compared with that of the positive control (p-values < 0.05). No inhibition was evident in the presence of 10 mM of the L-form of either, compared to the positive control (L-Nor: 38.18 ± 4.03 mM Fe^{2+}, p > 0.05; L-AABA: 41.01 ± 4.34 mM Fe^{2+}, p-value > 0.05). Addition of 10 mM of DL-Nor did not significantly affect MIR (41.16 ± 3.46 mM Fe^{2+}, p-value > 0.05), whereas the addition of the same concentration of DL-AABA did (7.80 ± 2.59 mM Fe^{2+}, p-value < 0.05).

In the absence of any amino acids, *Geopsychrobacter electrodiphilus* reduced 36.76 ± 9.82 mM Fe^{2+} over the course of 29 days. As shown in Figure 6.3b, MIR was significantly lower in the presence of 10 mM of both D-Nor (4.57 ± 1.40 mM Fe^{2+}, p-value < 0.05) and D-AABA (3.92 ± 2.50 mM Fe^{2+}, p-value < 0.05). No inhibition was evident in the presence of 10 mM L-Nor (30.24 ± 9.07 mM Fe^{2+}, p-value > 0.05) or 10 mM L-AABA (39.24 ± 9.99 mM Fe^{2+}, p-value > 0.05), or in the presence of an equal concentration of both racemic forms (DL-Nor: 19.89 ± 8.89 mM Fe^{2+}, p-value > 0.05; DL-AABA: 16.76 ± 9.99 mM Fe^{2+}, p-value > 0.05). However, the addition of DL-AABA led to noteworthy differences in Fe^{2+} concentration between replicates ([1] 11.31 mM, [2] 10.74 mM and [3] 28.23 mM), omitting the highest of which gives a p-value less than the critical value of 0.05.

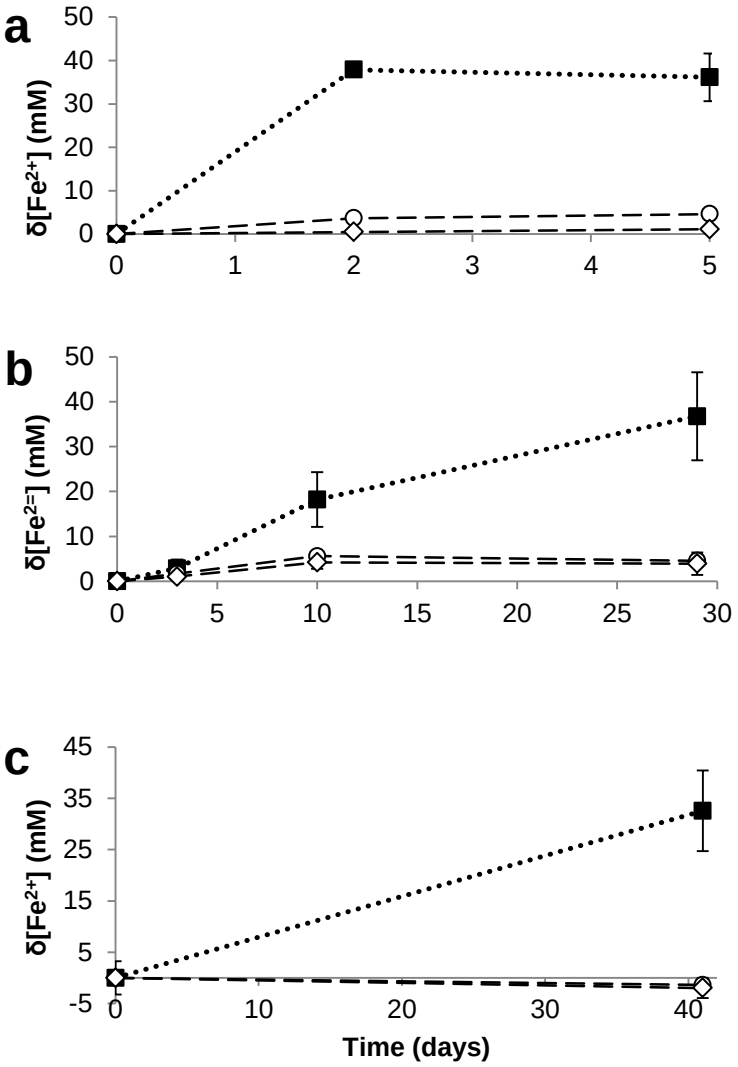

Figure 6.3: Inhibition of MIR by other iron-reducing microorganisms. Data represent mean change in Fe^{2+} concentration (mM) with time (days) when cultures of *G. metallireducens* (a), *G. electrodiphilus* (b) and the Borgarnes enrichment culture (c) are grown on a full MIR redox couple (dashed lines) in the presence of 10 mM D-Nor (empty circles), L-Norv (crosshatch-filled circles), D-AABA (empty diamonds) and L-AABA (crosshatch-filled diamonds). Dotted black line and black squares represent positive control (no amino acid added). Error bars represent standard deviation of triplicate measurements.

With the exception of L-Nor, the addition of 10 mM of all forms of amino acids inhibited MIR by the Borgarnes enrichment culture over the course of 41 days (Figure 6.3c). Compared with an increase of 32.56 ± 7.90 mM Fe^{2+} in the positive control, addition of 10 mM of D-Nor and D-AABA led to significantly lower Fe^{2+} production, with overall changes in concentration of -1.37 ± 0.87 mM (p-value < 0.05) and -1.97 ± 1.21 mM (p-value < 0.05), respectively. Similar changes were measured with the addition of DL-Nor (-0.86 ± 0.96 mM Fe^{2+}, p-value < 0.05) and DL-AABA (-2.31 ± 3.81 mM Fe^{2+}, p-value < 0.05). The presence of 10 mM L-AABA also led to significantly lower Fe^{2+} production, albeit to a lesser extent (5.27 ± 4.11 mM Fe^{2+}, p-value < 0.05), whereas the presence of 10 mM L-Nor did not significantly change Fe^{2+} concentration compared to the positive control (13.90 ± 25.03 mM Fe^{2+}, p-value > 0.05). However, an increase in Fe^{2+} concentration was detected in only one of the three replicates ([1] 43.58 mM compared with [2] -0.67 mM and [3] -1.22 mM).

The normalised effect of adding D- and L- forms of Nor and AABA to the different iron-reducing cultures is shown in Figure 6.4.

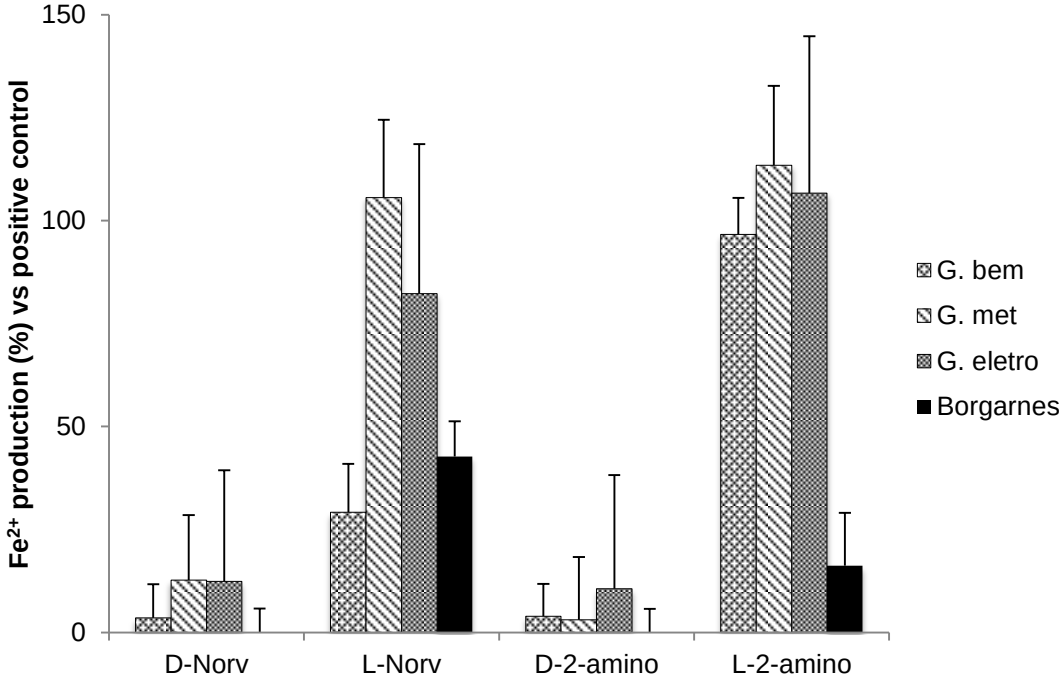

Figure 6.4: Normalised effect of adding D- and L-Norv and AABA on MIR by all strains. Media contained acetate (and lactate for Borgarnes enrichment culture) and Fe^{3+} as electron donor and acceptor, respectively. Results are expressed as the percentage of Fe^{2+} produced over the course of experiments compared to corresponding positive control (no amino acid added) for each strain, where 100% represents no inhibition. Error bars represent standard deviation of triplicate measurements.

6.3.3 Inhibition of MIR at different concentrations

Inhibition of MIR on *Geobacter bemidjiensis* was tested at the lower concentrations of 1 mM and 0.1 mM, the normalised results of which are summarised in Figure 6.5. Compared with the significant reduction in Fe^{2+} production in the presence of 10 mM discussed above, addition of D-Nor did not significantly effect the change in Fe^{2+} concentration at a concentration of 1 mM (p-value > 0.05) or 0.1 mM (p > 0.05). In contrast, the presence of 1 mM D-AABA did result in a significantly lower production of Fe^{2+}; resulting in a change of 2.08 ± 0.94 mM compared with 35.17 ± 2.98 mM in the positive control (p-value < 0.05) over the course of 17 days.

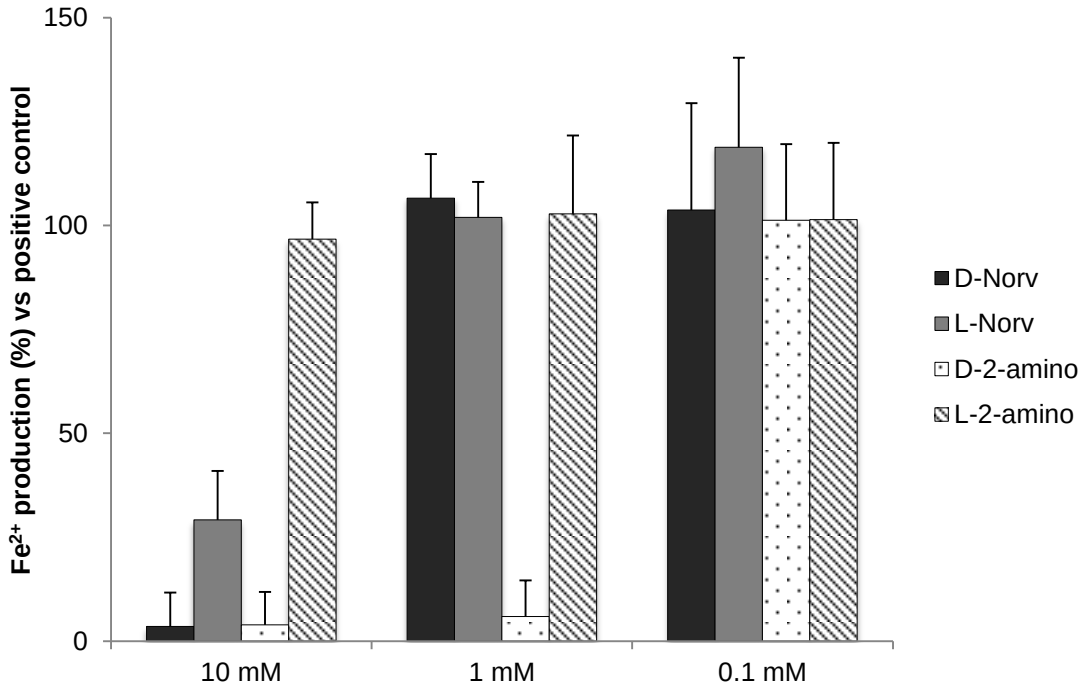

Figure 6.5: Normalised effect of adding D- and L-Norv and AABA at different concentrations on MIR by *G. bemidjiensis*. Final concentrations of amino acids were 10 mM, 1 mM and 0.1 mM. Results are expressed as the percentage of Fe^{2+} produced over the course of the experiment compared to the corresponding positive control (no amino acid added), where 100% indicates no inhibition. Error bars represent standard deviation of triplicate measurements.

6.3.4 Inhibition of microbial growth by D-enantiomers

To assess whether inhibition affected microbial growth as well as MIR, cells were enumerated in the 10 mM inhibition test with *Geobacter bemidjiensis*. The average number of cells ± standard deviation across all replicates at the start of the experiment was $1.14 \times 10^7 \pm 5.09 \times 10^6$ cells ml^{-1} sample. In the positive control the average number of cells increased from $1.48 \times 10^7 \pm 3.47 \times 10^6$ to $3.45 \times 10^7 \pm 6.91 \times 10^6$ cells ml^{-1}, the difference between the two numbers being statistically significant (p-value < 0.05). The average number of cells decreased in the presence of D-Nor from $1.09 \times 10^7 \pm 1.89 \times 10^6$ to $3.68 \times 10^6 \pm 1.47 \times 10^6$ cells ml^{-1}, representing a significant overall change of $-7.24 \times 10^6 \pm 2.39 \times 10^6$ cells ml^{-1} over time (p-value < 0.05). The number of cells in tests with added L-Nor also significantly decreased by $9.44 \times 10^6 \pm 3.41 \times 10^6$ cells ml^{-1} over the course of the experiment, from $1.37 \times 10^7 \pm 2.50 \times 10^6$ to $4.25 \times 10^6 \pm 2.32 \times 10^6$ cells ml^{-1} (p-value < 0.05). A similarly significant decrease in cell numbers was measured in the presence of D-AABA, with a reduction of $5.33 \times 10^7 \pm 1.34 \times 10^6$ cells ml^{-1} between the start and the end (p-value < 0.05). In each case, the change in cell numbers over the course of the experiment was statistically significant when compared with that of the positive control (p-values < 0.05). In contrast, there was a significant increase in the number of cells from $8.80 \times 10^6 \pm 4.41 \times 10^5$ to 6.33×10^7 cells ml^{-1} with the addition of L-AABA (p-value < 0.05), an overall change of $5.46 \times 10^7 \pm 2.93 \times 10^7$ cells ml^{-1} that is not significantly different to the change measured in the positive control (p-value > 0.05).

These results indicate that the presence of D-Nor, L-Nor and D-AABA inhibited microbial growth compared with the positive control, whereas addition of L-2-aminobutyric acid did not.

6.4 Discussion

The goal of this study was to address the hypothesis that nonproteinogenic amino acids common to carbonaceous meteorites can serve as electron donors for MIR. This was addressed by conducting experiments with different iron-reducing microorganisms and assessing 1) whether MIR occurs when the amino acids are supplied as the sole source of electron donors, and 2) whether the presence of D-forms of chiral amino acids inhibits MIR. The findings of this study with respect to these questions are discussed in detail below.

6.4.1 Use of meteoritic non-proteinogenic amino acids as electron donors for MIR

Of the amino acids tested in this study, only γ-aminobutyric acid (GABA) appears to serve as an electron donor for MIR by the Borgarnes enrichment culture (Figure 6.1b), albeit with variable effects on individual replicates. Two of the replicates gave rise to an increase in Fe^{2+} greater than the average of the positive control. Additionally, the increase in the least productive GABA replicate was still greater than the average of the negative control. Therefore the results support the conclusion that GABA was being used as an electron donor to support MIR by iron-reducing microorganisms in the Borgarnes enrichment culture.

Phylogenetic assessment of the Borgarnes enrichment culture revealed the enrichment to be dominated by members of the *Geobacter* genus (62.1%), with most of the remaining microorganisms belonging to the genus *Geothrix* (34.7%) (see 'BRT' in Figure 4.2). Members of both are known for the ability to conduct MIR. *Geothrix fermentans* is the only known species of its genus, however no amino acids were screened as electron donors in the type strain paper (Coates *et al*., 1999; see Table 2.2). Of the 62.1% *Geobacter* identified in the Borgarnes enrichment culture, 43.6% were resolved to species level, namely *G. pelophilus* (41.2%) and *G. psychrophilus* (2.4%). No amino acids were tested as electron donors in the characterisation of *G. pelophilus* either (Straub and Buchholz-Cleven, 2001). In contrast, *G. psychrophilus* was tested for the use of arginine, proline and serine as electron donors, but did not use any (Nevin *et al*., 2005). However, even with more information on the individual species present in this culture it would be difficult to predict their ability to utilise these compounds, owing to the differences in physiology between strains of the same species.

With the exception of norvaline, all the amino acids tested in this study share the same molecular formula (Table 6.1). That only γ-aminobutyric acid was used by the Borgarnes enrichment culture for MIR is therefore interesting, and serves to highlight the importance of structural isomerism in the use of organic compounds as electron donors. This finding is not novel, nor is it limited to amino acids. For example, both *Geobacter metallireducens* and *Tepidimicrobium ferriphilum* can use propanol but not isopropanol (Lovley *et al*., 1993a; Slobodkin *et al*., 2006). Similarly, *Deferribacter autotrophicus* can use maltose as an electron donor, but not sucrose (Slobodkina *et al*., 2009), both of which share the same molecular formula. In contrast, *Thermoanaerobacter siderophilus* can use both sugars for MIR (Slobodkin *et al*., 1999). Therefore, not only is structural isomerism a

contributing factor as to whether or not a compound can serve as an electron donor for MIR, but its influence also varies between individual iron-reducing microorganisms.

Of all the compound classes screened as electron donors in the characterisation of newly isolated strains, amino acids remain the most neglected. Furthermore, the compounds tested in these previous studies are limited to proteinogenic amino acids. This study therefore represents the first to move beyond the proteinogenic acids, and highlights the need for screening of a wider array of such compounds on more iron-reducing microorganisms. This will allow for better evaluation of the potential chemical energy sources available for MIR on Mars.

6.4.2 Inhibition of MIR by D-amino acids

Results of inhibition tests show that the presence of 10 mM D-norvaline and D-α-aminobutyric acid inhibit MIR by *Geobacter bemidjiensis* (Figure 6.2), *Geobacter metallireducens*, *Geopsychrobacter electrodiphilus* and the Borgarnes enrichment culture (Figures 6.3 and 6.4). The presence of D-α-aminobutyric acid inhibits MIR by *G. bemidjiensis* at 1 mM, but D-norvaline does not. Neither compound inhibits MIR by *G. bemidiensis* at 0.1 mM. In contrast, L-norvaline appears to inhibit MIR at 10 mM (Figure 6.5). MIR by the Borgarnes enrichment culture similarly appears to be inhibited by 10 mM L-norvaline, as well as L-α-aminobutyric acid (Figure 6.4).

Addition of these chiral amino acids in racemic form gave rise to variable results. The Borgarnes enrichment culture was inhibited by both DL-norvaline and DL-α-aminobutyric acid. Whether *G. electrodiphilus* was inhibited upon addition of DL-α-aminobutyric acid is ambiguous; although statistically it did not inhibit MIR compared to the positive control (p-value > 0.05), variability between replicates indicates inhibition occurred in two out of three. Similarly, MIR by *G. metallireducens* was inhibited by the addition of DL-α-aminobutyric acid but not DL-norvaline. This is intriguing, since the inhibitory effect of both chiral amino acids on this strain was evident in unsuccessful negative controls of initial electron donor tests. Specifically, the similarly lower magnitude of MIR observed in the presence of both chiral amino acids compared with the positive control suggested that they inhibit MIR equally (Figure 6.1c). A possible explanation for the conflicting results may lie in the fact that different stock cultures were used in the different experiments. Namely, a freeze-dried culture of *G. metallireducens*, later discovered to be contaminated

(see Chapter 7), was used for electron donor tests in which MIR was observed in the negative controls, whereas an actively growing (apparently uncontaminated) culture of the strain was used in inhibition tests.

Only two published studies have tested microbial inhibition by norvaline and α-aminobutyric acid. The first (Rowley, 1953a) involved testing the effects of adding racemic mixtures of both amino acids to 100 strains of the Gram-negative bacterium *Escherichia coli*, and demonstrated that 75 were inhibited by DL-norvaline and 7 by DL-α-aminobutyric acid, though the final concentrations of each were not reported. A follow-up study (Rowley, 1953b) extended these tests to 365 strains of *E. coli*, and reported inhibition by DL-norvaline (8.5 μM) in 261 strains and by DL-α-aminobutyric acid (9.9 μM) in 4. These inhibitory concentrations are much lower than those found to inhibit MIR in this study. Futhermore, unlike the results reported by Rowley (1953b), the racemic forms of both amino acids did not always inhibit MIR, yet individual D-isomers inhibited all iron-reducing cultures tested.

A number of other studies have reported the inhibition of microbial growth by D-amino acids, though these are limited to proteinogenic amino acids. Some of the earliest such accounts date back to the 1930's, when Gladstone (1939) reported the failure of a strain of the Gram-positive bacterium *Bacillus* to grow in the presence of the chiral amino acids valine (8.5 μM) and leucine (9.9 μM). Shortly after this, a study aimed at elucidating the effectiveness of the antibiotic gramidicin recognised the inhibitory effects of D-leucine on the similarly Gram-positive bacterium *Lactobacillus arabinosus* (Fox *et al*., 1944). Davis and Maas (1949) also reported the inhibitory effect of DL-serine on *E. coli*, where no inhibition was evident upon addition of the L-isomer only. Then followed the aforementioned studies conducted by Rowley (1953a, 1953b) in which several amino acids were found to inhibit different strains of the same species to varying degrees. In both studies, norleucine and norvaline inhibited the majority of strains. It was noted that some strains were inhibited by several amino acids, whilst others were only inhibited by one (Rowley, 1953a). Other studies reported inhibition by D-isomers on *E. coli* (Kuhn and Somerville, 1971; Caparrós *et al*., 1992), as well as strains of *Erwinia* (Grula, 1960), *Brucella* (Yaw and Kakavas, 1952), *Rhodospirillium* (Coleman, 1959), *Alcaligenes* (Lark and Lark, 1959), *Pseudomonas* (Eisenstadt *et al*., 1959), and *Coryneacterium* (Trippen *et al*., 1976). In the case of the latter, addition of 20-200 mM D-serine was found to cause only partial growth inhibition in seven different species of bacteria, though morphological alterations were also observed (Trippen *et al*., 1976). A more recent study reported the prevention of biofilm formation by *Staphylococcus aureus* in the presence of D-amino acids (Hochbaum *et al*., 2011). It is therefore clear that inhibition caused by D-amino acids is a well-known

phenomenon among Gram-positive and -negative bacteria, though the vast majority of studies test only proteinogenic amino acids. In accordance with the results of this study, it is also clear that different bacteria are inhibited to varying degrees, and at a range of concentrations.

In contrast to D-isomer inhibition, one study reported the inhibition of growth of strains of the chemolithotrophic bacterium *Thiobacillus* in the presence of 10 mM L-methionine and L-phenylalanine (Lu *et al*., 1971). Similar inhibition was detected in the Borgarnes enrichment culture in the presence of the same concentration of L-norvaline and L-α-aminobutyric acid. MIR by *Geobacter bemidjiensis* also appeared to be inhibited in the presence of L-norvaline (Figure 6.2).

A number of studies found inhibition to be either short-lived (Davis and Maas, 1949; Rowley, 1953b; Coleman, 1959) or completely preventable through the addition of other, seemingly antagonistic, amino acids (Davis and Maas, 1949; Rowley, 1953a, 1953b; Lu *et al*., 1971). The transient nature of inhibition in the former studies was not observed in these experiments, where the presence of the D-isomer resulted in total inhibition of MIR (Figure 6.3), or no inhibition at lower concentrations (Figure 6.5). However the antagonistic effects of adding different amino acids was not assessed (beyond adding the racemic form), hence it is possible that these MIR cultures would be affected differently in the presence of amino acids other than those shown to inhibit in isolation in this study.

6.4.3 Mechanisms of inhibition

The decrease in cell number of *G. bemidjiensis* over the course of the experiment in the presence of inhibitory amino acids may be the direct consequence of cell death from the inhibition of energy acquisition by MIR. Alternatively, it may result from interference of a more fundamental cellular process universal to all microorganisms. Although it is not possible to deduce which is responsible for the observed decrease in cells, the following review of the literature supports the latter explanation.

A number of mechanisms have been proposed to explain the inhibitory effects of D-amino acids. Early speculation attributed inhibition to interference with ‘proteosynthetic enzymes’ (Kobayashi *et al*., 1948), with hydrolysis of peptide bonds (Yaw and Kakavas, 1952), or with growth factors in the medium (Lark and Lark, 1959). However, the prevailing theory is that D-amino acids interfere with the production of peptidoglycan, the major

component of bacterial cell walls that is known to contain D-alanine (Izaki *et al*., 1986). Evidence to support this hypothesis was presented by Trippen *et al* (1976) who demonstrated that D-serine and other D-amino acids replaced D-alanine in nucleotide-activated peptidoglycan precursors, which led to less efficient incorporation into peptidoglycan. Decreased cross-linking within peptidoglycan was also apparent (Trippen *et al*., 1976). Caparrós and others (1992) also reported the incorporation of D-amino acids, including D-α-aminobutyric acid tested in this study, into peptidoglycan by *E. coli*, and found concentrations above 45 mM to be lethal, though incorporation of D-α-aminobutyric acid was observed in the presence of 20 mM. Their results echoed those of Trippen *et al* (1976) in that cross-linking was found to be reduced, leading them to propose that such affects may lead to less production of peptidoglycan per cell. Interestingly, β-aminobutyric acid was also assessed but was not found to be incorporated into peptidoglycan. In fact amino acids lacking an alpha-amino group, or achiral and diamino acids, were not found to be incorporated into macromolecular peptidoglycan (Caparrós *et al*., 1992), suggesting that inhibition is only caused by amino acids with alpha-amino groups, like the alanine that is integral to the formation of peptidoglycan in the first instance, in addition to the chiral amino acids tested in this study. It therefore seems probable that this interference with peptidoglycan formation is the cause of MIR inhibition observed in this study.

This hypothesis is further supported by the known presence of α-aminobutyric acid in the lantibiotic 'marsacidin' (Sass *et al*., 2008). Lantibiotics are naturally produced by, and act against, other Gram-positive bacteria, and are of two types. Type A lantibiotics are long flexible molecules that rapidly kill through the formations of pores, whereas Type B lantibiotics are globular in structure and inhibit peptidoglycan synthesis (Smith and Hillman, 2008). Marsacidin, a Type B lantibiotic, is ribosomally produced by *Bacilli*, and acts to complex the sugar phosphate head group of peptidoglycan precursor lipids, resulting in the inhibition of peptidoglycan biosynthesis (Sass *et al*., 2008). This strongly suggests that the inhibitory action of α-aminobutyric acid reported here and elsewhere is related to its role in this peptidoglycan-targeted lantibiotic.

Inhibition of MIR by *Geobacter bemidjiensis* was not observed in the presence of 1 mM D-norvaline or 0.1 mM D-α-aminobutyric acid, indicating that a threshold exists at which interference of peptidoglycan biosynthesis ceases. Recently, Zhang and Sun (2014) reported that soil microorganisms were capable of consuming D- and L-isomers of the proteinogenic amino acids alanine, aspartic acid and glutamic acid at equal or near-equal rates. They attribute this observation to the reverse racemisation of D-isomers into L, a phenomenon they propose all bacteria are capable of. They observed that utilisation of D-

glutamic acid, D-aspartic acid and D-leucine was prevented upon addition of rifampicin, which acts to inhibit RNA synthesis, whilst consumption of corresponding L-isomers and D-alanine continued unaffected. This led them to conclude that RNA synthesis and gene expression were essential to the bacteria utilising D-enantiomers, and supports their theory that enzymatic conversion of D- to L-isomers was responsible for the observed uptake (Zhang and Sun, 2014). The final concentration of amino acids they added to their experiments was 4 mM, which straddles the range of concentrations tested on *G. bemidjiensis* in this study. If all bacteria are indeed capable of the enzymatic conversion of D- to L-isomers, the results of this study indicate that such ability is concentration-dependent. For instance, at concentrations below the threshold at which they cause irreversible damage through interference with peptidoglycan formation (between 10 mM and 1 mM for D-norvaline and between 1 mM and 0.1 mM for D-α-aminobutyric acid), D-isomers are enzymatically converted to the non-inhibitory L-isomers, which in the case of nonproteinogenic amino acids then serve as a source of cellular nitrogen and carbon.

6.4.4 Implications for life on Earth

The inhibitory effect on growth of anaerobic iron-reducing microorganisms demonstrated in this study reveals an interesting paradox relevant to the emergence of life on the early Earth. The amino acids produced in Miller's landmark prebiotic chemistry experiments under simulated prebiotic reducing conditions thought to prevail at that time were present in racemic form (Miller, 1953; Miller and Urey, 1959). Although the conditions adopted in Miller's experiment are now thought to be too reducing (Heinrich *et al*., 2007), the abiotic production of chiral amino acids does not discriminate between stereoisomers. Extraterrestrial delivery of amino acids to the surface of the Earth in the form of meteorites would have further added to this racemic stock of chiral amino. Yet, in order for life to emerge and prosper, concentrated amino acids were required to form peptides. If the D-isomers of these amino acids were toxic, as the results of this study suggest, a coping mechanism must have emerged early enough for life to overcome the toxicity created in the biochemical selection of L- over D-enantiomers. In other words, the biochemical selection of L-amino acids appears to have rendered the corresponding D-isomers toxic.

It remains to be seen whether this inhibition also applies to *Archaea*. If the mechanism of inhibition is indeed interference with the formation of peptidoglycan, a

component of bacterial but not archaeal cell walls, the emergence of racemase enzymes is likely to be one of the earliest coping mechanisms. Investigations into the evolution and origin of racemase genes may shed light on this issue.

6.4.5 Implications for life on Mars

It is clear from this study that the inhibitory effects of D-amino acids on MIR are concentration-dependent. Therefore, whether or not the presence of such amino acids delivered exogenously to Mars will adversely affect habitability for iron-reducing and other life warrants further investigation. Although organic compounds have not yet unambiguously been detected on Mars, they are nonetheless expected to reside in the subsurface (Kanavarioti and Mancinelli, 1990). Using estimated rates of the accretion of meteoritic material on Mars, and concentrations of D-α-aminobutyric acid as measured in a number of carbonaceous chondrites, a first-order estimation of the concentrations of this compound and its potential to inhibit MIR can be reached.

Flynn (1996) calculated the present day accretion rate of meteoritic material to the surface of Mars with organic matter intact as 2.4×10^{6} kg per year. If one assumes this material is evenly distributed across the surface of Mars, this equates to 1.7×10^{-3} grams per square meter per year. Ehrenfreund and colleagues (2001) detected 13 ± 11 ppb and 914 ± 189 ppb α-aminobutyric acid per gram of Orgueil and Murchison, respectively. The concentrations reported for the same quantities of the carbonaceous chondrites Ivuna and Murray fall between the two at 12 ± 7 ppb and 463 ± 68 ppb, respectively. Using the lower bound concentration of 2 ppb in Orgueil and the higher bound of 1103 ppb in Murchison, and assuming that all organic matter in the meteoritic material accounted for in the accretion rate resembles that of these carbonaceous chondrites, the concentration of the D-isomer of α-aminobutyric acid that accumulates on the surface of Mars each year is in the range of 1.66×10^{-6} to 9.14×10^{-4} µg per gram bulk carbonaceous chondrite. Assuming a rock water ratio of 1:1 (1 gram meteorite in contact with 1 ml water), and complete disintegration of meteoritic material on impact (allowing exposure to the organic component within), it would take between 113 thousand to 62 million years for D-α-aminobutyric acid at any given point on Mars to accumulate to the inhibitory concentration of 1 mM found in this study. The lack of exposure of organic matter and the presence of large volumes of water would substantially dilute these concentrations.

However, the aforementioned accumulation rate of 2.4 x 10^6 kg per year is thought to be a lower limit, as the flux of meteoritic material to the surface of Mars during the first billion years of Solar System history is estimated to have been ten thousand times that of the present-day. During this period of heightened meteoritic in-fall, it could have taken little more than 10 years for the accumulation of D-α-aminobutyric acid to reach inhibitory concentrations, assuming the higher concentrations detected in Murchison (100 years gives rise to 0.91 mg D-α-aminobutyric acid per square meter; 8.86 mM in a 1:1 rock to water ratio scenario). Furthermore, the accumulation of D-α-aminobutyric acid over one hundred million years at the present-day accretion rate, and assuming the lower concentrations present in the Orgueil meteorite (Ehrenfreund *et al*., 2001), is still sufficient to produce inhibitory concentrations (1.61 mM at a 1:1 rock to water ratio).

The lack of conclusive detection of organics on the surface of Mars, coupled to the known presence of strong oxidising agents such as perchlorate salts (Hecht *et al.*, 2009), render these estimates inherently uncertain. It is therefore difficult to conclude whether the presence of such amino acids in the subsurface of Mars would indeed inhibit MIR and other modes of life. Furthermore, that some studies have shown inhibitory effects to be completely prevented in the presence of antagonistic compounds places additional uncertainty on the significance of this inhibition. Further research is thus warranted to shed light on these uncertainties. However, these results serve to highlight that amino acids, the very building blocks of all known life on Earth, may in fact restrict habitability on other planetary bodies. As such, it is important to regard the organic compounds that are found in meteorites and rare on Earth with caution when it comes to their role in the search for life on Mars and beyond.

6.4.6 Limitations

There are two main limitations to this study. The first pertains to the assessment of so few iron-reducing microorganisms for their ability to utilise nonproteinogenic amino acids as electron donors for MIR. This is in part attributable to issues encountered when trying to establish the negative control with *Geobacter metallireducens*, in addition to the shift in focus towards the apparent inhibition uncovered in these unsuccessful tests. The study would thus have benefited from testing a wider array of microorganisms, including

those that are reported to use amino acids in MIR already, such as *Geopsychrobacter electrodiphilus* (Holmes *et al*., 2004) and *Geothermobacter ehrlichi* (Kashefi *et al*., 2003).

An additional limitation concerns the concentration of DL-norvaline and DL-α-aminobutyric acid used in racemic tests. All amino acids were added to growth media at a final concentration of 10 mM, which means the concentration of each isomer in racemic mixes was in fact 5 mM each. Hence a true comparison of adding a single isomer compared to both isomers could not be made. Although conclusions can still be drawn from the effect of adding both isomers on MIR, addition of 20 mM of DL-amino acids would have allowed for a direct assessment of inhibition compared to the addition 10 mM of individual isomers.

6.5 Conclusions

This study has demonstrated for the first time that a nonproteinogenic amino acid, common to carbonaceous chondrites, can serve as an electron donor for MIR by an iron-reducing microbial enrichment culture. The initial hypothesis that such compounds can support MIR can thus be accepted. Furthermore, this is the first study to show that chiral nonproteinogenic amino acids are inhibitory to microbial growth of anaerobic microorganisms, and at concentrations relevant to those expected to have amassed on Mars. The following conclusions can be drawn:

- The use of γ-aminobutyric acid but not other amino acids of the same molecular formula for MIR highlights the importance of structural isomerism on the ability of iron-reducing microorganisms to utilise compounds as electron donors
- The presence of D-α-aminobutyric acid and D-norvaline caused the complete inhibition of MIR by all strains at the highest concentration, further highlighting the importance of (stereo)isomerism on such fundamental biochemical processes as peptidoglycan synthesis
- Inhibition of MIR is caused by inhibition of cell growth, with cell numbers decreasing over time in the presence of inhibitory amino acids
- Although concentration-dependent, the inhibitory effect of nonproteinogenic amino acids on microbial metabolism should be considered in the discussions of

habitability for life beyond Earth. Their presence on Mars and other planetary bodies considered conducive for life may render these environments less habitable

In order to further this research, two related scientific questions should be addressed. First, are the organic compounds known to serve as electron donors in bulk carbonaceous chondrite material sufficiently abundant and accessible to support MIR? Second, does the presence of inhibitory agents (such as D-isomers of alpha-amino acids) in bulk carbonaceous chondrite material inhibit growth and MIR of iron-reducing microorganisms? Both questions could be investigated simultaneously through growth experiments similar to those conducted in Chapter 5, using carbonaceous meteorite as the sole source of electron donors for MIR. The results would significantly add to the discussion of the habitability for, and feasibility of, MIR on Mars.

Chapter 7: Heterotroph-MIR syntrophy revealed by serendipitous contamination of a pure culture

Whilst conducting experiments to test the ability of pure strains to utilise amino acids common to carbonaceous chondrites as electron donors (Chapter 6), it became apparent that growth of a pure strain of *Geobacter metallireducens* was not occurring as expected. In particular, it was not possible to establish a negative control in these growth experiments despite several attempts. It soon became apparent that a number of different stocks of the strain exhibited the same behavior, and that the underlying reason was contamination. The following chapter outlines the original issues faced in attempting to establish negative controls, and the steps taken to identify the cause of the problem, culminating in the taxonomic identification of the contaminant responsible.

7.1 MIR in negative controls

In order to adequately assess the ability of a microorganism to use a compound as an electron donor for MIR, it is important to demonstrate the requirement of an electron donor in the first place. To this end, a washed and concentrated sample of the microorganism is inoculated into medium in which the electron acceptor is supplied, but a donor compound denied. The expected result is that little or none of the ferric iron is reduced to ferrous iron, thereby demonstrating that MIR is only possible where both donor and acceptor compounds are supplied.

In the first attempt to test the use of non-proteinogenic amino acids as electron donors for MIR by *G. metallireducens* (revived from a freeze-dried stock from DSMZ), a visual diagnostic indicated that MIR had occurred in several triplicate conditions, including the negative control in which no donor compound was added. After four days since inoculation, the growth medium had ‘bleached’ from dark brown to colourless in all but the blanks and racemic amino acid tests, the latter of which served as an indicator of inhibition (see Chapter 6). This bleaching is characteristic of complete or near-complete reduction of Fe(III)-citrate to ferrous iron, and hence serves as a useful visual indication of MIR. It was therefore evident that MIR had occurred in the negative control, as well as the positive. The

latter serves to demonstrate that the culture is capable of MIR when supplied with a complete redox couple known to support MIR. Hence, it appeared that a source of electron donor was present in the negative control afterall.

At the time it was thought that the number of possible explanations for this phenomenon were threefold. Firstly, it was feasible that through human error an electron donor had in fact been supplied to the negative controls. Repeating the experiment, taking extra care to avoid such a mistake, could easily test this. Secondly, although the cultures were twice washed in sterile anaerobic bicarbonate buffer ($NaHCO_3$, 30 mM), carry-over of residual acetate from the preceding culture may have resulted in the observed MIR. A quick stoichiometric calculation can be used to assess this possibility. The culture inoculated into the aforementioned experiment was grown in the recommended DSMZ *Geobacter* medium (no. 579), containing 55 mM Fe(III)-citrate and 30 mM acetate. For every mole of acetate oxidised, eight moles of soluble ferric iron are reduced; hence there is a 1:8 ratio requirement of acetate and ferric iron with which to carry out MIR. Accordingly, only 6.88 mM residual acetate would be required in medium containing no additional electron donor for the washed culture to fully deplete the 55 mM ferric iron available after inoculation, though in reality a small amount more would actually be consumed as a source of cellular carbon. It was therefore feasible that the carry-over of acetate from the initial culture was the reason for growth in the negative control, and required repetition of the experiment with a lower initial concentration of acetate. Thirdly, unlike later experiments that were carried out on the bench using the gas station, this early experiment was initiated within the anaerobic chamber. Whilst the majority of the atmosphere within the chamber is nitrogen, hydrogen is required to allow the palladium catalysts to mop up any unwanted oxygen. Although only present in small amounts (typically 1.8 – 2% v/v), hydrogen can be used as an electron donor by some iron-reducing microorganisms, and has been known to cause growth in negative controls in other experiments (Lloyd, 2012; personal communication). However, this seemed like an unlikely explanation, since the type strain paper for *G. metallireducens* (Lovley *et al*., 1993a) reported that hydrogen was not utilised. Furthermore, a substantial amount of hydrogen would be required to support MIR inline with that observed in the positive and negative controls. Nonetheless it was not completely ruled out.

The first explanation could be ruled out after the experiment was carefully repeated, and MIR was again observed in the negative control (see Figure 7.1). In this instance, the experiment was inoculated with a different stock culture of *G. metallireducens*, a new freeze-dried batch of which had been re-ordered since the first experiment. Furthermore, this experiment was set up outside of the anaerobic chamber, in which bottles of media were

purged with N_2/CO_2 prior to autoclaving. As such, with the exception of decanting the culture into falcon tubes within the anaerobic chamber prior to washing, contact with hydrogen had been largely avoided, indicating that hydrogen utilisation was an unlikely explanation. Although the late log-phase starter culture had been twice washed in bicarbonate buffer (as in the first instance), the concentration of acetate in the initial growth medium was reduced to 10 mM, limiting the amount of potential carry-over to fresh growth medium. Thus, although the potential for acetate carry-over had not been eliminated, it had to some extent been reduced.

Whilst it is clear that not all of the 55 mM ferric iron available had been reduced in the positive controls, MIR in the negative controls was equal to or greater than this in magnitude (see Figure 7.1).

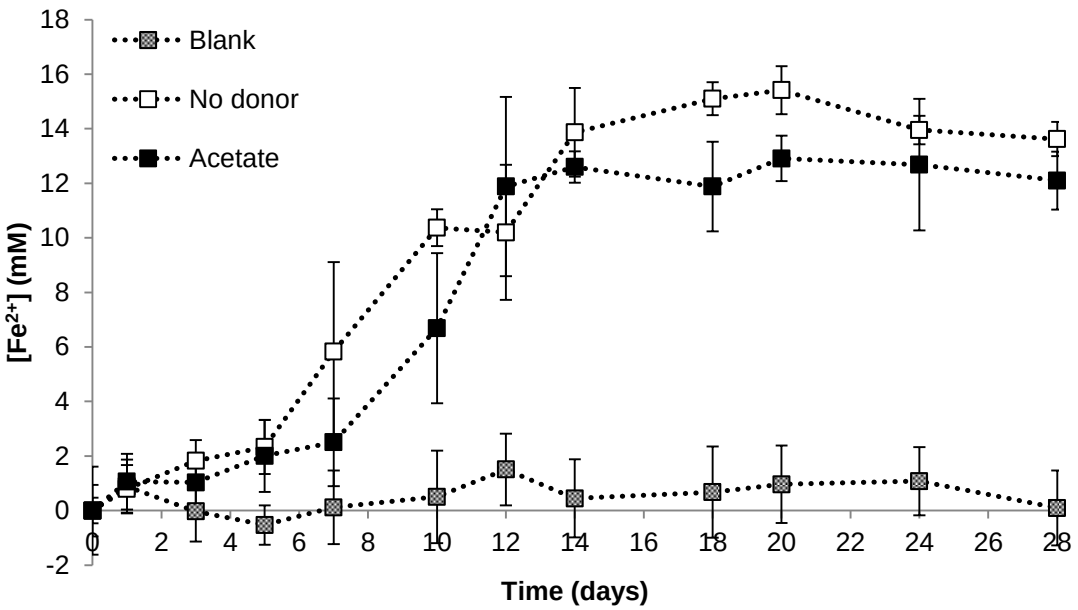

Figure 7.1: MIR in *G. metallireducens* negative controls. Data show change in Fe^{2+} concentration (mM) with time (days) in positive (full redox couple), negative (no donor added) and blank (full redox couple but not inoculated) controls from repeated amino acids experiment. Error bars represent standard deviation of triplicate measurements.

A further experiment was conducted, in which negative and positive controls alone were inoculated with an actively growing culture of *G. metallireducens*, this time thrice washed in bicarbonate buffer. In this instance the resulting concentrated cell suspension was transferred to the gas station from the anaerobic chamber in a sealed pre-sterilised 10 mL serum vial, and purged with N_2/CO_2 for approximately 10 minutes in order to rid the concentrated cell suspension of any residual hydrogen. Two different batches of medium were used to test growth in the presence and absence of supplied acetate. In the first instance the same acetate-free medium used in both previous amino acid experiments was used. In the second a fresh batch was used for comparison.

After incubating in the dark at 30°C for 6 days, MIR was apparent in negative controls with both batches of media. In the case of the older batch of medium, 13 mM Fe^{2+} was produced in the negative control compared with 7.5 mM in the positive. Similarly, where the fresh medium had been used 14.4 mM Fe^{2+} was produced in the negative control compared with 17.2 mM in the positive. That MIR was observed in this test, having purged the cell suspension, serves to unambiguously rule out the possibility of hydrogen acting as an alternative electron donor.

The negative control from the test with the older medium was transferred to fresh positive and negative controls, and after 4 days of incubation MIR was once again observed in both. This new negative control was further transferred to fresh negative and positive controls a further four times, with the same result observed in each case. This resulted in a culture that continued to proliferate when transferred to fresh medium devoid of any electron donors for five generations. This served to unambiguously rule out the possibility of MIR with residual acetate carried over through the washing stages.

Although these tests did not include replicates, the consistency of these findings with earlier results indicated reliable reproducibility. Having discovered that this phenomenon was occurring in cultures inoculated with two independent stocks of *G. metallireducens*, in two different batches of medium, and with and without exposure to hydrogen, the only explanation for the observed growth seemed to be contamination, in which an unwanted strain (or strains) was supplying *G. metallireducens* with a source of electron donors for MIR in negative controls.

It was hypothesised that the citrate component of the Fe(III)-citrate molecule was fuelling the observed growth in the absence of acetate, hence MIR with either ferrihydrite or Fe(III)-pyrophosphate ('Fe(III)-PPi') would offer the opportunity to test this. However several attempts to grow *G. metallireducens* on either were unsuccessful, despite reports of growth on ferrihydrite in the initial characterisation of the strain (Lovley *et al*., 1993a).

Fe(III)-PPi was not tested as an electron acceptor in this initial characterisation, and not all iron-reducing microorganisms are capable of using it. However in the case of ferrihydrite, it is possible that by solely maintaining the strain on Fe(III)-citrate in the laboratory, *G. metallireducens* has lost the physiological ability to reduce insoluble ferric iron, since the pili required for direct attachment of *Geobacter* strains to insoluble ferric iron are no longer required (Nevin and Lovley, 2000).

7.2 Systematic tests

Systematic tests of a third newly revived freeze-dried culture of *G. metallireducens* from DSMZ were initiated. In this case, the strain was revived in 10 mL sterile anoxic freshwater medium devoid of either electron donor or acceptor compounds inside the anaerobic chamber. As such, the strain had been denied both acetate and ferric iron upon hydration. A 10% (vol/vol) sample of this stock was transferred to both a fresh negative (‘–’ 50 mM Fe(III)-citrate, no acetate) and positive (‘+’ 50 mM Fe(III)-citrate, 10 mM acetate) control using sterile needles and syringes flushed with anoxic gas at the gas station. After cultures had been incubated in the dark at 30°C for one week, 51.1 mM Fe^{2+} was measured in the positive control compared with 19.4 mM in the negative. A subsample of both was transferred to duplicated negative and positive controls, and production of Fe^{2+} was monitored over a period of approximately 1 week. Production of Fe^{2+} was observed in all, however the ferrous iron concentration in the second negative originating from the first (‘– –’) was markedly lower. The negative controls of both sets (‘– –’ and ‘+ –’) were then transferred to new duplicate controls, the negative controls of which (‘– – –’ and ‘+ – –’) were again transferred. This process was repeated to five generations, and the results are summarised in Figure 7.2..

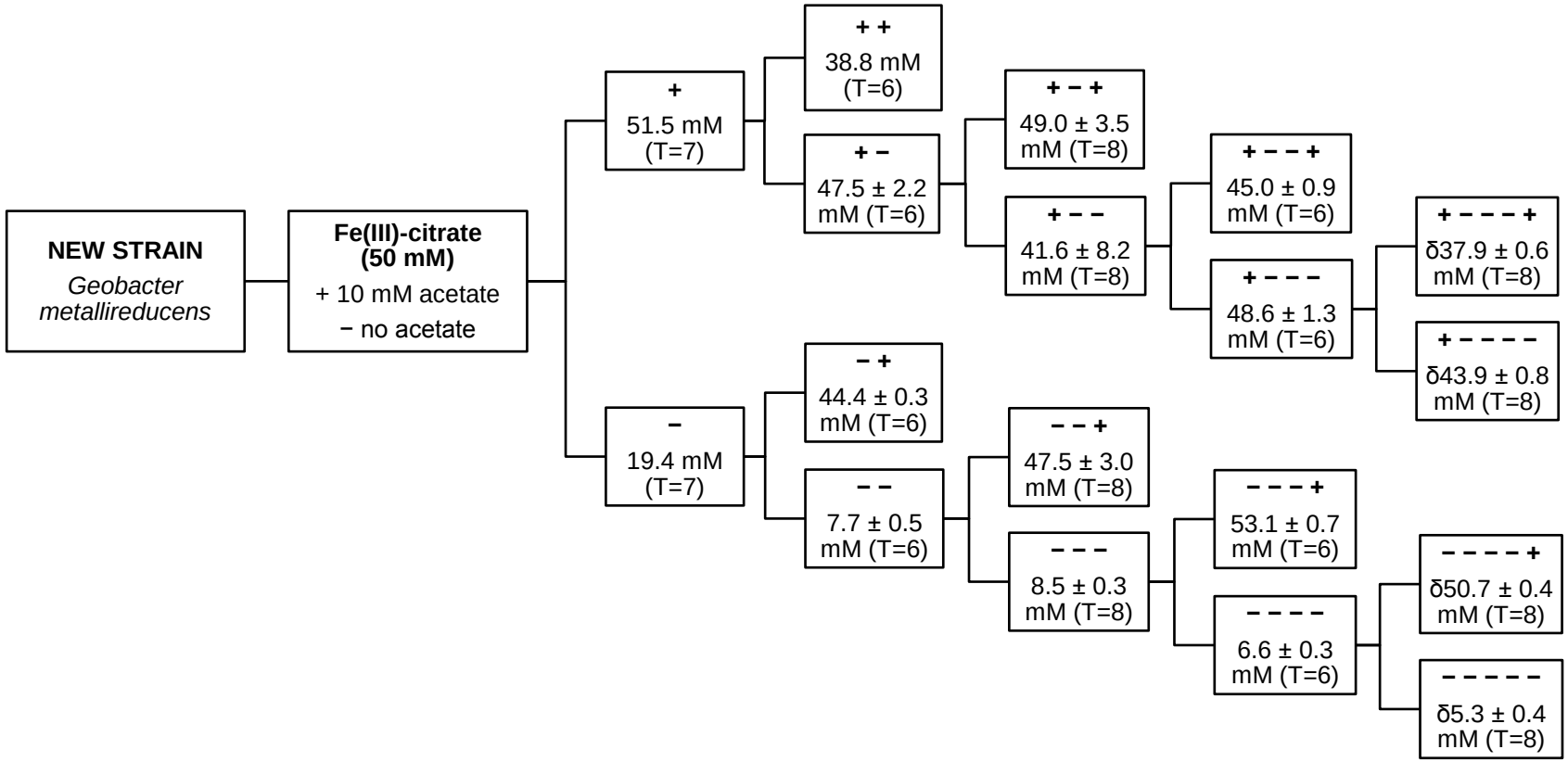

Figure 7.2: Results from systematic tests conducted on a new strain of *G. metallireducens*. The strain was denied acetate upon revival. Results represent Fe^{2+} concentration (mM) measured at the end point of each test. Duplicated tests are indicated by an average and standard deviation measurements. Fe^{2+} concentrations in the final generation represent net change in concentration since inoculation. Durations of incubations are indicated in parentheses.

The concentration of ferrous iron measured in all positive controls across all generations indicates complete or near-complete MIR. In the negative controls that were originally initiated from the first positive control ('+ –' to '+ – – – –'), MIR is also evident, and concentrations of ferrous iron are comparable to those measured in the positive controls. In contrast, very little ferrous iron was produced in negative controls originally initiated from the first negative control ('– –' to '– – – – –'). These results indicate that when the culture of *G. metallireducens* is denied acetate upon revival from a freeze-dried stock, no MIR occurs in negative controls. In contrast, when acetate is supplied for as little as one generation, it is not possible to establish a true negative control, even after four subsequent generations in growth medium devoid of acetate.

7.3 Isolation and taxonomic identification

In an attempt to enrich for and isolate potential contaminants, samples of '+ – – – –' were transferred to serum vials containing 1) sodium citrate (50 mM) and acetate (10 mM), and 2) Fe(III)-citrate (50 mM), acetate (10 mM) and 0.1% (wt/vol) yeast extract. The purpose of the former was to establish whether growth occurred in the absence of ferric iron, whereas in the case of the latter, addition of yeast extract was added specifically to stimulate heterotrophic activity. After incubation at 30°C in the dark for two days, no turbidity was evident in the first culture, however the second culture appeared turbid and 'thick'. In a further attempt to establish growth in the absence of ferric iron, a subsample of the culture stimulated with yeast extract (vial 2) was transferred to the sodium citrate/acetate vial (1), and supplemented with yeast extract. This culture thus contained 50 mM citrate, 10 mM acetate, 0.1% yeast extract and a subsample of the 'thick' culture from the ferric iron/acetate/yeast culture. An increase in turbidity was observed after 24 hours. In addition, growth was evident in a further four generations of this culture, in which each generation was transferred to fresh growth medium containing sodium citrate, acetate and yeast extract. In the case of the fourth and fifth generations, growth media were supplemented with 0.01% yeast extract, compared with 0.1% in preceding cultures. Attempts to grow this iron-independent culture anaerobically on agar, containing the same constituents as the initial growth medium, proved unsuccessful.

In order to assess dependency of the contaminant culture on various substrates, the fifth generation culture was subcultured into growth media containing different combinations

of sodium citrate, acetate and yeast extract. In each case, one substrate was omitted whilst the other two were included. After incubation as before for 48 hours, turbidity was observed only in medium containing both acetate and citrate. Growth media devoid of either of these substrates appeared not to support growth. Therefore this culture appeared to be dependent on citrate or acetate. This indicated that *G. metallireducens* was either absent or present in very small numbers, since growth of the contaminant in co-culture with *Geobacter* appeared unimpeded by the absence of acetate.

DNA was extracted from '+ – – – –', and the bacterial 16S rRNA gene sequenced (see Chapter 3 for details). The results indicated that all sequences present exhibited a 95-97% similarity with strains of the genus *Clostridium*, which comprises of obligately-anaerobic microorganisms that typically operate a heterotrophic mode of metabolism. From the 16S rRNA data, 20 randomly selected sequences of greater than 500 bp length were queried in the Blastn+ database, and the percentage identity matches to known species were averaged (see Table 7.1). In all cases, the closest match with an average identity match of 98.8 ± 0.4 % is an uncharacterised clone, *Clostridium* sp. CYP2. However, all other strains identified in Table 7.1 are characterised, and matched to at least species level identification (97% match). All but one of these strains are assigned to *Clostridium*, the exception being *Desulfomaculum guttoideum*, which is thought to be misidentified as such due to its extremely high 16S rDNA similarly with *Clostridium* species (Stackebrandt *et al*., 1997). Although none of the type strain studies tested citrate as a substrate for growth, a fermentation product common to all is acetate, a readily utilised electron donor for MIR by *G. metallireducens*. *Clostridium aerotolerans*, *C. saccharolyticum* and *C. algidixylanolyticum* also produce ethanol, with the latter additionally producing butyrate and butanol; all of which are known electron donors used by *G. metallireducens*. The pH and temperature optima for the strains listed in Table 7.1 are also close to the conditions under which *G. metallireducens* was being grown.

Species	% Identity	Reference
Clostridium sp. CYP2	98.8	[uncharacterised]
Clostrdium xylanolyticum	97.6	Rogers and Baecker, 1991
Clostridium aerotolerans	97.1	van Gylswyk and van der Toorn, 1987
Clostridium algidixylanolyticum	97.1	Broda et al., 2000
Desulfomaculum guttoideum	97.0	Stackebrandt et al., 1997
Clostridium saccharolyticum	97.0	Murray et al, 1982

Table 7.1: Most closely-associated species to the contaminant strain. The % identity was calculated from the mean % identities of 20 randomly-selected sequences of at least 500 bp in length using the Blastn+ database**.**

7.4 Source of contamination

Given that growth in negative controls was evident in cultures inoculated from three independent stocks of *G. metallireducens*, all of which had been obtained from DSMZ, it seemed feasible that the culture was contaminated at source. However to test this hypothesis it was important to eliminate the revival of freeze-dried cultures in the anaerobic chamber, a potential source of contamination. To this end, an actively-growing culture of *G. metallireducens* was obtained from DSMZ. Direct transfer of this culture into the same growth media with which the contaminant was previously isolated was conducted using sterile needle and syringe using the gas station, in close proximity to a flame. The stock was simultaneously transferred to media containing ferric iron and acetate, and MIR was evident after two days. In contrast, no turbidity was evident in the absence of ferric iron, with or without the addition of yeast extract. Therefore, in contrast to the freeze-dried stocks, this actively-growing strain appeared not to be contaminated.

Correspondence with DSMZ brought to light that a new batch of *G. metallireducens* DSM 7210 was preserved in June 2013, after all prior tests with freeze-dried stocks had been conducted but before the acquisition of the actively-growing culture. Whilst it cannot be ruled out that contamination was introduced within the non-sterile anaerobic chamber, the change in batch means that results may not be directly comparable. Therefore the source of contamination remains unclear.

7.5 Discussion and conclusion

Whilst the exact identification of *Clostridia* present in cultures of *G. metallireducens* remains unknown, it is clear that the contaminant was able to metabolise citrate, producing one or more compounds that could serve as electron donors for MIR. Although the closely associated strains listed in Table 7.1 were not assessed for citrate fermentation, Walther and colleagues (1977) reported the fermentation of citrate by *Clostridium sphenoides*, resulting in the production of acetate and ethanol, both of which are known to support MIR by *G. metallireducens* (Lovley *et al*., 1993a). It is therefore highly likely that similar products were produced by the isolated contaminant.

That *G. metallireducens* was able to prosper in the observed co-cultures indicates that heterotrophic microorganisms such as strains of *Clostridia* and iron-reducing microorganisms can coexist symbiotically. For instance, heterotrophic microorganisms in subsurface sediments containing iron-reducing microorganisms may serve to break down organic compounds that cannot serve as electron donors for MIR, in turn supplying iron-reducers with a readily available source of electron donors that would not otherwise exist. More relevant to this research, however, this observation should serve as a warning that *Clostridia* and other similarly anaerobic heterotrophs readily prosper in growth media containing ferric-citrate, commonly used to maintain and grow a number of iron-reducing microorganisms in the laboratory.

Chapter 8: Concluding discussion

The research conducted in this PhD research has sought to better constrain the feasibility for life sustained by microbial iron reduction (MIR) on Mars. The approach has focused on better defining the limits of iron-reducing microorganisms in Mars-like environments on Earth, and assessing the range of available organic electron donors in the subsurface of Earth and Mars. This final chapter summarises the main findings of the preceding studies, and discusses their implications with respect to the search for life on Mars, the driving force behind this research.

8.1 Summary of main findings

The study reported in Chapter 4 was conducted to answer Q1 posed in the Introduction: are iron-reducing microorganisms widespread in Mars-like environments on Earth? To address this, enrichment cultures selecting for MIR were initiated at different temperatures using sediments from a number of Mars-relevant environments on Earth. These environments include recent volcanic fields and iron-rich streams in Iceland; gravel plains, sand dunes and riverbed sediments from the extremely arid Namib Desert; stream water and sediment samples from the naturally acidic Río Tinto system in Spain; and sediments from beneath a number of geographically-distinct glaciers. The results indicate that MIR is widespread in subglacial and river/stream bed sediments, but not in recent volcanic and extremely arid environments. The detection of MIR in subglacial enrichments established at 4°C but not 15°C indicates that iron-reducing microorganisms in these environments are psychrophilic. Most subglacial enrichments appeared to be at least psychrotolerant, and were able to withstand extremely low storage temperatures for periods ranging from several months to years. Lack of both available redox constituents and stable anoxia are the dominant factors controlling the presence of viable iron-reducing microorganisms in the environments investigated. This is the first study to demonstrate the presence of active iron-reducing microorganisms in Río Tinto sediments, and represents the most comprehensive survey of MIR in subglacial and desert environments to date.

The study reported in Chapter 5 was carried out to answer Q2 stated in the Introduction: are iron-reducing microorganisms capable of using ancient recalcitrant

carbonaceous material in the subsurface of Earth as a source of electron donors for MIR? Microbial growth experiments were conducted with a MIR enrichment culture in which terrestrial carbonaceous material was supplied as the sole source of electron donors. Parallel experiments were carried out to test whether the presence of this material adversely affected the ability of this culture to conduct MIR when a full redox couple was supplied. Not only did none of the four kerogen and two shale samples tested give rise to measurable rates of MIR when supplied as the sole source of electron donors, but the presence of three types of kerogen was also found to completely inhibit MIR. Inhibition to a lesser degree was observed in the presence of remaining samples. The causes of inhibition are unknown, but are likely to be a combination of physical and chemical factors, such as adsorption of usable organics onto clay mineral surfaces and the presence of inhibitory polycyclic aromatic hydrocarbons. This study is the first to assess the ability of anaerobic microorganisms to utilise the organic material found in shales and kerogens. Furthermore, no other study has reported inhibition of microbial metabolism in the presence of bulk carbonaceous material.

The study outlined in Chapter 6 was designed to address Q3 posed in the Introduction: are iron-reducing microorganisms capable of using rare organic compounds found in carbonaceous meteorites as electron donors for MIR? A number of pure strains of iron-reducing microorganisms, in addition to a MIR enrichment culture, were assessed for their ability to use six nonproteinogenic amino acids common to carbonaceous meteorites as electron donors. Three of these amino acids were chiral. All except one amino acid shared the same molecular formula, yet only γ-aminobutyric acid was found to support MIR by the enrichment culture. Furthermore, the presence of D-forms of two of the chiral amino acids tested inhibited MIR in the presence of a full redox couple, and gave rise to a reduction in cell numbers over a short period of time. The observed inhibition is highly likely due to interference with peptidoglycan synthesis, in line with similar studies using aerobic microorganisms. These findings serve to highlight the importance of structural isomerism in both the ability of iron-reducing microorganisms to utilise organic compounds as electron donors, and the inhibitory effect compounds may have on fundamental biochemical cellular processes. Furthermore, calculations made using published accretion rates of extraterrestrial carbonaceous material onto the surface of Mars indicate that the accumulation of inhibitory amino acids may be sufficient to adversely affect habitability over geologic timescales. This study has demonstrated for the first time the inhibitory effects of D-amino acids on anaerobic microorganisms.

In the process of conducting amino acid growth experiments, numerous unsuccessful attempts to establish negative controls using the well-characterised iron-reducing

microorganism *Geobacter metallireducens* led to the discovery that the pure culture was contaminated (Chapter 7). In growth media lacking known electron donors, the contaminant was feeding on the citrate associated with the ferric iron-citrate electron acceptor, and liberating organic compounds that serve as electron donors for *G. metallireducens*. This contaminant was closely associated with strains of the genus *Clostridium*, and points to a syntrophic relationship in natural environments.

8.2 Synthesis

A number of findings from the individual studies summarised above complement one another. For instance, the presence of strains affiliated with the genus *Clostridium* in the MIR enrichment cultures reported in Chapter 4 concur with the syntrophic relationship uncovered between the contaminant and iron-reducing microorganism in Chapter 7. From this research, it seems highly likely that anaerobic heterotrophic microorganisms in subsurface environments play a central role in maintaining an available supply of organic electron donors for MIR and similar chemoorganotrophic redox-driven metabolisms, such as microbial sulphate- and nitrate-reduction.

In light of the results reported in Chapters 4 and 5, the hypothesis proposed by Wadham *et al* (2004) that microbially-mediated sulphate-reduction beneath the Finsterwalderbreen glacier in Svalbard is likely to be sustained by kerogens as the sole source of carbon and electron donors, can be refuted. Viable iron-reducing microorganisms are present in sediments from beneath this glacier, as evidenced by MIR measured in enrichments initiated with these sediments, and were found to be closely associated with strains of the sulphate-reducing *Desulfosporosinus* genus (Chapter 4). These findings confirm that microbially-mediated iron- (and sulphate-) reduction is likely to be occurring beneath this glacier, and that strains of *Desufosporosinus* are capable of operating both metabolisms. However neither the well-characterised kerogens, nor the shale samples that contain kerogen, were shown to serve as a source of electron donors for MIR in growth experiments reported in Chapter 5. Although the enrichment culture used in these experiments was not that of the Finsterwalderbreen subglacial sediments, the large overlap between the conventionally named iron- and sulphate-reducing microorganisms indicates that the same range of electron donors are drawn upon in both metabolisms. Therefore it is

highly unlikely that bedrock kerogen beneath the Finsterwalderbreen glacier is the source of electron donors for microbially-mediated sulphate- (and iron-) reduction.

Kerogens are chemically similar to the organic material in carbonaceous meteorites, and as such are often used as terrestrial analogues. The inhibition observed in the presence of both terrestrial carbonaceous material (Chapter 5) and D-forms of amino acids common to extraterrestrial carbonaceous material (Chapter 6) are therefore significant with respect to the feasibility of MIR in the subsurface of Mars. Although the findings of the amino acid growth experiments of Chapter 6 suggest carbonaceous meteorites may offer a wider range of electron donors for MIR than previously anticipated, the presence of inhibitory compounds that are rare on Earth, coupled to the indication that similar bulk carbonaceous material is itself inhibitory, places significant habitability constraints on MIR in the Martian subsurface. These findings indicate that the factors affecting whether such life could persist on Mars are much more complex than the availability of redox constituents highlighted in Chapter 4.

8.3 Implications

The dominance of many MIR enrichment cultures established in Chapter 4 by microorganisms conventionally thought of as sulphate-reducing microorganisms has significant implications for the way in which microorganisms are associated with metabolic function in a given microbial community. The findings in Chapter 4 clearly demonstrate that the characterisation and naming of a microorganism based on the metabolism initially selected for is not a sufficiently comprehensive approach for microorganisms capable of numerous metabolisms. It is the complementary combination of culture-dependent and -independent tools employed in this study that brought this issue to light. Future studies concerned with the presence or absence of certain metabolisms should consider this issue, and draw upon a similarly complementary approach of culture-based and molecular techniques in order to avoid bias created by conventional nomenclature. Ultimately, environmental microbiology studies would significantly benefit from a nomenclature independent of microbial metabolism.

The presence of recalcitrant carbonaceous material, such as kerogens, in the terrestrial subsurface should not be assumed to represent an available source of carbon and electron donors for anaerobic microbial communities, as previously suggested (e.g. Grasby *et al*., 2003; Wadham *et al*, 2004). However, this material is more likely to support microbial

communities in the presence of oxygen and, if so, may represent a more significant contributor to the global carbon cycle than previously thought. Whether or not such material can support aerobic microbial communities is of great importance to global climate models, where the predicted melting of glaciers and ice sheets underlain by kerogens and similar carbonaceous material may indirectly affect the magnitude of future temperature increases through microbial utilisation.

The syntrophic relationship between anaerobic heterotrophic and iron-reducing microorganisms revealed in Chapter 7 indicates that a single-metabolism approach to constraining the habitability for, and feasibility of, life on Mars is overly simplistic. Although conceptually neat, experiments involving microorganisms capable of only the plausible metabolism proposed may be to the detriment of the microorganisms being tested. In particular, the presence of heterotrophic microorganisms in enrichment cultures for this purpose should not be deliberately avoided, since they may play a crucial role in the functioning of other microbial metabolisms. The experiments conducted in this study that employed a MIR enrichment culture as the inoculum should serve as a model for similar future studies.

Finally, this study demonstrates that the habitability of an environment is greater than the sum of its parts. Although energy is the most fundamental requirement of life, the presence of suitable chemical energy sources in an environment is not a sufficient cause to assume that an environment is habitable, even if all other habitability factors are thought to be non-limiting. Extraterrestrial organic material is relatively rare on Earth, and components of this carbonaceous material have been demonstrated to be inhibitory to microbial metabolism. It is therefore essential that future discussions concerning the habitability of environments on Mars take these findings into consideration, and exercise caution in predictions of the feasibility of life on Mars.

8.4 Remaining knowledge gap and future research

Based on the results of this research project, the remaining knowledge gap can be updated. Unanswered questions arising from this work are outlined below along with recommendations for future research:

- Is MIR active *in situ* in the Mars-like environments studied? Although viable iron-reducing microorganisms were found to be widespread in a number of sediments from streams, rivers and beneath glaciers, whether or not this metabolism occurs, and at what rates, remains unknown. Future studies should address this through microbial incubations, where the prevailing conditions of the environment in question are recreated without addition of a MIR-selective redox couple

- Does hydrogen serve as an electron donor for MIR in subglacial environments? Given that sedimentary carbonaceous material does not appear to support MIR, the prevailing electron donor(s) used by iron-reducing microorganisms in subglacial environments lacking readily available organic electron donors are yet to be defined. One plausible explanation is that hydrogen, liberated from freshly crushed underlying bedrock, serves as an inorganic electron donor, a hypothesis that could be tested through microbial growth experiments

- Do shales and kerogens contain known electron donors for MIR, regardless of whether their presence results in inhibition? Although kerogen samples were assessed for their major organic components, neither this nor any other published study has reported a full organic inventory, including the smaller compounds such as amino acids and alcohols most likely to serve as electron donors for MIR. It is therefore not clear whether these materials do indeed represent a source of electron donors. This should be investigated through the application of more appropriate organic analysis tools with the view to characterise the full organic inventory of the shale and kerogen samples utilised in this study

- Do carbonaceous meteorites serve as a source of electron donors for MIR? Although bulk terrestrial carbonaceous material, recognised analogues of the organic material in carbonaceous meteorites, does not appear to represent an electron donor source (and in some cases inhibits MIR), it remains to be seen whether the same holds true for bulk carbonaceous meteoritic material. Furthermore, it is not clear whether the D-enantiomers common to these meteorites are sufficiently concentrated in bulk material to inhibit MIR. These uncertainties should be addressed through microbial growth experiments using bulk carbonaceous meteorite as the sole source of electron donors for MIR.

8.5 Conclusion

This thesis has demonstrated that MIR is more widespread on Earth than previously thought, particularly in subglacial environments. Colder and previously more acidic environments on Mars are therefore within the bounds of habitability for MIR on Mars. In light of this research, MIR remains a compelling microbial metabolism to operate in the subsurface of Mars, past or present. However, the use of meteoritic carbonaceous material in the Martian subsurface for this metabolism is called into doubt. Experiments using meteoritic carbonaceous material should be prioritised in the lead up to near-future life detection missions to Mars, such as the ExoMars rover. Such experiments are essential to better constrain the potential energy sources available on Mars for microbial iron reduction and other plausible microbial metabolisms.

Appendix

Two first-authored review papers were published during the course of this PhD. The full references of these are listed below, and a copy of each can be found at the back of this document.

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