This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.
A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.
This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.
The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.
When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.
Microbial Responses to Extreme Radiation Environments

Jennifer Louise Wadsworth

Doctor of Philosophy
The University of Edinburgh
2018
Abstract

Microorganisms are known to tolerate a variety of extreme environments, such as high and low pH, desiccation and a wide range of temperatures that would prove uninhabitable for most eukaryotic cells. However, extreme radiation exposure is a ubiquitous hazard to pro- and eukaryotic viability. Ionising and non-ionising radiation, and their associated high energies, cause damage to a cell in the form of DNA double-strand breaks, membrane deterioration, and lethal mutations. Radiation also induces secondary effects such as the production of reactive oxygen species, which attack and degrade organic compounds. It is therefore not surprising that radiation is considered by the scientific community to be one of the main influencing factors when regarding habitability on the early Earth, as well as other planets, such as present-day Mars. This thesis explores the response of select microbes that have been exposed to extreme radiation environments, i.e. both high and ultra-low radiation.

Understanding how radiation affects the geochemical environment is key to the assessment of its potential to support life and to harbour molecules associated with life. The effect of radiation-induced photochemistry on the early terrestrial and present-day Martian surface is explored in conjunction with Fenton chemistry. Iron oxides are abundant on both Earth and Mars and act as catalysts in Photo-Fenton reactions, enabling the production of free radicals. The resulting consequences for habitability are shown to be antagonistic, with iron oxide enabling both the protection or destruction of cells, depending on the local geochemistry. In addition, the photo-reactivity of perchlorate is investigated. The UV-induced activation of the strong oxidant, and recently confirmed Martian surface constituent, is demonstrated, revealing its severe bacteriocidal effect on microbes. It is also shown to significantly reduce microbial viability when combined with further Martian soil constituents and components required for Photo-Fenton chemistry.
In order to accurately analyse the effects of low earth orbit radiation on prokaryotic life, cyanobacterial samples were attached to the outside of the International Space Station as part of the EXPOSE-R2 mission for 1.5 years. The samples were subjected to various conditions, including exposure to a minimally filtered space radiation environment and simulated Mars conditions. The experiment is designed to test the protection that biogenic and non-biogenic substances may provide to cells. The results in this work present the post-flight analysis of the samples and demonstrate the ability of these substances to maintain cyanobacteria viability. They also show that the cyanobacterial cells themselves can effectively act as a shield for a secondary, co-cultured bacteria species.

On the other end of the radiation dose scale, this work addresses the gaps in knowledge with regard to the little-understood effects of low, sub-background radiation on prokaryotes. Using the Boulby Underground Lab in the functioning Boulby Mine, Cleveland UK, microbes are cultivated under regulated, extremely low radiation environments to test multiple dose-response models. The results show no change in cell’s growth rates or gradients in low radiation exposure when compared to surface-dose controls. They also fail to exhibit any enhanced susceptibility to stress factors, such as UV irradiation, as suggested by previous work in the field. These experiments mark the first extensive and tightly controlled research into microbial responses in the near-absence of radiation.

This work illustrates the importance of understanding both primary and secondary effects of radiation on microbes and begins to bridge the knowledge gap from both ends of the dose axis. These approaches show the far-reaching influence radiation has on astrobiologically relevant topics, such as habitat geochemistry and life detection, and demonstrate the capacity of life to survive in extreme radiation environments.
Lay summary

Radiation is the emission of energy in the form of waves and particles. It is ubiquitous to the environment on Earth and is present throughout the universe. At high doses, radiation can be harmful to life, causing multiple forms of damage to vital molecules, such as DNA and cell membranes. This thesis studies the response of bacteria that have been exposed to extreme (i.e. very high or low) radiation doses in various environments. Research of this nature not only informs us on how radiation contributes to the limiting parameters of life, but also on the potential for non-terrestrial life, if it exists, to survive in similar extreme radiation environments.

The first section of the thesis is dedicated to investigating high radiation environments, such as space and the surface of Mars, and the chemistry that results from those environments being irradiated. The chemical effects of irradiated soil compounds found on Earth, but particularly on Mars, are studied with regard to how they influence the survival of bacteria in that environment. The findings predominantly highlight the lethal chemical species that are produced as a result of irradiation, that can be more destructive to life than radiation on its own.

The high-radiation work also encompasses the analysis of bacteria that have been exposed to the space environment on the outside of the International Space Station for 1.5 years. Despite the multiple extreme conditions, the bacteria are still able to be cultured once back on Earth. The best survival rates are obtained from bacteria that have been exposed to space in large groups as opposed to single cells, as well as bacteria that have been exposed with a thin layer of Mars-like dust on top of them. These results show the resilience of certain bacterial lifeforms to high radiation exposure and other extreme conditions. This has implications for understanding the contamination of other planets with life from Earth (planetary protection), but also gives insight into the probability of similar life surviving in
extreme, non-terrestrial habitats.

To gain a holistic understanding of how radiation affects life, it is necessary to also consider the other end of the dose range: sub-background radiation dose rates, i.e. radiation doses lower than normal radiation exposure on the surface of the Earth. In response to the lack of scientific consensus on the effects of very low radiation exposure on life, various bacteria species are grown in a specially built lead chamber in a 1.1 km-deep active mine. Their growth is measured as an indicator for overall fitness and shows no difference when compared to the fitness of bacteria exposed to surface radiation doses. Although such extremely low dose rates are unlikely to have any realtime influence on life, these results contradict much of the previous literature in this field.

These findings collectively inform on the ability of life to survive various radiation extremes, which help shape our understanding of the physical limits of life not only on Earth, but also elsewhere in the universe.
Declaration

This thesis has been solely composed by the candidate. Unless clearly stated, this work is the candidate’s own. Where results have been obtained through collaborations with other researchers, their precise contributions are made clear in the text as appropriate. Parts of this thesis have been published in two first-authored peer-reviewed papers listed below. This work has not been submitted for any other degree or professional qualification.

Parts of this work have been published in [1, 2].

(Jennifer Louise Wadsworth, 2018)
Acknowledgements

The work presented in this thesis would not have been possible without the diverse and generous support of many people; I would like to acknowledge the UK Space Agency for providing the funding for this project by means of the Aurora Science Programme.

I would particularly like to thank Professor Charles S. Cockell (aka the indefatigable Moth-Man) for his seemingly limitless patience and contagious enthusiasm whilst guiding me through my work. I also thank him for introducing me to the delights of “Captain Tardigrade” and for sharing his unquenchable appreciation for dairy-based frozen confections. He is the reason I felt able and still feel able to pursue astrobiological research.

I would also like to extend my thanks to the team at the Boulby Underground Laboratory (Sean, Chris (my no. 1, never my no. 3, and disputed Twister “champion”), Emma, Barbara, Paul and Lou), who have been a part of my PhD from the beginning. I am indebted to you for your exhaustive support, which has not only enabled me to perform my experiments, but has also made it an incomparable pleasure to do so. Boulby has a special place in my memories, which is not at all influenced by the outstanding quality of the local cuisine.

Thank you to all the past and present members of the Edinburgh PIPC and Astrobiology group for your input, encouragement and of course for providing all-important baked goods at lab meetings. Your daily support and the uncountable laughs have enabled me to see this through. I will gladly share my fondue cheese and Kirsch with you any time.

I would also like to say a massive “Merci vill mol!” to all my friends in Switzerland, who have always had my back and been my omni-present rock during my studies. An especially big shout out to those who have braved the Scottish weather to come visit me.

Finally, I would like to thank Tasha (Tashkins), Dr. Ben, Mum, Dad, Richie and the rest of the Wadsworth-McCutcheon clan for your unconditional love and unwavering support. I dedicate this work to you.
# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>i</td>
</tr>
<tr>
<td>Lay summary</td>
<td>iii</td>
</tr>
<tr>
<td>Declaration</td>
<td>v</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vii</td>
</tr>
<tr>
<td>Contents</td>
<td>ix</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xv</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xix</td>
</tr>
</tbody>
</table>

1 Introduction

1.1 Thesis focus and outline ................................. 3

2 Background

2.1 Introduction ...................................................... 5

2.2 Types of radiation .............................................. 6

2.2.1 Biological radiation damage ............................... 9

2.3 High-radiation environments ............................... 11

2.3.1 Radiation environment of the early Earth ............... 11

2.3.2 Radiation environment of Mars ........................... 12
2.3.3 Radiation in low Earth orbit ........................................ 14

2.4 Radiation-induced chemistry .................................................. 16
  2.4.1 Fenton photochemistry ................................................ 16
  2.4.2 Perchlorate photochemistry .......................................... 19

2.5 Sub-background radiation ..................................................... 22
  2.5.1 Radiation-response models ........................................... 23

3 Methodology  ...................................................................... 25
  3.1 Introduction ................................................................. 25
  3.2 Bacterial strains ............................................................. 25
    3.2.1 Long-term bacterial storage ....................................... 26

3.3 Microscopy ........................................................................ 27
  3.3.1 Transmission electron microscopy (TEM) ....................... 27
  3.3.2 Scanning electron microscopy (SEM) .............................. 28

3.4 Raman spectroscopy ............................................................ 29

4 Protective and destructive photochemical properties of iron oxide to life ........................................................................ 31
  4.1 Introduction ................................................................. 31
  4.2 Background ................................................................. 32
    4.2.1 Fenton reaction ....................................................... 32
    4.2.2 Reactive oxygen species (ROS) ................................. 33
    4.2.3 Protective and destructive properties of iron ............... 33

4.3 Methods ........................................................................... 34
  4.3.1 Model organism selection .......................................... 34
  4.3.2 Bacterial preparation .................................................. 34
5 Perchlorates on Mars enhance the bacteriocidal effects of UV light

5.1 Introduction ................................................................. 53

5.2 Background ................................................................. 54
  5.2.1 Perchlorate characteristics ........................................ 54
  5.2.2 Perchlorates on Mars ............................................... 54

5.3 Methods ........................................................................ 56
  5.3.1 Model organism selection ........................................ 56
  5.3.2 Perchlorate UV irradiation ....................................... 56
  5.3.3 Rock analogue system ............................................. 57

5.4 Results ........................................................................ 57
  5.4.1 Bacteriocidal effect of UV-irradiated perchlorate ........... 57
  5.4.2 Bacteriocidal effect under Martian analogue conditions 58
5.4.3 Interactions of other Martian soil components ............... 64
5.5 Discussion ........................................................................ 68
5.5.1 Limitations .................................................................. 71
5.5.2 Future work ............................................................... 72
5.6 Conclusions ..................................................................... 73

6 Survival of cyanobacteria after extended exposure to space and simulated Martian environments
6.1 Introduction .................................................................... 75
6.2 Background ....................................................................... 76
6.3 Methods ........................................................................... 78
6.3.1 Model organism selection ............................................. 78
6.3.2 EXPOSE-R2 platform ................................................ 80
6.3.3 Post-flight culturing ................................................... 85
6.3.4 Staining ..................................................................... 86
6.3.5 TEM preparation ....................................................... 87
6.3.6 SEM preparation ....................................................... 87
6.3.7 Raman spectroscopy ................................................... 88
6.4 Results ............................................................................. 88
6.4.1 Growth assays ........................................................... 88
6.4.2 Fluorescent microscopy ............................................... 92
6.4.3 Transmission electron microscopy ............................... 101
6.4.4 Scanning electron microscopy .................................... 103
6.4.5 Raman spectroscopy ................................................ 105
6.4.6 Bacterial co-culture .................................................... 110
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2.1)</td>
<td>The ionising and non-ionising electromagnetic spectrum</td>
<td>6</td>
</tr>
<tr>
<td>(2.2)</td>
<td>Overview of radiation types</td>
<td>7</td>
</tr>
<tr>
<td>(2.3)</td>
<td>SI radiation dose units</td>
<td>8</td>
</tr>
<tr>
<td>(2.4)</td>
<td>Direct and indirect mechanisms of DNA double strand breaks</td>
<td>11</td>
</tr>
<tr>
<td>(2.5)</td>
<td>Sources of ionising radiation of low Earth orbit</td>
<td>14</td>
</tr>
<tr>
<td>(2.6)</td>
<td>Overview of oxychlorine photochemistry on Mars</td>
<td>22</td>
</tr>
<tr>
<td>(2.7)</td>
<td>Radiation dose-risk relationship models</td>
<td>24</td>
</tr>
<tr>
<td>(3.1)</td>
<td>Transmission electron microscopy electron beam</td>
<td>28</td>
</tr>
<tr>
<td>(3.2)</td>
<td>Virtual electronic transitions of Rayleigh and Raman scattering</td>
<td>30</td>
</tr>
<tr>
<td>(4.1)</td>
<td>Effects of lower and higher hydrogen peroxide concentrations on cell viability under UV and dark conditions</td>
<td>36</td>
</tr>
<tr>
<td>(4.2)</td>
<td>Modelled Mars UV irradiance vs. measured polychromatic lamp irradiance 200 - 400 nm</td>
<td>37</td>
</tr>
<tr>
<td>(4.3)</td>
<td>The protective effects of iron oxides</td>
<td>39</td>
</tr>
<tr>
<td>(4.4)</td>
<td>Detail at 60 s exposure comparing hematite data with and without hydrogen peroxide</td>
<td>41</td>
</tr>
<tr>
<td>(4.5)</td>
<td>Heterogeneous Photo-Fenton reactions in rock analogue discs</td>
<td>42</td>
</tr>
<tr>
<td>(4.6)</td>
<td>Photo-Fenton reaction in the liquid and solid systems containing B. subtilis under anaerobic conditions, detail at exposure time of 60 s</td>
<td>43</td>
</tr>
<tr>
<td>(4.7)</td>
<td>Photo-Fenton reaction at 4°C</td>
<td>44</td>
</tr>
<tr>
<td>(4.8)</td>
<td>Photo-Fenton reactions after polychromatic light exposure</td>
<td>45</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>5.1</td>
<td>Effects of UVC-irradiated Mg(ClO$_4$)$_2$ on B. subtilis viability</td>
<td>58</td>
</tr>
<tr>
<td>5.2</td>
<td>Dark controls for M9 and perchlorate</td>
<td>59</td>
</tr>
<tr>
<td>5.3</td>
<td>Effects of UVC-irradiated Mg(ClO$_4$)$_2$ in rock analogues, under anaerobic conditions, polychromatic light and low temperature.</td>
<td>60</td>
</tr>
<tr>
<td>5.4</td>
<td>UV spectrophotometric absorbance of irradiated perchlorate 250 - 400 nm</td>
<td>62</td>
</tr>
<tr>
<td>5.5</td>
<td>Perchlorate photoprodut concentration</td>
<td>62</td>
</tr>
<tr>
<td>5.6</td>
<td>Effects of low concentrations of UVC-irradiated Mg(ClO$_4$)$_2$ on cell viability, 60 seconds exposure</td>
<td>63</td>
</tr>
<tr>
<td>5.7</td>
<td>Influence of increased perchlorate concentration on bacteriocidal effects under UV irradiation</td>
<td>63</td>
</tr>
<tr>
<td>5.8</td>
<td>Effects of UVC-irradiated sodium/calcium perchlorate and sulphate on B. subtilis viability</td>
<td>65</td>
</tr>
<tr>
<td>5.9</td>
<td>Perchlorate-induced bacteriocidal effects in the presence of other components of the Martian surface (hematite and hydrogen peroxide) after 30 and 60 seconds UV exposure</td>
<td>67</td>
</tr>
<tr>
<td>5.10</td>
<td>Perchlorate reduction pathway</td>
<td>72</td>
</tr>
<tr>
<td>6.1</td>
<td>Experimental layout of BOSS and BIOMEX</td>
<td>77</td>
</tr>
<tr>
<td>6.2</td>
<td>Gloecocapsa samples on sintered discs</td>
<td>80</td>
</tr>
<tr>
<td>6.3</td>
<td>Positioning of cells in BOSS and BIOMEX set up</td>
<td>81</td>
</tr>
<tr>
<td>6.4</td>
<td>EXPOSE-R2 in and on the ISS</td>
<td>83</td>
</tr>
<tr>
<td>6.5</td>
<td>EXPOSE-R on the ISS</td>
<td>83</td>
</tr>
<tr>
<td>6.6</td>
<td>Live/Dead stained BIOMEX/BOSS cells</td>
<td>94</td>
</tr>
<tr>
<td>6.7</td>
<td>FDA stained BIOMEX/BOSS cells</td>
<td>96</td>
</tr>
<tr>
<td>6.8</td>
<td>DiBAC$_4$(3) stained BIOMEX cells</td>
<td>98</td>
</tr>
<tr>
<td>6.9</td>
<td>DiBAC$_4$(3) stained BIOMEX/BOSS cells</td>
<td>99</td>
</tr>
<tr>
<td>6.10</td>
<td>DiBAC$_4$(3) stained BOSS cells</td>
<td>100</td>
</tr>
<tr>
<td>6.11</td>
<td>TEM of ultra-thin BIOMEX/BOSS cell sections</td>
<td>102</td>
</tr>
<tr>
<td>6.12</td>
<td>SEM of BOSS cell sample</td>
<td>104</td>
</tr>
<tr>
<td>6.13</td>
<td>Raman spectra of ground control</td>
<td>105</td>
</tr>
</tbody>
</table>
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2.1)</td>
<td>Examples of primary and secondary effects of radiation damage</td>
<td>10</td>
</tr>
<tr>
<td>(2.2)</td>
<td>Archaean and present-day Earth UV flux</td>
<td>12</td>
</tr>
<tr>
<td>(6.1)</td>
<td>BIOMEX &amp; BOSS sample nomenclature</td>
<td>84</td>
</tr>
<tr>
<td>(6.2)</td>
<td>BIOMEX/BOSS sample growth three months</td>
<td>89</td>
</tr>
<tr>
<td>(6.3)</td>
<td>BIOMEX/BOSS sample growth nine months</td>
<td>90</td>
</tr>
<tr>
<td>(6.4)</td>
<td>Raman peak heights of three characteristic carotenoid peaks</td>
<td>107</td>
</tr>
<tr>
<td>(7.1)</td>
<td>Activities of nuclide series in the bacterial sample</td>
<td>128</td>
</tr>
<tr>
<td>(7.2)</td>
<td>SELLR experiment nomenclature</td>
<td>133</td>
</tr>
<tr>
<td>(A.1)</td>
<td>Numerical results of <em>B. subtilis</em> viability</td>
<td>156</td>
</tr>
<tr>
<td>(A.2)</td>
<td>Continuation of numerical results of <em>B. subtilis</em> viability</td>
<td>157</td>
</tr>
<tr>
<td>(B.1)</td>
<td>Internal dose summary of all components</td>
<td>162</td>
</tr>
<tr>
<td>(B.2)</td>
<td>Bacterial growth experiment numerical values</td>
<td>164</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

Radiation can be defined as the emission of energy in either wave or particle form and is present throughout the universe. Exposure to radiation has been ubiquitous to life since its emergence in the first billion years after the formation of the Earth [3, 4]. From the high-radiation environment of the ozoneless early Earth, to the intrinsic radioactive decay of specific elements in our planet’s lithosphere, life has evolved alongside radiation of various doses and sources. However, it has been shown that heightened radiation exposure can rapidly lead to severe damage of an organism’s vital structures, such as cell membranes and DNA [5–7]. These effects have prompted considerable research into how radiation exposure compounds human health. Examples of research topics include the carcinogenic nature of the ultraviolet (UV) component of sunlight [8], the ionising radiation risk faced by astronauts [9], as well as the exploitation of radiation effects for the treatment of cancer [10]. Deleterious radiation-induced effects on prokaryotic life have also been well-established [11, 12] and are routinely used in sterilisation processes. In addition, radiation has been identified as a key limiting factor to microorganisms exposed to the multiple extreme conditions of space [13] and is predicted to massively impact the habitability of potential non-terrestrial environments, such as Mars [14]. Because of this, understanding the limiting nature of radiation on life has become a significant topic of astrobiological research.

There have been multiple Earth [15–17] and space-based [18–20] experiments dedicated to investigating the effects of extreme radiation environments on a variety of organisms and compounds. Similarly, the high-radiation work in the
thesis makes use of both Earth and space-based experimental approaches to investigate the response of prokaryotic organisms to various radiation conditions. In spite of the multiple orbit and ground-based instruments on Mars [21, 22], there is still a lack of understanding of how radiation interacts with the Martian environment and how the resulting conditions influence its habitability. This work aims to address the gap in knowledge regarding Martian radiation-induced chemistry, as well as to investigate the ability of various biogenic and abiogenic compounds to confer protection to lifeforms in deleterious radiation conditions. The results of these endeavours shed light on the probability of life and life signatures persisting in a heightened radiation environment and provide insight into how life on the early Earth survived similar extreme radiation exposure. These results also aid in guiding the appropriate design of life sign-detecting planetary exploration equipment, as well as establishing necessary planetary protection protocols to avoid the accidental contamination of non-terrestrial environments with terrestrial life.

An additional extreme radiation condition that is often overlooked is the lack of radiation. With radiation being omnipresent for the origin of life and throughout its evolution, its absence warrants investigation into its relevance for life. Removing it as an environmental factor can offer insight into why and how it influences life in high-radiation conditions. Although the topic of life in low and sub-background radiation is not novel [23–26], the current literature is pervaded by controversy and confusion. The final section of this work presents an attempt to establish a thorough basis for future research on the effects of ultra-low radiation on prokaryotes.

Understanding the effects of radiation on biology and the geochemical environment is crucial to establishing parameters for the physical limits of life. Moreover, if these limits are universal they can not only aid in our understanding of the persistence of life on our own planet, but can also be applied to assessing habitability of environments elsewhere in the universe.
1.1 Thesis focus and outline

The focus of this thesis is to investigate the effects of both high and low radiation environments on prokaryotic organisms and to gain insight into the limits they present, not only to life on Earth, but also to potential habitats on other non-terrestrial bodies.

Chapter 2 provides background knowledge of the different kinds of radiation environments and radiation-induced chemistry referenced in this work. It also highlights the wide range of conditions and factors associated with extreme radiation environments, as well as the differing challenges they bring with them with regard to the survival and proliferation of bacterial life. Chapter 3 briefly provides an overview of the general methods and their basic principles that were employed while conducting this research. In-depth, specific methods are discussed in each results chapter, respectively.

The research presented in this thesis is divided into two sub-topics: high radiation and low radiation microbial responses. The first three results chapters (4 - 6) present high-radiation investigations, in which the effects of radiation-induced photochemistry on habitability and the effects of the low Earth orbit (LEO) radiation environment on microbial viability are addressed. Chapters 4 & 5 present research concerning the protective and destructive characteristics of iron oxide and the reactivity of perchlorate, all driven by UV irradiation. Although applicable to environments on the early Earth, experiments were carried out with a specific focus on Mars, as both iron oxides and perchlorates are prominent surface regolith components. Ramifications of their photochemical products are investigated regarding the effect on microbial viability and, by extension, the consequences for Martian habitability and planetary protection.

Chapter 6 presents the post-flight data analysis of microbial responses to actual space-like (low Earth orbit) and simulated Martian conditions. Microbes previously isolated in a low Earth orbit mission were subjected to the multiple extreme conditions of low Earth orbit, such as high radiation, vacuum and severe temperature fluctuations, on the outside of the International Space Station (ISS) for 1.5 years. The focus of the post-flight analyses was on biofilm and Mars simulant soil protection of cells as well as protection conferred by the cells to a secondary species included in the flight samples. This not only provides insight into what protection is necessary for the preservation of viability
under multiple extremes including radiation, but also ties in to understanding the protective/destructive nature of Martian soil and its radiation induced-photochemistry covered in the earlier chapters.

The second part of the thesis covers low-radiation research, exploring microbial responses at the little-understood, sub-background levels of radiation. Chapter 7 presents data taken in a novel experimental set-up at 1.1 km underground in the Boulby Underground Lab (Cleveland, UK). Here, a rigorous assessment of all experimental components was carried out in order to establish a well-characterised low radiation environment, in which representative strains of bacteria were grown. Results from the experiments provide perspective on current and much disputed radiation-response models, as well as contributing to the formation of a new model of low-dose prokaryote responses.

Chapter-specific limitations, conclusions and future work statements are located at the end of each results chapter.

The general conclusions of the research are summarised in chapter 8, whereupon a general outline of future research questions and directions is presented.
Chapter 2

Background

2.1 Introduction

The aim of this thesis is to explore the scope of habitability under different radiation extremes. Initially, the focus will be on the challenges that life forms must overcome when faced with survival in high-radiation environments, such as the surface of the early Earth or Mars. The focus will then shift to the opposite end of the dose range: sub-background radiation environments. While much research is invested in establishing the dangerous effects of high radiation, few experiments have been successful in advancing our understanding of biological effects in the absence of radiation. This chapter discusses the background literature relevant to understanding these topics and their context. I discuss the different types of radiation and their characteristics, including biological damage, and introduce the relevant high and low radiation environments. Additionally, I will provide an overview of the two radiation-driven chemical reactions examined in this work, in the context of high-radiation early Earth and Martian environments.
2.2 Types of radiation

Radiation is generally defined as an emission of energy in particle and wave form (e.g. neutrons, helium nuclei, photons; see fig. 2.1), which propagate through a medium. There are multiple units used to describe radiation. Relevant SI units include:

- Gray (Gy, [J/kg]) describing the physical absorbed dose.
- Sievert (Sv, [J/kg]) describing the biological equivalent & effective dose.
- Becquerel (Bq, [s\(^{-1}\)]) describing radionuclide activity.

Other historical, non-SI units appearing in radiation literature include roentgen (R; exposure dose for X-rays or \(\gamma\)-rays), rad (radiation absorbed dose) and rem (roentgen-equivalent-man). These will not be used in this work.

Radiation can be categorised as either “ionising” or “non-ionising” (fig. 2.2) with respect to its ability to ionise an atom by stripping it of a valence electron, resulting in a net-positive charge. The required ionisation energy is 4 - 25 eV [27], which is also substantial enough to break chemical bonds, although the US Federal Communications Commission material defines ionising energy as above 10 eV (equating to a light wavelength of \(\sim\) 124 nm) [28]. Both ionising and certain kinds of non-ionising radiation have the potential to cause a substantial amount of biological damage (see section 2.2.1).

![Figure 2.1](image-url)  
**Figure 2.1** The ionising and non-ionising electromagnetic spectrum [29].
Ionising radiation can be either directly ionising or indirectly ionising. High-energy, charged particles directly ionise atoms or molecules without requiring an intermediate step. Examples of directly ionising particles are electrons or α-particles (\(^4\)He\(^2+\) nuclei). Indirect ionisation occurs when an uncharged particle, such as a photon or neutron interacts with an atom or molecule, causing the release of an electron. Although most of the ultraviolet (UV) spectrum is considered non-ionising, extreme UV (124 - 10 nm) falls under the energy category of ionising radiation. Non-ionising radiation has an energy lower than 10 eV, such as most of the UV spectrum and lower electromagnetic wavelengths. Though unable to induce electron loss, non-ionising radiation can still cause electron excitation, i.e. its movement to an energetically higher state. Thereby, higher energy non-ionising radiation retains its ability to inflict biological damage. An example thereof is UV-induced production of carcinogenic, reactive oxygen species (ROS) [8].

Radiation not only differs in its constituents but also in its ability to deposit energy. Linear energy transfer (LET) is a property of ionising radiation regarding its energy deposit per distance travelled, described in either newton or keV/\(\mu\)m units. Radiation with high-LET, such as α-particles, incur densely ionising tracks through material, whilst low-LET particles like X-rays are sparsely ionising. This means that high LET particles cause more ionisation events and by extension more biological damage, than low LET particles at the same dose [30].
### Figure 2.3: SI Radiation Dose Units

<table>
<thead>
<tr>
<th>SI Unit</th>
<th>Definition</th>
<th>Quantity</th>
<th>Dose</th>
<th>Effective Dose</th>
<th>Equivalent Dose</th>
<th>Absorbed Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rem</td>
<td>1 Rem = 1 R absorbed by tissue</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Sievert (Sv)</td>
<td>1 Sv = 1 J/kg (absorbed dose)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Gray (Gy)</td>
<td>1 Gy = 1 J/kg (absorbed dose)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Effective Dose**
- Sum of products of exposure and tissue weighting factor
- Summation over all tissues and organs
- Units: Sievert (Sv)

**Equivalent Dose**
- Effective dose x tissue weighting factor
- Units: Sievert (Sv)

**Absorbed Dose**
- Units: Gray (Gy)

---

**Meaning**
- **Joule/Kg (J/kg)**: Physical energy absorbed by mass
- **Rem**: A physical unit of radiation risk
- **Sievert (Sv)**: A physical unit of radiation risk
- **Gray (Gy)**: A physical unit of absorbed dose

---

**SI units**
- Conversion factor: 1 Rem = 0.01 Sv = 0.01 J/kg
- Conversion factor: 1 Sv = 100 Rem = 100 J/kg
- Conversion factor: 1 Gy = 1 J/kg

---

**Deviating Radiation Protection Dose Quantities in SI Units**
- Effective dose (E) = dose x tissue weighting factor
- Equivalent dose (H) = effective dose x tissue weighting factor
- Absorbed dose (D) = dose

---

**Tissue and Organ Weighting Factors**
- Different tissues and organs have different risks due to radiation exposure.
2.2.1 Biological radiation damage

This section presents a brief overview of the main kinds of cellular damage caused by irradiation.

Although life on Earth has originated and evolved in the constant presence of radiation, cells are highly prone to sustaining lethal damage when exposed to heightened doses. As mentioned in section 2.2, radiation can be ionising and non-ionising, both of which can result in significant damage to cell constituents. The affected structures and severity of damage depend on many factors, such as dose rate, LET characteristics and cell radio-sensitivity. As a result, lethal doses for various prokaryotes range from $10^1$ to $10^3$ Gy [32], while radiation doses of up to 30 Gy cause death in humans within 48 h. Both prokaryotic and eukaryotic cells are affected by radiation damage to their DNA, proteins and membranes and have analogous repair mechanisms, such as for DNA damage-repair [33]. However, there are differences between pro- and eukaryotic susceptibility and responses. An example of this is the ability of eukaryotic cells to undergo programmed cell death. The biological radiation damage described in this chapter is focussed on effects in prokaryotes, but is also generally applicable to eukaryotes.

Section 2.2 describes the units used to measure biological effects of radiation (Sv, [J/kg]). The “equivalent dose”, denoting the biological effect of a specific radiation type, is calculated using the physical adsorbed radiation dose in gray (Gy, [J/kg]), which is multiplied with the radiation type-dependant weighting factor ($W_R$) and measured in sievert. This can be further multiplied with the tissue weighting factor ($W_T$), resulting in the “effective dose”, which is solely relevant for higher eukaryotes and is therefore not used in this research. For consistency, the radiation unit used in the thesis will be gray (Gy), which is relevant for chapters 6 & 7. In chapter 6, doses of radiation received by the model organism are indicated in Gy, as there is no known weighting factor for the organism used. Furthermore, irradiation of model organisms in chapter 7 is done with $\gamma$-radiation, which results in an equal absorbed and equivalent dose (i.e. the weighting factor for low-LET gamma-rays is 1, therefore 1 Gy $\gamma$-radiation = 1 Sv $\gamma$-radiation). The effective dose is only applicable to higher organisms and will therefore not be utilised in this work. Cited work may contain the non-SI adsorbed dose unit “rad”, which is equivalent to 0.01 Gy (1 Gy = 100 rad).
Both directly and indirectly ionising radiation can cause primary or secondary effects in target structures (tab. 2.1).

**Table 2.1** Examples of primary and secondary effects of radiation damage by direct and indirect ionisation. DSB = Double strand break; ROS = Reactive oxygen species.

<table>
<thead>
<tr>
<th></th>
<th>Direct ionisation</th>
<th>Indirect ionisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary effects</td>
<td>DSB</td>
<td>DNA photoproducts</td>
</tr>
<tr>
<td>Secondary effects</td>
<td>ROS</td>
<td>ROS</td>
</tr>
</tbody>
</table>

**Primary effects**

DNA damage is a frequent result of high radiation exposure and can be caused by both primary and secondary effects of irradiation. Ionising radiation is known to directly induce DNA double strand breaks (DSBs, fig. 2.4), which have been shown to be produced with equal efficiency in both eukaryotes and prokaryotes (∼ 0.005 DSB/Mbase/Gy irradiation) [34]. While point mutations and single strand breaks can be more readily repaired, DSBs are the primary cause of radiation-induced cell death [35] and can additionally result in carcinogenesis or aberrations if improperly repaired. DSB occurrence is particularly prominent in high-LET or high-dose rate exposure. Primary damage to DNA induced by UV is mainly characterised by the formation of dimeric photo-products. These lesions are predominantly formed by exposure to UVB, as bases in DNA directly absorb photons at this wavelength range [36]. Additionally, membrane proteins and lipids are also directly affected by radiation by way of cleavage or ionisation and subsequent degradation. Heavy, charged ions in particular are known to easily penetrate tissue and have been shown to cause cell lysis [37].

**Secondary effects**

Aside from direct, primary effects, secondary reactions can have equally destructive effects on cell viability. A prominent example of this is radiation-induced oxidative stress, of which DNA, lipids and membranes are major targets. Low levels of oxidative stress are constantly present in aerobic organisms, that in response, have developed strategies to counteract oxidative effects, such as intrinsic repair mechanisms, antioxidant enzymes and radical scavengers [38, 39].
However, irradiation can heighten oxidative stress by increasing the production of free radicals. Radicals are predominantly produced when radiation interacts with water, the primary constituent of the cell, causing radiolysis and creating reactive oxygen species (ROS) such as the hydroxyl (•OH) or alkoxy (RO₂•) radicals. These are highly reactive chemical species containing an unpaired electron, which are responsible for significant molecular structural damage, such as DNA cleavage, lipid and protein degradation leading to lysis or apoptosis [40–42]. Because of this, ROS have been recognised as the leading cause of radiation-induced cellular damage [43].

![Figure 2.4](image.png)

**Figure 2.4** Direct (i.e. primary) and indirect (i.e. secondary) mechanisms of DNA double strand breaks [44].

### 2.3 High-radiation environments

This section will discuss the relevant terrestrial and non-terrestrial high-radiation environments to preface the high-radiation experiments in chapters 4 - 6.

#### 2.3.1 Radiation environment of the early Earth

The radiation environment of the early, Archaean Earth (3.9 - 2.5 Gya) was very different from that of the present-day Earth. The Archaean eon marks what researchers believe to be the the first time in the history of the Earth that the environment was stable and favourable enough to sustain life [45], though
origin of life could have occurred much earlier. Although it has been calculated that the sun would have been 23-26% less luminous during the Archaean [46], the contribution of high-energy radiation, namely UV and X-rays, would have been greater. In addition to solar luminosity and spectrum contribution, the Earth’s early atmosphere also differed from its present state. The dominant constituent is estimated to also have been nitrogen [47], and it is assumed that the level of greenhouse gases was higher to solve the “faint young sun paradox” [48]. The paradox is created by the confirmed presence of liquid water on the surface of the Earth in spite of the lack of sufficient energy emission from the sun at that time. One of the most notable differences in the early atmosphere was the low concentration of oxygen [49] and, by extension, the absence of an ozone layer. This allowed a high flux of shortwave UV radiation to reach the surface of the Earth [50], shown in table 2.2. As described in section 2.2.1, shortwave UV is known to severely damage key molecules of life, such as DNA, proteins and lipids. Although organisms in subsurface environments would not have been exposed to these conditions, early phototrophs would have had to develop strategies to avoid serious damage. Examples of strategies include physical (endolithic habitats) and biogenic protection (pigmentation) as well as efficient cellular repair mechanisms.

<table>
<thead>
<tr>
<th>UV radiation</th>
<th>Present Earth (W/m²)</th>
<th>Archaean Earth (W/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVC</td>
<td>∼0</td>
<td>3.1</td>
</tr>
<tr>
<td>UVB</td>
<td>3.7</td>
<td>11.7</td>
</tr>
<tr>
<td>UVA</td>
<td>71.3</td>
<td>56.3</td>
</tr>
</tbody>
</table>

2.3.2 Radiation environment of Mars

The greater distance of Mars from the sun than the Earth results in a 57% decrease in solar radiation reaching the top of Mars’s atmosphere, in comparison to the amount reaching that of the Earth [15]. Despite the lower solar irradiance, the Martian surface is subjected to higher radiation levels than the Earth. Although the 95% CO₂ Martian atmosphere screens wavelengths below 190 nm, the lack of an ozone shield allows for a high flux of shortwave UVB and UVC (190 - 315 nm) radiation to reach the surface [51]. This results in a UV flux in Martian equatorial regions comparable to that of mid-latitudes on the Archaean.
Earth and is thought to contribute to the highly oxidising surface chemistry on Mars. The atmosphere and magnetosphere of the Earth effectively shield its surface environment from ionising radiation such as solar energetic protons (SEP), produced by solar flares and coronal mass ejections, as well as galactic cosmic rays (GCR). However, Mars lost its internal dynamo action, and consequently its magnetosphere, \(\sim 4 \text{ Gya}\) \([52]\). This, in addition to its thin atmosphere, resulted in the bombardment of its surface with unattenuated high-energy charged particles. The Radiation Assessment Detector (RAD), on board the Mars rover Curiosity, made \textit{in situ} measurements of the surface radiation environment for \(\sim 300\) days during the solar maximum. Data obtained at Gale Crater showed a GCR dose rate of \(0.210 \pm 0.040\) mGy/day (\(\sim 26\times\) global daily average radiation dose on Earth \([53]\)) and a total SEP dose contribution of \(50\) \(\mu\)Gy \([21]\).

Although surface data can be extrapolated to estimate the subsurface radiation environment, \textit{in situ} measurements are yet to be carried out. GCR and SEP are known to have a greater penetration depth than UV, which is attenuated millimetres into the regolith \([54]\), while secondary particle cascades from ionising radiation can penetrate the subsurface on an order of metres. Adjusting previous models \([14, 55]\) by using the \textit{in situ} surface radiation data and local estimates of regolith composition and density can give more precise estimates of the subsurface radiation dose. In addition, the intrinsic subsurface rock radiation must also be taken into account. Regolith radionuclide decay produces ionising radiation and has been estimated to result in a dose rate of \(4 \times 10^{-4}\) Gy/year (0.4 mGy/y) \([56]\).

In conclusion, the RAD team estimate that below a depth of 3 m, the intrinsic rock radiation becomes the dominant source of radiation over GCR \([21]\). The Martian high radiation environment has significant implications for predicted microbial survival times and for biomarker preservation. Estimates for the survival of \textit{Deinococcus radiodurans}, one of the most radioresistant known organisms, do not reach beyond the timeframe of a few million years if the organism were located in the top meters of Martian regolith \([57]\). Equally, organic compounds in the top centimetres of Martian soil would be degraded in a 100-million-year timescale. Although these data provide low expectations of finding preserved biomarkers within drilling depth of rovers, organic constituents of carbonaceous chondrites in the subsurface may survive degradation on a billion-year timescale \([58]\). When considering the preservation of biomarkers on Mars it is also crucial to take the oxidising surface chemistry into account. This is discussed in sections 2.4.1 & 2.4.2.
2.3.3 Radiation in low Earth orbit

When discussing the radiation environment in space, it is important to distinguish between numerous environments with their own specific radiation conditions. The relevant space environment for this thesis is that of low Earth orbit (LEO), which will be briefly presented in this section.

LEO is defined as the region extending from 160 - 2000 km above the surface of the Earth and is still within the atmosphere and magnetosphere. The radiation environment of LEO is therefore not to be equated with that of interplanetary space beyond the Earth’s atmosphere and magnetosphere. Despite this, regions above 100 kM in altitude, including LEO, are defined as “outer space” [59]. The International Space Station (ISS) orbits within LEO at an altitude of ∼ 400 km, motivating a thorough understanding of the LEO radiation conditions and their effects on building materials and human health. The primary constituents of ionising radiation in LEO are charged particles from galactic cosmic radiation (GCR), Earth’s radiation belts (ERB) solar particle events (SPE) (fig. 2.5) [60].

Figure 2.5 Sources of ionising radiation of low Earth orbit: particles from galactic cosmic rays, Earth’s radiation belts and solar particle events [60].
GCRs consist of charged particles originating from beyond the solar system, while energetic electrons and protons trapped in the Earth’s magnetic field create radiation belts around the planet (Van Allen belts). GCR and ERB radiation each contribute to \(\sim 50\%\) of the radiation dose onboard the ISS. Although SPEs can cause a significantly increased flux in ionising radiation (up to 40 mGy), their occurrence is sporadic and dependent on the solar cycle stage.

As GCRs consist of charged particles - mostly protons and heavier ions, such as carbon, oxygen and iron - they are affected by the Earth’s magnetic field and are pulled towards the North and South Poles. Therefore, objects in LEO receive the highest GCR dose when closest to the Poles. Electrons trapped in the Earth’s geomagnetic field create inner and outer radiation belts, with the former comprised of 5 MeV electrons and the latter of 7 MeV electrons. Trapped protons decrease in energy with increasing distance from the Earth and range from 150 - 250 MeV. Most of the protons are located above the ISS orbital altitude. However, because of the axis displacement of the magnetic field in comparison to the Earth’s axis of rotation there exists a region called the “south Atlantic Anomaly”, in which the magnetic field drops to a lower altitude. When the ISS traverses this region it is subjected to the higher trapped proton energies, which contribute to 50\% of its total received radiation [61]. Mean measured LEO radiation dose rates have shown a typical dose rate not exceeding 500 \(\mu\)Gy/day, regardless of factors such as space craft orientation and solar cycle, although high-altitude missions have experienced dose rates of 2000 \(\mu\)Gy/day [60]. This mean dose rate is \(\sim 60\times\) higher than the yearly global average dose rate on the surface of the Earth [53]. Because of this, prolonged exposure to LEO still poses a real threat to astronaut health. Conversely, it can also be used to test upper radiation exposure limits of organisms and compounds in conditions that would be almost irreproducible on Earth, demonstrated in chapter 6.
2.4 Radiation-induced chemistry

The following section presents two photochemical reactions of relevance to both the Earth and Mars, which will be the focus of the first two results chapters (4 & 5).

2.4.1 Fenton photochemistry

Fenton photochemistry is known on Earth for its use in sterilising wastewater but it also has significance for Martian habitability, as the required reagents are present on its surface. The Fenton reaction was first described in the late 19th century by H.J.H. Fenton [62]. It involves the oxidisation of the ferrous iron (Fe$^{2+}$) catalyst (also called the “Fenton reagent”) to ferric iron (Fe$^{3+}$) in the presence of hydrogen peroxide (H$_2$O$_2$). The products of this reaction are reactive oxygen species (ROS), such as hydroxyl and hydroperoxyl radicals $\cdot$OH and $\cdot$OOH. This is known as the Fenton reaction (eq. 2.4.1, 2.4.2).

\[
\text{Fe}^{2+}_{(aq)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+}_{(aq)} + \cdot \text{OH} + \cdot \text{OOH} \quad (2.4.1)
\]
\[
\text{H}_2\text{O}_2 + \cdot \text{OH} \rightarrow \cdot \text{OOH} + \text{H}_2\text{O} \quad (2.4.2)
\]

The production of ROS is exploited for the degradation of organic material, most often in wastewater treatment facilities [63–65]. The Fe$^{3+}$ is then recycled back to Fe$^{2+}$ either by additional H$_2$O$_2$ or $\cdot$OOH in the “Fenton-like reaction” (eq. 2.4.3, 2.4.4).

\[
\text{Fe}^{3+}_{(aq)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+}_{(aq)} + \cdot \text{OOH} + \text{H}^+ \quad (2.4.3)
\]
\[
\text{Fe}^{3+}_{(aq)} + \cdot \text{OOH} \rightarrow \text{Fe}^{2+}_{(aq)} + \text{O}_2 + \text{H}^+ \quad (2.4.4)
\]

This step is the rate-limiting step, as the reduction reaction rate is significantly lower than the first step. Precluding the addition of extra Fe$^{3+}$ or H$_2$O$_2$, the production of reactive oxygen species will be drastically reduced. Despite the apparent simplicity of the reaction, its precise mechanisms and products are still subject to debate. The main two mechanisms believed to be involved in Fenton chemistry are the “radical” and “non-radical” mechanisms involving either the
production of the hydroxyl radical (\(\text{•OH}\)) or ferryl ion (FeO\(_2^+\)), respectively [66, 67]. Though conclusive evidence is still required, and may indeed indicate that both radical and non-radical models co-exist [68], the general literature consensus is that the radical mechanism is the correct one at this point in time. Therefore, all Fenton reactions portrayed in this work illustrate the “radical” mechanism.

One variation of the Fenton reaction is the Photo-Fenton reaction (eq. 2.4.5), of which UV/vis light is an additional component, and has been shown to accelerate the degradation of organics due to the additional reactions that irradiation enables.

\[
\text{Fe}^{3+}(\text{aq}) + \text{H}_2\text{O} \xrightarrow{h\nu} \text{Fe}^{2+}(\text{aq}) + \text{•OH} + \text{H}^+ \quad (2.4.5)
\]

At the optimal pH for the Fenton reaction (\(\sim\) pH 3) the predominant Fe\(^{3+}\) species is Fe(OH)\(^{2+}\), which absorbs in most of the UV spectrum [69] and is efficiently reduced to Fe\(^{2+}\) (eq. 2.4.6) [70]. Furthermore, there is an increase in radical production at wavelengths of < 320 nm due to the photolysis of H\(_2\text{O}_2\) (eq. 2.4.7) [71].

\[
\text{Fe(OH)}^{2+} \xrightarrow{h\nu} \text{Fe}^{2+} + \text{•OH} 
\]

\[
\text{H}_2\text{O}_2 \xrightarrow{h\nu} 2\text{•OH} 
\]

The Fenton reaction can be self-limiting due to intermediate products such as oxalic acid (H\(_2\text{C}_2\text{O}_4\)), that further lower the pH and can chelate Fe\(^{3+}\), preventing complete mineralisation. However, in the case of Photo-Fenton reactions oxalic acid catalyses mineralisation in the form of ferrioxalate. These complexes can absorb light up to 570 nm and decompose to Fe\(^{2+}\) and CO\(_2\) [72]. Due to its increased efficiency over the Fenton reaction and requirement of only small amounts of catalyst, the Photo-Fenton reaction is an environmentally friendly and widely-used method of industrial decontamination [73].

Despite its effectiveness, homogenous Photo-Fenton chemistry, using dissolved iron, also has its limitations. In the presence of excessive H\(_2\text{O}_2\), \text{•OH} radicals are scavenged. This still leaves \text{•OOH} species, however these are less reactive resulting in a lower degradation rate of organics and additional scavenging of remaining \text{•OH} (eq. 2.4.8, 2.4.9) [74].
\[ \text{H}_2\text{O}_2 + \cdot\text{OH} \rightarrow \text{HOO} \cdot + \text{H}_2\text{O} \]  
\[ \cdot\text{OH} + \text{HOO} \cdot \rightarrow \text{O}_2 + \text{H}_2\text{O} \]  

Homogenous Fenton chemistry is also susceptible to the influence of other ions. Chloride ions (Cl\(^-\)) had been thought \[75, 76\] to cause the preferential production of Cl\(_2\)\(^-\), a less reactive radical than the hydroxyl radical, which was proven to occur by Machulek \textit{et al.} \[77\]. The presence of sulphate (SO\(_4^2-\)) and phosphate (PO\(_4^{3-}\)) ions have also been shown to terminate the Photo-Fenton reaction by forming photo-inert Fe\(^{3+}\) complexes \[78\].

There are additional drawbacks to the use of homogenous (Photo-)Fenton chemistry in industry. Working with dissolved iron requires the need to work at a low pH to avoid iron precipitation, which can often occur regardless of low pH in the case of Fe\(^{3+}\) that goes on to form "iron sludge". The recovery of the iron from treated solutions is also uneconomical. In lieu of this there has been increased research into use of iron oxides as Fenton reagents in the "heterogeneous Photo-Fenton reaction". The use of iron oxides allows for work at neutral pH and easy post-treatment removal of the catalyst. In conjunction with research showing iron oxides’ ability to act as highly efficient Fenton reagents \[79, 80\], specialised design and synthesis of iron crystals can allow for a tailor-made, optimised catalyst \[81\].

**Fenton photochemistry on Mars**

The presence of iron oxide on the Martian surface is responsible for its unmistakable red colouring. However, it may also be responsible for multiple photochemical reactions with other Martian soil constituents, thereby contributing to the chemical environment of the surface, in addition to its distinctive pigmentation. Iron is the second most abundant metal and fourth most abundant element in the Earth’s crust. It is also abundant on Mars, which has twice the amount of iron oxides in its outer layers as the Earth \[82\]. Data from the Opportunity Mars Exploration Rover showed that \(\sim 90\%\) of the total sampled Martian surface iron was in the form of Fe\(^{3+}\)-oxides such as hematite and goethite \[83\]. The other Fenton constituent, hydrogen peroxide, remained undetected on Mars until atmospheric observations, made by two independent teams in the last decade, confirmed its presence \[84, 85\].
All of the components necessary for the heterogeneous Photo-Fenton reaction have been detected on Mars (iron oxide & hydrogen peroxide), including a high flux of shortwave (200 - 400 nm) UV radiation due to the lack of shielding by the thin atmosphere \[86\]. This additional, probable source of free radical production is an important part of the complex surface chemistry taking place on the Martian surface, which is vital for assessing the degradation of possible extant life signatures as well as terrestrial contaminants.

### 2.4.2 Perchlorate photochemistry

An additional component of the Martian surface is perchlorate, a powerful oxidant that was spectrally identified in flowing liquid brines on the surface of Mars in September 2015 by NASA’s Mars Reconnaissance Orbiter \[87\]. The perchlorate ion (ClO$_4^-$) is known to be highly stable and non-reactive at ambient temperatures and usually requires high temperatures for its thermal degradation. This is especially the case in aqueous environments due to the large kinetic barrier of its reduction. However, since the mid-20\textsuperscript{th} century it has been shown to be susceptible to radiolysis, i.e. degradation by ionising radiation, without a change in temperature \[88, 89\]. Due to the high flux of ionising radiation reaching the Martian surface, perchlorate radiolysis and its products have become the focus of much recent research with regard to their ability to degrade organics.

There is still uncertainty with regard to the mechanism by which perchlorate is reduced by radiation. Heal \[88\] proposed a possible degradation mechanism with regard to X-ray irradiated KClO$_4$, consisting of the electronic “excitation” of the perchlorate ion, (ClO$_4^-\star$) (eq. [2.4.10]) which consequently was reduced to chlorate and atomic oxygen (eq. [2.4.11]).

\[
\text{ClO}_4^- \rightarrow \text{ClO}_4^-\star \quad \text{ (2.4.10)} \\
\text{ClO}_4^-\star \rightarrow \text{ClO}_3^- + \text{O} \quad \text{ (2.4.11)}
\]

However, it was argued that this would only occur in sites of lattice imperfections. Additionally, due to the size of the oxygen atom and low temperatures, the probability of the oxygen diffusing out and the perchlorate not reforming after reduction was very low.

Over the years, empirical evidence has accumulated showing the various oxychlo-
rine products obtained after perchlorate irradiation. Prince et al. [89] described the formation of various oxychlorine species such as chlorate (ClO$_3^-$), chlorite (ClO$_2^-$), hypochlorite (ClO$^-$) and chlorine dioxide (ClO$_2$) as well as molecular oxygen (O$_2$) after irradiating perchlorate at room temperature with a $^{60}$Co source's $\gamma$-rays. More recent studies have shown the capacity of UV-irradiated perchlorate to generate chlorite [90] as well as the first in situ measurement of chlorine dioxide production [91]. In 2016 Turner et al. [92] proposed an altered mechanism of degradation. This followed on from their infrared spectroscopic detection of chlorate and atomic oxygen, and mass spectrometric detection of atomic oxygen, after irradiation with galactic cosmic-ray (GCR) particles. They suggested the cleavage of the oxygen-chlorine bond to form an electronically “excited” atomic oxygen (O(1$D$)) or an energetically more favourable ground state atomic oxygen (O(3$P$)) (eq. 2.4.12, 2.4.13).

$$\text{ClO}_4^- \rightarrow \text{ClO}_3^- + \text{O}(1D) \quad (2.4.12)$$
$$\text{ClO}_4^- \rightarrow \text{ClO}_3^- + \text{O}(3P) \quad (2.4.13)$$

They proposed that once cleaved, the oxygen remains in the solid or diffuses out and eventually forms molecular oxygen (O$_2$), which has also been observed as a product of perchlorate irradiation [89, 91, 93].

**Perchlorate photochemistry on Mars**

Perchlorate photochemistry is of particular interest with regard to understanding the chemical environment of the Martian surface and subsurface. The failure of the Viking landers to detect organics on the Martian surface, coupled with the detection of chloromethane, resulted in the first speculation of the presence of strong oxidants. Perchlorate was later measured in situ in 2008 by the Phoenix lander [94] and has since been observed spectroscopically in brine seeps [87]. Perchlorate’s organic-degrading products are of particular interest. Hypochlorite has been shown to chlorinate amino acids [93], atomic oxygen can degrade nucleobases [95] and chlorine dioxide is a well-known strong oxidant used for disinfection [12]. It has also recently been suggested that perchlorate degradation by GCR may result in the production of hydrogen peroxide, another strong oxidant, albeit in ultra-high vacuum conditions [96]. The confirmed presence of perchlorate on Mars, in addition to its strongly oxidising properties, make it
a subject of astrobiological significance concerning habitability and biomarker detection, as well as planetary protection.

With this knowledge, it is no surprise that scientists researching extinct and extant life detection on Mars are interested in perchlorate’s effect on the geochemical environment and degradation of organic molecules, such as biomarkers. Solar cosmic ray (SCR) and UV-induced photo-degradation primarily occurs on the surface, as solar rays and UV photons are efficiently absorbed by the Martian soil [57, 97]. Yet any degradation products may diffuse through the soil and extend the reach of deleterious molecules. Concurrently, with their higher energy than solar photons, GCR particles have been shown in simulation to penetrate multiple metres [98]. This may enable them to degrade organics and induce secondary degradation via the reduction of oxychlorine species. Additionally, the calculated intrinsic regolith radiation emitted by radionuclide decay [56] is also a source for ionising radiation in the Martian subsurface, which may be capable of inducing radiolysis.

Photochemistry is also thought to be involved in the formation of perchlorates. Most terrestrially available perchlorate is synthetically produced for use in rocket propellant and fireworks [99], while naturally occurring perchlorate is scarce, with the highest concentrations found in the Atacama desert at \( \sim 0.6 \text{ wt\%} \) [100]. Catling et al. [101] argued that isotopic fractionation observed in Atacama perchlorate [102] suggested atmospheric origin and went on to propose synthesis pathways involving the atmospheric oxidation of chlorine-containing species by ozone. This may be the mechanism for natural terrestrial perchlorate synthesis, yet it fails to account for the high yields (\( \sim 0.6 \text{ wt\%} \)) measured on Mars [103]. Kim et al. [104] suggest GCR as means of perchlorate synthesis on Mars. However, this seems unlikely in light of the aforementioned studies showing the GCR-induced degradation of perchlorate. Formation pathways have also been put forward that do not restrict reactions to the atmosphere. Shuttlefield et al. [105] proposed the oxidation of chloride by UV-irradiated semiconductors, such as titanium dioxide or iron oxides, which are found in Martian soil. Carrier et al. [106] additionally suggest a ground-based reaction, whereby chloride-containing mineral surfaces are oxidised by oxidants either produced in the Martian atmosphere or by photochemical reactions in the soil. This continuous photochemical formation mechanism would explain the regeneration of perchlorate amidst the evidence of its concurrent-photochemical degradation. However, many of the proposed formation pathways (see fig. 2.6)
raise the issue of depth distribution of perchlorate, as they suggest its formation is restricted to the Martian surface. Although perchlorate may become buried in the Martian regolith, radiation simulation data suggest that it would be degraded by ionising radiation, even in the subsurface, and may not be readily replenished as the synthesis pathways suggest. This ultimately shows the persisting gaps in understanding regarding the formation and degradation of perchlorate as well as its reactivity, which may impact the evaluation of Martian habitability.

![Figure 2.6](image)

**Figure 2.6** Overview of potential photochemical reactions induced by ionizing and non-ionizing radiation involved in the formation and degradation of oxychlorine species, radicals and other oxidants on the Martian surface. [107]

### 2.5 Sub-background radiation

This section will provide an overview of the sub-background radiation environment on Earth and current models of its effects on life, which is the focus of research presented in chapter 7.

Much radiation research is dedicated to understanding the effects and dangers of high doses on organisms. Such high-radiation analysis is focused on doses that are orders of magnitude higher than the dose range experienced by most organisms on the surface and near-surface environment of Earth. The “background” radiation on Earth is mostly comprised of naturally occurring radiation such as radon in the air, solar UV and rock radionuclides. All of this naturally occurring radiation
adds up to a global average of 2.8 mGy/y. Including artificial radiation sources, the global average is estimated to be 3.01 mGy/y [53]. To put this into context, human exposure to 10 Gy over a short time scale is classed as severe radiation exposure, 10 - 30 Gy causes certain death within 48 h [108]. The surface radiation dose in one year is four orders of magnitude below the lethal ranges for humans and approximately six orders of magnitude below damaging doses for radio-resistant organisms such as Deinococcus radiodurans [109]. Sub-surface radiation levels are generally classed as below $\sim 10^{-3}$ Gy/y.

### 2.5.1 Radiation-response models

Up until the mid-20\textsuperscript{th} century, the predominant model used for estimating the radiation dose-response or dose-risk (risk of damage to organism) was a thresholded one (yellow line, fig. 2.7). The risks of higher radiation exposure had been discovered after encouraged daily exposure and ingestion of radioactive material in the early 20\textsuperscript{th} century lead to multiple deaths [110]. Yet it was generally considered that doses below a certain range were not harmful. Work done by Muller in the late 1920s and his conclusion of a linear dose-risk relationship between radiation dose and mutations in Drosophila melanogaster [111], and consequent work [112], provided the basis on which a new model, the linear no-threshold (LNT) model, was built. The LNT model stipulates the harmful effect of any radiation dose without a dose threshold and is independent of dose rate (red line, fig. 2.7). The LNT model became, and remained, the basis for consequent radiation safety guidelines from the mid-20\textsuperscript{th} century onwards. Its acceptance was additionally helped by initial analyses of cancer in Japanese atomic bomb survivors, indicating linearity of dose and risk. However, more recent analysis for the data does not support the LNT model [113, 114].

Since the mainstream introduction of the LNT model, there has been vehement criticism [115, 116] of its use and supporting data, including documented accusations of deliberate misleading and scientific misconduct [117], as well as a passionate exchanges of views [118, 119]. The most prominent counter-model currently being suggested by the scientific community is the hormesis (hormetic) model (green line, fig. 2.7). It is consistent with LNT in that it predicts high-dose inhibition (i.e. damage) but it also propounds a low-dose “negative risk”, (i.e. stimulation, beneficial effects). The hormetic effect has been reported at varying low dose-rates and in various organisms [120, 123]. Although the concept
pre-dates LNT, first being suggested in the late 19th century [124], it has yet to be accepted as the predominant radiation-risk response model. An additional response observed, especially with regard to cancer cells, was the hypersensitive response to certain ranges of low to intermediate radiation doses (blue line, fig. 2.7), which could be exploited as a means on cancer treatment [125, 126]. While there is still no scientific consensus on a mechanistic radiation-risk model, it is becoming apparent that the LNT model may not apply to lower doses and that the configuration of a future model may heavily depend on the type of cell/organism, dose-rate and radiation type in question.

Figure 2.7 Proposed models for experimental and theoretical correlations of radiation dose and the risk of damage to organisms, including the widely accepted LNT model. Models are based on high-dose data, extrapolating responses for lower dose ranges [127].
Chapter 3

Methodology

3.1 Introduction

This chapter will briefly present the general methods used in this work, all of which are standard techniques in the field of microbiology and are therefore not described in detail. Further information on these methods can be found in references [128–130]. Protocols of utilised methods and specific experimental execution are presented in detail in the methodology section of each results chapter.

3.2 Bacterial strains

Four bacterial species were used in this work and will briefly be described along with their culturing requirements.

*Escherichia coli* is a Gram negative, facultatively anaerobic bacterial species of large genomic and phenotypic diversity. It is a commonly used model organism for laboratory-based studies due to its ease of culturing and genetic manipulation. Laboratory strains (e.g. K12) are non-pathogenic, with a doubling time of ~20 minutes under optimal conditions. *E. coli* can be cultured in a variety of laboratory media and achieves optimal growth at a temperature of 37°C, although growth still occurs up to 49°C [131].

*Bacillus subtilis* is a facultatively anaerobic, heavily flagellated, Gram positive
bacterial species found in soil and the gastrointestinal tract. It is a well-studied, frequently used model organism in laboratory-based research and thereby considered the Gram positive equivalent of *E. coli*. It is able to form endospores that are resistant to multiple extreme conditions, such as desiccation and temperature fluctuations [132]. *B. subtilis* spores have also been shown to be 5-50 times more resistant to 254 nm UV exposure than its vegetative form [133, 134], making them frequent inhabitants of cleanrooms and of interest regarding planetary protection. Spore resistance is further discussed in section 4.5.

*Gloeocapsa sp.* are a genus of cyanobacteria that produce a gelatinous sheath (note: not classed as a traditional biofilm, as described in section 6.2) that envelop single cyanobacteria cells, as well as whole cell clusters. The ability of certain species of *Gloeocapsa* to tolerate harsh conditions, such as hypersaline environments [135], is attributed to this sheath. The species used in this work is an environmental sample retrieved from the upper greensand layer of limestone cliffs in Devon, UK, after which it was isolated by exposure to low Earth orbit for 10 days [136]. The isolate was formally identified as part of the *Gloeocapsa* genus by its morphological features and sequence analysis. Optimal growth conditions consist of 26°C in cyanobacterial media (e.g. BG-11) exposed to 2000 - 3000 lux of light and result in a doubling time of 65 h.

*Geminicoccus roseus* are α-proteobacteria. The bacterial strain used in this work was isolated from the same cell culture as the above-mentioned *Gloeocapsa*, during the exposure to low Earth orbit conditions, and was identified as an isolate related to *Geminicoccus roseus*. It is described as a diplococcoid bacterium containing carotenoids and low amounts of bacteriochlorophyll a, giving its colonies a pink colouration [137]. Although stated to grow at 30°C on nutrient agar [138], it was unable to be isolated under lab conditions on nutrient agar, in nutrient broth or BG-11.

### 3.2.1 Long-term bacterial storage

When necessary, bacteria aliquots were prepared for long term storage. This enables experiments to be carried out using isogenic bacteria to enhance comparability of results over extended periods of time. Isogenic stationary phase bacterial cultures were centrifuged at 10,000 × g/min for five minutes, after which the supernatant was removed. The pellet was resuspended in 25% glycerol
solution in a 1:1 ratio. Aliquots were then stored at −80°C for future use, which enables cell preservation for multiple years.

3.3 Microscopy

3.3.1 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) enables microscopic imaging and analysis on the micro to nanometre scale ($10^{-6} - 10^{-9}$). As TEM is able to resolve single columns of atoms, it can determine chemical and structural properties of crystals and is widely used in the field of biology, as well as chemistry and physics. Unlike a light microscope it utilises a magnetically focused beam of high-energy electrons in vacuum, which allows for high-resolution and high-magnification of microstructures. Optical microscopes are limited to a maximum resolution $\sim 200$ nm, while TEM can achieve a resolution of under 0.2 nm [139], as the wavelength of an electron (2.5 pm at 200 keV) is far smaller than that of a photon (visible light: 390 - 700 nm). The general principle of TEM is to resolve the sample structures by allowing electrons to be scattered by the sample, as well as to transmit it (fig. 3.1). The transmitted electrons are then collected by a phosphorescent screen or on camera, creating an image of the “shadow” that the sample structure casts when electrons are backscattered. The amount of scattered electrons is inversely proportional to sample thickness. The backscattered secondary electrons can be collected in a similar set up for scanning electron microscopy (SEM), used to highly resolve surface structures. In order for the electrons to pass through the sample, it has to be cut into ultra-thin slices no more than 100 nm thick, usually achieved using a diamond-tipped knife. To achieve greater contrast, samples are coated with elements such as osmium or gold that have a high-atomic number and more effectively absorb or scatter electrons. Additional stains such as uranyl acetate and lead citrate can be used to enhance contrast. Biological sample preparation involves the fixation of cells and their embedment in resin before cutting them into ultra-thin sections [128]. While TEM is invaluable for high-resolution images of microbial and molecular structures, the required pre-TEM preparation and exposure to the electron beam may damage the sample and result in a non-representative image.
3.3.2 Scanning electron microscopy (SEM)

The initial principle of SEM is the same as the previously described TEM. A focused beam of high-energy electrons is directed at the sample in a vacuum chamber. The narrow electron beam enables a large depth of field, resulting in the production of 3-D images [129]. The electron-sample interactions provide information on the sample. Scatter patterns of secondary electrons (i.e., electrons ejected from the sample after initial exposure to incident electrons) and backscattered incident electrons give topographical information, as well as sample composition (fig. 3.1). Characteristic X-rays and sample current absorption can provide additional information on the sample such as crystal structure and orientation. Although the resolution is not as powerful as in TEM, resolution to 5 - 10 nm is possible as well as a magnification of $\sim 30,000 \times$. Biological samples are fixed, dehydrated and sputter coated with a gold-palladium alloy. The higher atomic number of the surface coating allows for a higher emission of secondary electrons, enhancing the signal to noise ratio [141].
3.4 Raman spectroscopy

Raman spectroscopy is a non-destructive, no-contact spectral analysis technique for identifying molecular and crystal structures. The main principle of Raman exploits the characteristic inelastic scattering of light by the sample, which is then collected using a CCD camera. A monochromatic laser light source is used to irradiate the sample, which scatters the light. A small fraction of the scattered light results in Raman scattering (inelastic) and has a different energy from the laser. When irradiated, the electron cloud of the sample’s molecules absorbs the incident energy and become excited, after which they re-emit the energy and return to their ground state. This process results in elastic Rayleigh scattering, which is the predominant scattering effect of light and involves no energy change. However, a small percentage of photons undergo inelastic scattering, whereby a change in energy occurs. This can either be in the form of Stokes Raman scattering or anti-Stokes Raman scattering. The former involves the excitement of an electron that then falls to a vibrational level and the partial absorption of energy by the molecule, resulting in the scattered light having lower energy. The latter involves the opposite reaction where an electron in a vibrational level absorbs light and falls back to the ground level, producing higher energy scattered light. Raman scattering occurs due to virtual electronic-vibrational transitions. Such virtual states are short-lived, unobservable quantum states and shouldn’t be confused with fluorescence which involves real electronic transitions (fig. 3.2). For Raman spectroscopy, Rayleigh scattering is filtered out and only Stokes Raman scattering is measured. Due to differences in Stokes and anti-Stokes Raman scattering at various temperatures, the ratio can be used to verify the temperature of a sample. The “shift” in energy of the Raman-scattered light is known as the “Raman shift” [cm$^{-1}$] and is used to identify characteristics of the sample as it is dependent on atom mass, bond strength and proximity to other atoms.
Figure 3.2 Virtual electronic transitions of Rayleigh (a), Stokes (b) and anti-Stokes Raman light scattering (c).
Chapter 4

Protective and destructive photochemical properties of iron oxide to life

4.1 Introduction

As described in the background chapter 2, the surface of the early Earth was most likely subjected to a higher flux of ultraviolet (UV) radiation than today. UV radiation is known to severely damage DNA and other key molecules of life. Some extremophiles have evolved strategies to cope with high radiation environments, including adopting endolithic lifestyles. On other planets such as Mars, which lacks a significant ozone shield, the surface also experiences high levels of UV radiation. Although it is not known whether Mars ever hosted life, understanding the biological effects of UV radiation are important for assessing its surface habitability and the chances of survival of contaminant microorganisms on spacecraft (i.e. planetary protection) [144]. Radiation affects the environment, not only directly, but also indirectly through photo-induced chemical reactions and their secondary products such as reactive oxygen species. However, many aspects of how high radiation fluxes interact with the surrounding geochemical environment, and how this could affect its habitability, are still obscure.

This chapter explores the photochemical activity of iron oxide, an abundant component of the Earth and Mars, and well-characterised photo-catalyst. It
is frequently used for removing compounds from wastewater on Earth by aiding in the production of reactive oxygen species in the Fenton reaction. Conversely, iron is also known to reflect and absorb light, and therefore may be capable of protecting organics from shortwave radiation. The aim of the experiments described in this chapter is to determine whether iron oxides could sufficiently protect cells exposed to UVC, and at what point the iron’s protective ability is eclipsed by its destructive trait as a component of the Photo-Fenton reaction. Using a liquid culture and a rock analogue system, the interplay of protective and deleterious effects of iron oxides was investigated, as part of the heterogeneous Photo-Fenton reaction, on the viability of the model organism *Bacillus subtilis*. These results shed light on the surface habitability of anoxic worlds and how a high UV flux leaves life precariously poised between the beneficial and deleterious effects of iron oxides. These results are not only relevant for understanding the habitability of the early Earth, but also that of the Martian surface. Both environments are characterised by high levels of UV radiation and contain iron oxides and hydrogen peroxide - all components of the Fenton reaction. The work presented in this chapter was published in FEMS Microbiology Ecology [1].

4.2 Background

4.2.1 Fenton reaction

The Photo-Fenton reaction is a photochemical reaction known to produce harmful photoproducts caused by UV radiation interactions with the geochemical environment. The general Fenton reaction consists of the oxidation of a catalytic ferrous iron ion (Fe$^{2+}$) to ferric iron (Fe$^{3+}$) in the presence of hydrogen peroxide (H$_2$O$_2$), which produces reactive oxygen species (ROS). These radical products can react to reduce further Fe$^{3+}$ (eq. 4.1, eq. 4.2, eq. 4.3) [62].

\begin{align*}
\text{Fe}^{2+}_{(aq)} + \text{H}_2\text{O}_2 &\rightarrow \text{Fe}^{3+}_{(aq)} + \bullet\text{OH} + \text{OH}^- \quad (4.1) \\
\text{Fe}^{3+}_{(aq)} + \text{H}_2\text{O}_2 &\rightarrow \text{Fe}^{2+}_{(aq)} + \bullet\text{OOH} + \text{H}^+ \quad (4.2) \\
\text{Fe}^{3+}_{(aq)} + \bullet\text{OOH} &\rightarrow \text{Fe}^{2+}_{(aq)} + \text{O}_2 + \text{H}^+ \quad (4.3)
\end{align*}
An extension thereof is the Photo-Fenton reaction. This enhanced version of the reaction is more efficient, as it utilises UV light to accelerate the iron catalyst’s recycling back to ferrous iron, without additional hydrogen peroxide. In addition, it also produces more radicals by inducing the photolysis of hydrogen peroxide at wavelengths shorter than 300 nm (eq.4.4, eq. 4.5) [145, 146].

\[
\begin{align*}
\text{Fe}^{3+}(aq) + H_2O & \xrightarrow{h\nu} \text{Fe}^{2+}(aq) + \cdot OH + H^+ \quad (4.4) \\
H_2O_2 & \xrightarrow{h\nu} 2 \cdot OH \quad (4.5)
\end{align*}
\]

Standard Fenton and Photo-Fenton reactions utilise homogenous Fenton reagents i.e. dissolved iron ions. In recent years, scientists have also studied solid iron oxides as catalysts (heterogeneous Fenton reagents) [147]. These reagents have various advantages over dissolved iron such as more economical catalyst recovery and effectiveness over a broad pH range, while still functioning at a high efficiency.

### 4.2.2 Reactive oxygen species (ROS)

Reactive oxygen species can be detrimental to all forms of life as they ionise molecules and cleave membranes, lipids, saccharides and importantly, DNA. Both prokaryotes and eukaryotes have evolved pathways that can repair damage and/or mop up the free radicals and transform them into a less destructive chemical species [40]. ROS are also produced intracellularly by auto-oxidation events: an intracellular ROS source involving the Fenton reaction “the Haber-Weiss reaction” is a cornerstone of free radical biochemistry [148]. However, if the ROS influx is too great, the cellular repair mechanisms cannot keep up with the damage and the cell or organism dies.

### 4.2.3 Protective and destructive properties of iron

It has previously been shown [149] that biogenic iron(III) minerals from iron-oxidising microbes can act as a shield against radiation. Iron is known to absorb in the UV spectrum and could thereby not only act as shield for cells but also reduce the incident UV environment [150]. However, the reactivity of iron as a Fenton reagent, resulting in the production of free radicals, raises a fundamental question about the trade-off between these beneficial effects and potentially deleterious
effects caused by photochemical reactions. Understanding the interplay between shielding and radical production gives insight into conditions experienced by microbes on the early Earth, as well as how this could affect life in similar conditions in non-terrestrial habitats. Exploring the impact of these inherent properties of iron oxides on bacterial viability is the focus of the following experiments presented in this chapter.

4.3 Methods

4.3.1 Model organism selection

*Bacillus subtilis* was chosen as model organism to test the effects of the Photo-Fenton reaction. It is a Gram-positive bacteria capable of forming resistant spores, and is most often found in the soil or gastro-intestinal tract. It is one of the best studied Gram positive bacteria species, easy to consistently grow in the lab and has a short doubling time of \( \sim 20 \) minutes.

4.3.2 Bacterial preparation

Vegetative cells of *Bacillus subtilis* strain 168 (DM 402) were obtained from spores provided by the radiobiology group at the Deutsches Zentrum für Luft- und Raumfahrt (DLR), Cologne. To obtain aliquots for experiments, a monoculture was grown at 38°C before being frozen into 25% glycerol 1 mL aliquots and stored at \(-80^\circ\)C. To obtain overnight cultures for experiments, 200 mL of nutrient broth was inoculated with 200 \( \mu \)L of thawed aliquot and cultured overnight at 38°C before use in the experiments. The concentration of organisms in each culture was determined by serial dilution and plating on Oxoid (LP0011) nutrient agar. In preparation for the radiation exposure experiments, 1 mL of the culture was centrifuged twice at 10,000 \( \times \) g/min for five minutes and washed in between with Phosphate Buffered Saline (PBS). The pellet was resuspended in 1 mL Minimal Media (M9) and transferred into a 12-well plate. In this chapter, this is referred to as the “liquid system”. M9 media was used in place of an undefined medium containing multiple organics to rule out any secondary products being produced by irradiated organic molecules, which could additionally affect the cell viability. One litre of M9 contained the following components: 10.5 g M9 salts (Sigma
Aldrich) in 988 mL of distilled water; 2 mL of 1M MgSO$_4$; 10 mL of 20% glucose; 100 µL of 1M CaCl$_2$. The bacterial concentration was kept below a maximum of 8 × 10$^7$ cells/mL, so that the theoretical surface area of the bacteria would not exceed the surface area of the bottom of the well, even if they settled there, causing self-shielding from multiple cell layers. As B. subtilis is a motile bacterium and could move away from the iron on the well floor, multiple samples were taken with a pipette from the top half and the bottom half of the M9-bacteria suspension in the well after preparation. Cell concentration of the top and bottom samples was estimated using microscopy. Results indicated that samples taken from the bottom of the well contained 75% more bacteria than samples taken from the top of the well. It was therefore assumed that most of the bacteria were located on or very near to the well floor for the duration of the experiments and were most likely in close contact with the iron oxides. After irradiation, the samples were always taken from the well floor for plating.

4.3.3 UV irradiation experiments in liquid system

Hematite (product nr. 310050) and magnetite (product nr. 310069) with a grain diameter of 5 µm and goethite (product nr. 371254) with a grain diameter of < 300 µm (Sigma Aldrich) were added in appropriate concentrations to the bacterial samples in M9. Hematite was predominantly used as a representative iron oxide in most of the experiments, as it is abundant on both Mars and the Earth. Controls were also run containing only bacteria in M9. One microlitre of hydrogen peroxide (30%) was added to the 1 mL aliquots of M9 per well, resulting in a final concentration of 10 mM. This concentration of hydrogen peroxide was used, as a decrease below 1 mM leads to inefficient catalyst function, and an increase to 100 mM causes sterilisation in the dark controls (fig. 4.1).

The concentration of 1 g/L, i.e. 1 mg per well containing 1 ml of M9, of iron oxide catalyst (when used) was chosen, as UV light could still penetrate it and reach the cells; iron concentrations on Mars would vary substantially depending on location, so this concentration was chosen as an example. Additionally, this concentration allows for resulting data to be compared to that of Mammeri et al. [147], as they tested a similar iron oxide catalyst and hydrogen peroxide concentration in their heterogeneous Photo-Fenton reactions. The 12-well plate was placed on a shaker for one minute to mix the components as homogeneously as possible. For the experiments at 4°C the plate was placed on ice for 30 minutes.
Figure 4.1 Effects of lower and higher hydrogen peroxide concentrations on cell viability under UV and dark conditions: 1 mM and 100 mM concentrations of H$_2$O$_2$ under light (UVC) and dark conditions in the presence of 1 g/L hematite in M9 media containing B. subtilis, sampled at given time points. H = 1 g/L hematite; HP = H$_2$O$_2$ at given concentration. UV = 254 nm; dark = covered. No statistically significant difference ($p > 0.05$) was found between or amongst samples; error bars are + s.e. ($n = 3$).

before and during irradiation. M9 media was stored at 4°C prior to use.

UV irradiation of experiments was carried out using two sources. In all experiments, except when stated otherwise, wells were irradiated with a monochromatic UVC lamp ($\lambda = 254$ nm; $I = 11.2$ W/m$^2$) at distance of 5 cm for the indicated length of time. This was performed aerobically and at 25°C unless otherwise indicated. The effect of the monochromatic UVC was made comparable to Martian radiation by producing an absolute fluence, which is similar to the fluence expected from the UVC to UVB regions (200 - 315 nm) on Mars [144], albeit that biological effectiveness varies across the UV radiation spectrum. Nevertheless, as the biological effectiveness of processes, such as DNA damage, is similar across the short-wavelength region, this monochromatic flux can be considered a reasonable proxy for short-wavelength UV radiation damage. To produce a more realistic UV radiation environment, a polychromatic light was also used for irradiation when stated, generated by a 150 W Xe-Arc UV lamp ($\lambda = 200 - 2500$ nm; $I = 0.010$ W/m$^2$/nm). The spectral irradiance on the surface of Mars was calculated
using the model from Cockell et al. [144] and compared to the measured Xe lamp irradiance in the Mars chamber (fig. 4.2).

**Figure 4.2** Modelled Mars UV irradiance vs. measured polychromatic lamp irradiance 200 - 400 nm. Mars UV irradiance was calculated using the model from Cockell et al. [144] with following parameters: OD = 1 (τ = 1.0); Sunlight angle 45°.

### 4.3.4 Rock analogues

In order to recreate a more realistic environment for microorganisms on the surface of a planet, rock analogues were used. These consisted of sintered discs of silica grains, commercially produced to give a pore size of 100 - 160 μm (largest pore size available at Scientific Glass, UK); they were 10 mm in diameter and 3 mm thick. The discs were soaked in the M9 medium and bacterial suspension, with the 1 mM hydrogen peroxide already dissolved in the M9 medium for up to a minute before irradiation. After irradiation, the discs were gently crushed with a sterilised mortar and pestle in added sterile water to extract the cells.
4.3.5 Anaerobic experiments

To test whether the observed effects also occur under anaerobic conditions some experiments were carried out in a Coy anaerobic chamber in a nitrogen atmosphere. For the anaerobic experiments, 10 mL of M9 media was placed in a sealed, sterile serum bottle and degassed. This was achieved by purging the bottle with sterile, 0.22 µm-filtered N$_2$, for 20 min/L using sterile needles to introduce the gas and relieve the pressure. After purging, the bottle was filled with 100% N$_2$ headspace to maintain anaerobic conditions. The bacteria were then resuspended in the purged M9 in the anaerobic chamber and transferred into 12-well plates, in which the UV irradiation experiments were carried out, as described in “UV irradiation experiments in liquid system”.

4.3.6 Analysis

Ten microlitre samples were taken from the wells or discs at the given time points and plated on nutrient agar at a dilution of 1:100 and incubated overnight at 30°C. Colonies were then counted and viability was expressed in terms of percentage of the starting cell concentration (N/N$_0$ × 100%) where “N$_0$” = starting concentration and “N” = surviving cells at the point of sampling. “Viability” is defined as any number of cell colonies greater than zero on the plate. Consequently, “sterility” is defined as zero cells. Results are shown on a log scale in all figures. Therefore, cases that are strictly zero are not represented on the log plot but should be interpreted as strictly zero. All experiments were performed in triplicate; numbers in figures show averages, error bars show standard error (s.e.) between triplicates. Non-irradiated, dark controls were run in all cases. Statistical analysis was performed using one-way ANOVA and two-tailed unpaired equal variance Student’s t-tests, where p < 0.05 was considered significant.

4.4 Results

4.4.1 Protective properties of iron oxide

In the first experiment, the protective properties of iron oxides were established. A selection of iron oxides consisting of hematite, magnetite and goethite were
added to the bacteria in M9 and irradiated with UVC radiation (fig. 4.3). At a low concentration (1 g/L), the finer grained (hematite and magnetite) iron oxides provided protection, shielding the cells for at least 120 minutes, whilst the larger-grained goethite only provided sufficient protection for 30 minutes before viability was lost completely at 120 minutes. When the concentration of the iron oxides was increased, so that they covered the well floor to a thickness of 1 mm, hematite provided the cells with sufficient protection so that, even after 120 minutes UV exposure, there was no significant change in the cell count from the first time point (p > 0.05). Similar to hematite, increased concentrations of magnetite provided protection, causing the cell count to decrease only after 120 minutes of exposure. Although cells’ viability in the presence of a higher concentration of the coarse-grained goethite dropped even after 10 minutes of UV exposure, viability remained unchanged between 30 and 120 minutes. In the controls exposed to UV radiation without any iron oxides, viability was completely lost by the first time point of 10 minutes.

![Figure 4.3](image)

**Figure 4.3** The protective effects of iron oxides. Cell-protecting properties of different types of iron oxides exposed to UVC (λ = 254 nm) in M9 media containing *B. subtilis*: 1 g/L (hematite, magnetite, goethite and ground goethite); higher concentration of iron oxides resulting in a 1 mm layer (hematite, magnetite and goethite). UV = UVC control, cells exposed to UVC in only M9. Y-axis is log scale. Letters shared in common between or among the groups indicate no significant difference (p > 0.05); error bars are + s.e. (n = 3).
4.4.2 Destructive properties of iron oxide

The introduction of hydrogen peroxide to the system dramatically changed the interactions. All the reagents necessary for the heterogeneous Photo-Fenton reaction were present: iron oxide, hydrogen peroxide and UV radiation. Hematite was added in concentrations ranging from 0.5-10 g/L. For the first 25 seconds (fig. 4.4 a) viability under the various experimental conditions was generally reduced. However, at the 60-second time point (fig. 4.4 b), when viability across all treatments was reduced to below 1%, significant differences between treatments were observed. There was a significant ($p < 0.05$) 2.7-fold reduction in cell viability at 1 g/L + 10 mM H$_2$O$_2$ compared to both the 0.5 g/L + 10 mM H$_2$O$_2$ and 5 g/L + 10 mM H$_2$O$_2$ treatments (either side of 1 g/L in fig. 4.4 a). Additionally, a significant 6.2-fold decrease in viability was observed when 1 g/L hematite samples were compared to cells irradiated just in the presence of 10 mM H$_2$O$_2$. The reduction in viability observed at 1 g/L hematite shows that viability was more negatively affected in the presence of the mineral compared to cells exposed only to UV radiation or UV radiation and hydrogen peroxide, despite the protective properties of 1 g/L hematite in fig. 4.3. Dark controls with hydrogen peroxide and dark controls with hydrogen peroxide and hematite showed no reduction of cell viability when compared to the dark controls with no additions for the duration of the experiment.

To determine if this result was replicable in a more realistic environment, the same experiment was carried out using the rock analogues. As shown in fig. 4.5, the overall cell count was higher across all conditions compared to the liquid system and the reduction in viability at a range of hematite concentrations under UV irradiation with hydrogen peroxide was replicated as observed in fig. 4.4 (a) and (b). The only difference was the more efficient bacteriocidal effect of hydrogen peroxide, which sterilised the cells after 60 seconds of irradiation when compared to the liquid system, in which viability was retained (0.14 %) after 60 seconds.
(a) Iron oxides as catalysts in the heterogeneous Photo-Fenton reaction

(b) Detail at 60 s exposure comparing hematite data with and without hydrogen peroxide

**Figure 4.4** Iron oxides as catalysts in the heterogeneous Photo-Fenton reaction. Different concentrations of hematite exposed to UVC in the presence of 10 mM H$_2$O$_2$ in M9 media containing *B. subtilis*. 0.5 H + HP = 0.5 g/L hematite + H$_2$O$_2$; 1 H + HP = 1 g/L hematite + H$_2$O$_2$; 5 H + HP = 5 g/L hematite + H$_2$O$_2$; 10 H + HP = 10 g/L hematite + H$_2$O$_2$; UV = UVC control, cells exposed to UVC in only M9; HP = H$_2$O$_2$ control, cells exposed to UVC in 10 mM H$_2$O$_2$. Y-axis is log scale. Letters shared in common between or among the groups indicate no significant difference (p > 0.05); error bars are ± s.e. (n = 3).
Figure 4.5  Heterogeneous Photo-Fenton reactions in rock analogue discs. Hematite exposed to UVC for 60 s in the presence of 10 mM H$_2$O$_2$ in M9 media-soaked discs containing *B. subtilis*. 0.5 H + HP = 0.5 g/L hematite + H$_2$O$_2$; 1 H + HP = 1 g/L hematite + H$_2$O$_2$; 5 H + HP = 5 g/L hematite + H$_2$O$_2$; UV = UVC control, cells exposed to UVC in only M9-soaked-discs; HP = H$_2$O$_2$ control, cells exposed to UVC in 10 mM H$_2$O$_2$. Y-axis is log scale. Letters shared in common between or among the groups indicate no significant difference (p > 0.05); error bars are ± s.e. (n = 3).
(a) Photo-Fenton reaction in the liquid system containing *B. subtilis* under anaerobic conditions, detail at exposure time of 60 s

(b) Photo-Fenton reaction in the solid system containing *B. subtilis* under anaerobic conditions, detail at exposure time of 60 s

**Figure 4.6** Hematite exposed to UVC in the presence of 10 mM H$_2$O$_2$ in M9. 0.5 H + HP = 0.5 g/L hematite + H$_2$O$_2$; 1 H + HP = 1 g/L hematite + H$_2$O$_2$; 5 H + HP = 5 g/L hematite + H$_2$O$_2$; UV = UVC control, cells exposed to UVC in only M9 (A) or M9-soaked-discs (B); HP = H$_2$O$_2$ control, cells exposed to UVC in 10 mM H$_2$O$_2$. Y-axis is log scale. Letters shared in common between or among the groups indicate no significant difference (p > 0.05); error bars are ± s.e. (n = 3).
The same experiment was carried out in the liquid and rock analogue systems under anaerobic conditions, to establish whether the Photo-Fenton reaction would take place to the same extent in the absence of atmospheric oxygen, as would be the case on the early Earth and present-day Mars. In fig. 4.6 (a) and (b) the reduction in viability at an iron oxide concentration of 1 g/L was again observed. The rock analogue system sample (fig. 4.6(b)) containing only hydrogen peroxide showed a greater loss of viability when compared to the same condition in the liquid system (fig. 4.6(a)). Overall however, the rock analogue system showed a higher number of viable cells than in the liquid system, a trend that was also observed in the aerobic experiments.

**Figure 4.7** Photo-Fenton reaction at 4°C: Different concentrations of hematite exposed to UVC in the presence of 10 mM H₂O₂ in M9 media containing *B. subtilis*. Samples chilled on ice before and during exposure. 0.5 H + HP = 0.5 g/L hematite + H₂O₂; 1 H + HP = 1 g/L hematite + H₂O₂; 5 H + HP = 5 g/L hematite + H₂O₂; UV = UVC control, cells exposed to UVC in only M9; HP = H₂O₂ control, cells exposed to UVC in 10 mM H₂O₂. Y-axis is log scale. Letters shared in common between or among the groups indicate no significant difference (p > 0.05); error bars are + s.e. (n = 3).
Figure 4.8  Photo-Fenton reactions after polychromatic light exposure. Detail at exposure to polychromatic light source at the time point of 10 s. Hematite exposed to UVC in the presence of 10 mM H₂O₂ in M9 media containing B. subtilis. 0.5 H + HP = 0.5 g/L hematite + H₂O₂; 1 H + HP = 1 g/L hematite + H₂O₂; 5 H + HP = 5 g/L hematite + H₂O₂; 5 H + HP (monochromatic) = 5 g/L hematite + H₂O₂ under monochromatic exposure, taken from graph 4a at 10 s; UV = UVC control, cells exposed to UVC in only M9; HP = H₂O₂ control, cells exposed to UVC in 10 mM H₂O₂. Y-axis is log scale. Letters shared in common between or among the groups indicate no significant difference (p > 0.05); error bars are + s.e. (n = 3).

All of the aforementioned experiments were performed at 25°C; in the following experiments, samples were chilled on ice to 4°C prior to and during irradiation. This was to determine whether Photo-Fenton reaction rates and photoprodut diffusion still showed the same effects observed at higher temperatures (fig. 4.3- 4.6). Figure 4.7 shows that the reduction in viability was still observable at 1 g/L iron oxide after 60 seconds irradiation, with a two-fold reduction of cell viability in comparison to the UV controls. Although the hydrogen peroxide had a more bacteriocidal effect than the Photo-Fenton reaction, the overall cell viability, including that of the UV-irradiated control, was higher than when irradiated at 25°C.

Experiments recreating environments that more realistically simulate the UV radiation environments of early Earth and Mars, such as the anaerobic rock analogue systems, were all performed under a monochromatic UVC source at 254 nm. A polychromatic source was used to simulate a more realistic exposure
to multiple wavelengths; the measured irradiance vs. Martian UV irradiance is shown in fig. 4.2. Initially, the first time point was measured at 30 seconds. However, the cultures had already been completely sterilised after this exposure time. Figure 4.8 shows data after an adjusted time point of 10 seconds of exposure. The dip in viability was again observed at 1 g/L, and the hydrogen peroxide-treated samples showed the lowest cell count. Even after 10 seconds, the UV-irradiated controls were killed to just above 1% of the original cell count. Even the shielding from the hematite at 5 g/L could not protect the cells as effectively as demonstrated under monochromatic light.

4.5 Discussion

Ultraviolet radiation is known to be severely damaging to all forms of life, as its high energy damages key molecules such as lipids, DNA and proteins [151]. Furthermore, radiation can produce secondary products such as reactive oxygen species that are detrimental to organisms [40, 152]. Iron oxides are known to absorb in the ultraviolet (UV) spectrum [150] and could therefore be considered a shield for microorganisms. Iron is the second most abundant metal and fourth most abundant element in the Earth’s crust, and is often found in the form of iron oxides. It is a ubiquitous substrate and is the component of banded iron formations, which formed primarily in the late Archean (2.7 - 2.5 Ga) [153]. Mars also has high levels of iron: it has twice the amount of iron oxides in its outer layers as the Earth, giving its surface the distinct rust colour [82]. Despite the protective properties of iron oxides, this work demonstrates that iron oxides can also negatively contribute to the habitability of an environment. This detrimental effect is the result of a subtle interaction between a biota, UV radiation and the geochemical environment and depends on the concentrations of the compounds involved. Experimental parameters were chosen to be analogous to the Archean Earth and the Martian surface i.e. the presence of iron oxides, anoxic conditions and a high UVC flux (monochromatic and polychromatic). To test for the effects on the Martian surface, low temperatures were also studied.

The first experiment shows that iron oxides can efficiently act as a physical shield for bacteria under damaging UVC radiation, with smaller (5 µm, hematite and magnetite) iron oxide grain sizes providing a more homogenous and effective coverage than larger (< 300 µm, goethite) grains at low concentrations. Once the concentrations increase to produce a 1 mm thick layer on the well floor, all
oxides provided substantial protection even after over an hour of exposure to UV radiation. The model organism used in this work is not photosynthetic and is additionally a facultative anaerobe \[154\] and would therefore probably not be limited by the thickness of radiation shielding provided by regolith. However, photosynthetic bacteria on the Archean Earth and possibly on present-day Mars would face a trade off between being protected from radiation, while receiving enough light to be photosynthetically active. Olsen and Pierson \[155\] show that iron compounds are able to screen radiation without blocking so much visible light as to prevent photosynthesis, making iron oxides a beneficial screen to such organisms. Furthermore, Gauger \textit{et al.} \[149\] show that phototrophic iron(II)-oxidising bacteria produce their own biogenic iron(III) minerals that screen from UVC whilst allowing enough photosynthetically viable wavelengths to penetrate.

In spite of this protection, the results demonstrates that, once iron oxides are in the presence of a small concentration of hydrogen peroxide, the interactions with biota are considerably more complex due to iron’s role in ROS production. The experiments show that, under various conditions and liquid/rock analogue systems, there is a consistent drop in cell viability at an iron oxide concentration of 1 g/L. These data are very different from the prediction that would result from a simple relationship between higher iron oxide abundance and greater biological protection. Instead, it corresponds to the predicted optimal iron oxide and hydrogen peroxide concentration (1 g/L and 10 mM, respectively) required for the heterogeneous Photo-Fenton reaction to take place \[147\]. Once this concentration has been reached, a subsequent increase in iron oxide concentration once more results in the protective effects of iron becoming more prominent.

The data from the rock analogue discs, designed to simulate an endolithic habitat, show an overall increase in cell viability. This may be because the cells are initially being protected by the disc structure. Additionally, this may be caused by a protective distance between most of the cells and the layer of iron oxide on the top of the disc, which is not the case in the liquid system. This separation between iron oxides and microorganisms has been observed by Edwards \textit{et al.} \[156\] in natural cryptoendolithic communities. Hypothetically, this observation in cryptoendolithic communities may demonstrate the optimal distance that photosynthetic microorganisms adopt as a trade-off between protection that iron oxide offers while minimising its detrimental effects. The organisms position themselves in the rock to allow for adequate light exposure for photosynthetic activity and to benefit from the UV protection that the iron oxides provide,
while remaining far enough away from any ROS production catalysed by the UV-irradiated iron. These experiments are relevant to the early Earth, when UV fluxes were likely higher, and under such fluxes oxidative products of water dissociation may have been present, such as hydrogen peroxide. The results are also relevant to the surface of Mars today. Hydrogen peroxide has long been suspected as an oxidant on Mars based on photochemical models [157, 158]. These models predict the production of hydrogen peroxide on the Martian surface through the interaction of atmospheric hydrogen peroxide and Mars’ large electric fields causing it to precipitate out [159, 160]. Its existence was suggested from surface data such as the Viking experiments [161]. Its presence was only confirmed in 2004 by two independent teams [84, 85].

The experiments conducted in anaerobic and low temperature conditions show that the Photo-Fenton reaction is unaffected by the lack of atmospheric oxygen, as is the case for Mars and the early Earth, and still takes place at temperatures close to 0°C. The comparison of the higher viability of the UV-irradiated controls in the low temperature experiment and cell viability in the experiments at a higher temperature suggests that UV radiation is not solely responsible for directly killing the cells. Rather, that secondary products, such as hydroxyl radicals created by the radiolysis of water, play a part. At lower temperatures, diffusion and chemical reactions would be expected to occur at a slower rate, as a result of the Arrhenius relationship. This rate reduction would explain the reduced loss of viability in samples when exposed to the same compound doses used in higher temperature experiments. Although the average surface temperature on Mars is approximately 218 K (−55°C), the Mars rover Opportunity measured a daily maximum of 295 K (21.85°C) [162]. Therefore, a range of reaction rates varying with latitude, season and time of day would be expected.

The polychromatic radiation exposure experiments show that, after exposure to a broader and more environmentally realistic wavelength range, the interactive effects of the Photo-Fenton reactions and protective screening are still observable. The polychromatic spectrum mimics the solar spectral range and is filtered to 200 nm. This is representative of the UV radiation cut-off point on Mars and the expected wavelength range on early Earth, both due to CO₂ in the atmosphere. Although the Martian total integrated UV flux is comparable to Earth’s (200 - 400 nm), its lack of ozone over most latitudes allows the shorter wavelengths (200 - 315 nm) to reach the surface, which would proportionally contribute more to biological damage [51]. As the early Earth is also thought to have lacked an ozone
layer, and thus may have similarly been subjected to higher UV fluxes of shorter wavelengths, it makes present-day Mars a potential analogue for the radiation environment of early Earth. Cockell [15] suggested that the biologically effective irradiance experienced by DNA on the equator of Mars would be similar to that experienced by DNA on equatorial regions on the Archean Earth.

Spores of *B. subtilis* are known as common clean room and spacecraft contaminants, making it of interest with regard to planetary protection. The responses of its vegetative cells, after exposure to Photo-Fenton products, provide insight into how such an organism might survive on the Martian surface, if it is transferred there as a contaminant and able to germinate [163, 164]. Spores of *B. subtilis* can be 5 - 50 times more resistant to 254 nm UV exposure than in its vegetative cells [133, 134]. This is attributed to the unique DNA photochemistry under UV exposure resulting in the production of a spore photoproduct (SP), which has been shown to prevent the formation of thymine dimers in irradiated DNA [165]. In addition to SP production, the spores possess two distinct repair mechanisms for nucleotide excision repair (NER) and SP-thymine monomerisation [166, 167]. The results show that for contaminant organisms, such as *Bacillus subtilis* transferred to Mars on spacecraft, the iron-rich surface dust could provide a certain amount of protection, consistent with other findings [168, 169]. However, under thin layers of dust, or when exposed in atmospheric circulation, the combination of iron oxides and hydrogen peroxide would allow for Photo-Fenton chemistry to destroy organisms much more quickly than UV radiation or oxidants alone. While the DNA photochemistry of *B. subtilis* spores confers enhanced protection from UV damage, it would be interesting to test whether such mechanisms may lend them enhanced protection from other harmful environments such as exposure to ROS. If so, it would have serious implications for planetary protection guidelines. It should generally be noted that, although the experiments show sensitivity to the Photo-Fenton reaction in the model organism, a more extensive study of various organisms, especially extremophiles, would be necessary to establish whether the reaction has a more wide-range implication for Martian habitability and planetary protection requirements.

Finally, these results also have implications for the human exploration of Mars. The homogenous and heterogeneous Photo-Fenton reactions are already used efficiently to treat waste water on Earth [63, 65]. With the naturally high UV-flux and abundance of iron oxides, the heterogeneous Photo-Fenton reaction should be considered as a method of sterilising water for future expeditions and
settlements on Mars using the environmental UV flux. This could be achieved by circulating waste water through quartz tubes, which allow short wavelengths of light to penetrate, unlike standard window glass and many plastics on the outside of habitats. Another beneficial property of iron is its ability to treat water to remove toxic perchlorate \cite{170,171}, which is also present in the Martian environment.

### 4.5.1 Limitations

#### Chemical limitations

Despite the effectiveness of the Photo-Fenton reaction at producing ROS, there are limitations to its chemistry as presented in the background chapter (fig. 2.4.1), which are also applicable here. One such limitation of the homogenous reaction is the inhibition of the reaction in the presence of the chloride ion (Cl$^-$), which is abundant on Earth and has also been identified on Mars \cite{172}. It was hypothesised \cite{75,76} that the less reactive Cl$_2$$^•$ is preferably formed over the hydroxyl radical, preventing the recycling of Fe$^{3+}$ back to Fe$^{2+}$. This assumption was confirmed by Machulek et al. \cite{77}, although this was only confirmed for the homogenous Photo-Fenton reaction. Additional ions that may disrupt the reaction are sulphate (SO$_4^{2-}$) and phosphate (PO$_4^{3-}$) \cite{78}, which react with Fe$^{3+}$ to form photo-inert iron sulphates or phosphates. Despite the lack of studies on heterogeneous Photo-Fenton chloride, sulphate or phosphate inhibition, they remain possible factors for inhibition, especially if there is any amount of dissolved iron present in the environment.

Another chemical component, which could limit the reaction, is the H$_2$O$_2$ concentration. Mammeri et al. \cite{147} reported an increase in H$_2$O$_2$ concentration resulted in a decrease in the heterogenous Photo-Fenton reaction’s efficiency at degrading organics. This is due to the ability of excess H$_2$O$_2$ to act as a •OH scavenger, leaving the less reactive HOO• species \cite{71} (eq. 4.6, eq. 4.7).

\[
\begin{align*}
H_2O_2 + •OH &\rightarrow HOO• + H_2O \quad (4.6) \\
•OH + HOO• &\rightarrow O_2 + H_2O \quad (4.7)
\end{align*}
\]
Just as the overabundance of \( \text{H}_2\text{O}_2 \) may disrupt reaction efficiency, the lack thereof also poses a problem, which may be the case on Mars. \( \text{H}_2\text{O}_2 \) detection on Mars has proven to be difficult and has so far only been successfully confirmed in the atmosphere. This doesn’t necessarily imply the reaction wouldn’t take place on Mars, but that it may not be a planet-wide phenomenon, except in places where \( \text{H}_2\text{O}_2 \) has condensed out of the atmosphere or where there is \textit{in situ} production.

**Physical limitations**

Oxidation state, surface area, and temperature are some of the factors that can influence the catalytic ability of iron. Counterintuitively, it was shown [173] that an increased surface area (in this case of hematite) did not result in an increase in catalytic activity. Amorphous hematite with a larger surface was a less efficient catalyst than crystalline hematite with a smaller surface area. This indicates that catalyst morphology is more decisive than amount of reaction area and may vary in naturally occurring hematite. Magnetite and goethite, which were used in the initial protection experiment (fig. 4.3), have also been shown to be equally as effective Fenton catalysts [174]. Additionally, other transition metals, such as copper, can act as catalysts in Fenton-like reactions (eq. 4.8, eq. 4.8), albeit with their own individual limitations [175, 176].

\[
\begin{align*}
\text{Cu}^+ + \text{H}_2\text{O}_2 & \rightarrow \text{Cu}^{2+} + \cdot \text{OH} + \cdot \text{OH} \\
\text{Cu}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Cu}^+ + \text{HO}_2\cdot + \text{H}^+ 
\end{align*}
\]

(4.8) (4.9)

**4.5.2 Future work**

Further work is necessary to have a holistic understanding of the Fenton photochemistry and its implications for life and exploration on Mars. Work is ongoing to properly understand the precise mechanisms involved in the Fenton reaction, the outcome of which may change predictions of subsequent chemical interactions.

From an astrobiological standpoint, further experiments involving the exposure of \textit{B. subtilis} spores and other extremophiles to products of Photo-Fenton chemistry, would give insight into the extent of the reaction’s sterilisation properties.
Additionally, research into how much of an inhibitory impact local concentrations of Cl\(^{−}\), SO\(_4^{2−}\) and PO\(_4^{3−}\) would impart on Martian Photo-Fenton chemistry would also be required to make accurate predictions with regard to the reaction’s bacteriocidal effectiveness. Experiments using various types of iron oxides such as magnetite or goethite and differing crystal structures may also lead to further insight into how the reaction’s bacteriocidal effect could be enhanced and used on Earth in waste water treatment. Research into these topics would in turn be valuable information that could feed into planetary protection protocols, and may enable Fenton-photochemistry to be used as a sterilisation mechanism for future missions to Mars.

4.6 Conclusions

Iron oxides absorb strongly in the biologically damaging UV radiation spectrum and can provide physical shielding to microbial cells. However, they can also act as catalysts in the heterogeneous Photo-Fenton reaction, resulting in the generation of reactive oxygen species. This reaction can take place in aerobic and anaerobic conditions, at temperatures just above the freezing point of water, and in rock analogue environments. Microbes on anoxic planets such as the early Earth and Mars are therefore faced with this two-sided nature of iron oxides, and are precariously poised between the beneficial effects of iron oxides, and their damaging catalytic properties.
Chapter 5

Perchlorates on Mars enhance the bacteriocidal effects of UV light

5.1 Introduction

Perchlorates have been identified on the surface of Mars and in flowing water brine seeps on its surface [87]. This discovery has raised the question of how perchlorates contribute to the habitability of the Martian surface. This chapter presents evidence of perchlorates having bacteriocidal properties when irradiated with a UV flux simulating that expected on the Martian surface. The photolysis of perchlorate and its potential reactivity with organics was investigated by irradiating perchlorates at Martian concentrations under UV in the presence of the model organism, Bacillus subtilis and observing the effect on its cell viability. This chapter reports the significant bacteriocidal effects of UV-irradiated perchlorate on life at ambient temperatures and under Martian conditions. Additionally, the combined effects of perchlorate and other components of the Martian surface, including components of the previously discussed Photo-Fenton reaction (chapter 4), are investigated to give a more representative picture of the Martian chemical environment. Not only do these results contribute to the understanding of Martian surface photochemistry, but they also shed light on the conditions that extant life, such as biological contaminants from robotic and human exploration missions, would have to face in order to survive and reproduce on Mars. This in turn allows for a more precise design of life detection equipment, as well as more comprehensive planetary
5.2 Background

5.2.1 Perchlorate characteristics

Consisting of a negatively charged chloride, surrounded by a tetrahedral formation of oxygen atoms, perchlorates (ClO$_4^-$) represent the highest oxidation state of chlorine (+7) and are powerful oxidants when heated, but remain stable at room temperature and lower temperatures. In biology, the high oxidation state of perchlorates means that they can be used as an electron acceptor by microorganisms to provide energy for growth. Bacteria, especially proteobacteria known to reduce chlorate and perchlorate such as *Dechloromonas aromatica* [177–179], have even been used to experimentally remove perchlorate from wastewater to avoid drinking water contamination [180]. Although perchlorate sources are limited and mostly found in the Atacama desert [181], perchlorate-reducing bacteria are widely distributed in the environment on Earth [178]. Perchlorate has been shown to decay into reduced oxychlorine species such as chlorate (ClO$_3^-$), chlorite (ClO$_2^-$) and hypochlorite (ClO$^-)$ when irradiated with ionising or UV radiation [88–90]. Photochemistry is also thought to be involved in the production of naturally occurring perchlorate on Earth and on Mars. It has been speculated that atmospheric ozone is able to oxidise chlorine-containing species to form perchlorate [101].

5.2.2 Perchlorates on Mars

Perchlorates have been detected on Mars both *in situ* [94] and inferred in brine seeps [87], which raises questions on their effects on the habitability of the planet. The presence of oxidants in the Martian soil was first suspected during the Viking Lander missions [182 183]. The results of the missions suggested low levels of reactive oxidising substances, which were thought to explain why no evidence for organics was found [161 184]. The landers were equipped with molecular analysis experiments, which subjected Martin soil samples to thermal volatilisation-gas chromatography-mass spectrometry (TV-GC-MS), whereby the
samples are heated to fragment molecules for analysis. In addition to the lack of organics, the results showed the presence of chloromethane and dichloromethane. The detection of chloro-hydrocarbons was initially considered to be terrestrial contamination but, in retrospect, these results make sense given that oxychlorine species, such as perchlorate, were detected upon re-analysis [185]. In 2008, the NASA Phoenix Lander’s onboard Wet Chemistry Lab eventually discovered perchlorate anions, at a concentration of 0.4-0.6 wt%. This finding was recently supported by the Sample Analysis at Mars instrument (SAM) on the Curiosity rover [186]. In September 2015, the Mars Reconnaissance Orbiter spectroscopically detected hydrated salts of NaClO₄, Mg(ClO₄)₂ and Mg(ClO₃)₂ in locations thought to be brine seeps [87]. Despite the stability and lack of reactivity of perchlorate at ambient temperatures, once heated, it becomes a well-characterised and highly effective oxidising agent [187], used as solid rocket fuel [188].

The implications of the presence of perchlorate are significant, as their detection suggests the existence of other oxychlorine species, which may negatively impact the habitability of Mars and interfere with the preservation and detection of organic material [93]. Conversely, the presence of such salts lowers the freezing point of water [189, 190], thereby potentially allowing for a contemporary active hydrological system on Mars, which could enhance the habitability of the near-surface environment. This has prompted recent research into the use of perchlorates as a potential energy source for bacteria on Mars [191, 192]. Considering the high ionising radiation and UV flux on the surface of Mars, the research in this chapter asks whether perchlorate can become activated by Martian radiation conditions and thereby be detrimental to life or biosignatures, rendering its potential as an energy source redundant when irradiated.
5.3 Methods

The methods used in these experiments are identical to those in chapter 4.3. The following section presents additions or alterations to the methodology that are specific to this chapter.

5.3.1 Model organism selection

*Bacillus subtilis* was chosen as model organism to test the effects of irradiated perchlorate at Martian concentrations. *B. subtilis* is discussed in more detail in section 4.5. It was chosen as a model organism for these experiments for the same reasons as discussed in chapter 4, namely that it is a well-characterised organism as well as a candidate for forward contamination.

5.3.2 Perchlorate UV irradiation

Six milligrams of magnesium perchlorate hexahydrate (Sigma Aldrich) were added to each well of the previously described “liquid system” in the 12-well plate to obtain the final weight percentage of 0.6 wt%, unless another weight percentage is stated. When used, six milligrams of sodium and calcium perchlorate (Acros Organics) were also added to give a final concentration of 0.6 wt%. Three hundred milligrams of magnesium sulphate (99.5%, Acros Organics) were added to each well to give a final concentration of 30 wt% when used. If stated, 1 mg of hematite with a grain diameter of 5 µm (Sigma Aldrich) was added to each well giving a final concentration of 1 g/L (1 mg per 1 ml M9 in each well); 1 µL of 30% hydrogen peroxide solution (H₂O₂) was added in each well of 1 mL M9 to give a final concentration of 10 mM. As previously described in chapter 4.3, this concentration of hydrogen peroxide was used because a decrease below 1 mM leads to inefficient Photo-Fenton catalyst function and an increase to 100 mM causes sterilisation in the dark controls (see fig. 4.1 in chapter 4.3). The amount of 1 mg (1 g/L) of iron oxide catalyst (when used) was chosen, as UV light could still penetrate it and reach the cells; iron concentrations on Mars would vary substantially depending on location so this concentration was chosen as an example.
5.3.3 Rock analogue system

The same discs used in chapter 4.3 were soaked in the M9 medium and bacterial suspension, with the 6 mg (0.6 wt%) of magnesium perchlorate already dissolved in the M9 medium before irradiation. This is referred to as the “rock analogue system”.

5.4 Results

5.4.1 Bacteriocidal effect of UV-irradiated perchlorate

To determine if perchlorate had an effect on cell viability, Bacillus subtilis cells in minimal media M9 were irradiated in the presence of dissolved magnesium perchlorate (Mg(ClO$_4$)$_2$) at a concentration (0.6 wt%) typical of the Martian surface. Magnesium perchlorates were used in experiments, as they have been directly detected in Martian regolith [94] and thought to be a putative component of Martian brine seeps [87]. However, as it is in solution, the focus of the experiments is the perchlorate ion and its affects of cell viability.

Experiments were conducted under a monochromatic UVC radiation source emitting at a wavelength of 254 nm. Mars is subjected to UVC (200 - 280 nm) radiation, on account of the lack of a significant oxygen concentration or ozone shield and a lower cut-off caused by CO$_2$. The chosen flux of 254 nm radiation was similar to the absolute flux of radiation between 200 and 315 nm (UVC and UVB radiation), the most damaging region of the UV radiation spectrum to DNA [144]. The harmful effect on viability was quantified by calculating the ratio of surviving cells, $N$, with regard to the starting concentration $N_0$. If the number of plated colonies was greater than zero, it was defined as viable; if it was zero it is referred to as sterile. Results are shown on a log scale in all figures, therefore cases that are strictly zero are not represented on the log plot but should be interpreted as strictly zero. Statistical significance was defined as $p < 0.05$. Numerical results are summarised in appendix A.

Irradiated perchlorate had a significant bacteriocidal effect (fig. 5.1). Cell viability was completely lost after 30 seconds exposure. By contrast, the control cells exposed to UV radiation without perchlorate took 60 seconds to
be completely sterilised. Non-irradiated controls consisting of cells in M9, and cells in M9 in the presence of 0.6 wt% perchlorate, showed no significant difference in viability when left for up to one hour (fig. 5.2).

5.4.2 Bacteriocidal effect under Martian analogue conditions

To test if this result was reproducible in an environment more representative of Mars, experiments were carried out under a number of Martian analogue conditions. To simulate a rocky Martian habitat, the experiment was carried out using a simple system to more accurately simulate a rock environment, in which cells were deposited within silica discs. Although the overall cell survival was higher than in the liquid system, fig. 5.3 (a) shows a significant 9.1-fold drop in viability in the irradiated perchlorate-treated samples after 60 seconds, whilst the UV-irradiated controls show a two-fold viability decrease after the same exposure time.

Experiments were then carried out to investigate whether the perchlorate
Figure 5.2 Dark controls for M9 and perchlorate: *Bacillus subtilis* in M9 medium, covered to control for light and sampled at given time points. M9 = cells in M9 medium; M9 + perchlorate = cells in M9 containing 0.6 wt% Mg(ClO$_4$)$_2$. Error bars are + s.e. (n = 3).

Sterilisation effect would still be observed under the influence of other environmental parameters relevant to Mars, namely anaerobic conditions, polychromatic irradiation and low temperature, the results of which are also shown in fig. 5.3 (a). Firstly, the liquid system and rock analogue systems were irradiated under anaerobic conditions. Both systems showed that perchlorate-containing samples irradiated with UV experienced a greater loss of viability than the UV-irradiated controls. In the liquid system, cells remained viable (0.12%) after 60 seconds under just UV irradiation, but in the presence of perchlorate, viability was completely lost after 60 seconds. By contrast, in the rock analogue system, under UV irradiation greater cell viability was retained after 60 seconds (8.23%), but irradiated perchlorate still caused a significant loss of cell viability (9.7-fold decrease) compared to the UV-irradiated-only control after 60 seconds.

Secondly, experiments in the liquid system were replicated with the addition of a polychromatic light source to more accurately simulate a natural light spectrum. Under polychromatic light, cells in the presence of perchlorate showed a significant 10.8-fold decrease in viability compared to the polychromatic UV-irradiated only controls. Figure 4.2 from the previous chapter (chapt. 4) shows the total UV irradiance on Mars compared to the experimental absolute UV irradiance from 200 - 400 nm.
(a) Effects of UVC-irradiated Mg(ClO$_4$)$_2$ in rock analogues, under anaerobic conditions, polychromatic light and low temperature.

(b) Effects of UVC-irradiated Mg(ClO$_4$)$_2$ at low temperature, 1-4 minute exposure.

Figure 5.3 Rock analogues exposed to aerobic environment (Rock 30 60 seconds); Liquid and rock analogue exposed to anaerobic environment (Anaerob. Liquid/Rock 60 s); Liquid system exposed to polychromatic light (polychrom. 10 s); Liquid system chilled to 4°C whilst irradiated (4°C 30 60 seconds); UV = UVC irradiated control; Perchlorate = 0.6 wt% Mg(ClO$_4$)$_2$. Liquid system chilled to 4°C whilst irradiated. $p < 0.05$ was considered statistically significant (*) between or among groups; error bars are + s.e. (n = 3).
Additionally, the effect of low temperature on the reaction was investigated. The experimental system was chilled to 4°C before and during monochromatic irradiation and after 60 seconds it was observed that the cell viability of the UV-irradiated perchlorate samples did not drop significantly below that of the UV-irradiated controls (fig. 5.3 (a)). To test whether this was because UV-irradiated perchlorates were no longer effective at low temperatures or whether the chemical reaction was delayed, the same experiment was performed for 10 minutes, measuring at each minute (fig. 5.3 (b) shows minutes 1 - 4). Once again, after one minute of UV exposure both samples displayed no significant differences in viability. However, after two minutes of irradiation the irradiated perchlorate-treated samples showed a significant 11.4-fold decrease in viability in comparison to the UV controls; both UV and perchlorate samples were sterile after three minutes. It is unclear if the low temperature reduced perchlorate activation, reduced the diffusion of photoproducts to the cells or reduced the rate of cellular damage. Nevertheless, even at low temperatures, irradiated perchlorates proved bacteriocidal.

To confirm the production of potential biologically damaging photoproducts during perchlorate irradiation, the absorption spectrum of an irradiated solution of perchlorate was measured in the UV radiation range (fig. 5.4 shows absorbance from 250 - 400 nm). Potential photoproducts produced during irradiation could be hypochlorite (absorbance maximum = 290 nm), chlorite (absorbance maximum = 260 nm) and chlorine dioxide (absorbance maximum = 360 nm). Small peaks indicating an increase in absorbance were observed at 260 nm and at 290 nm. There was no observable increase in absorbance at 360 nm. A control sample containing non-irradiated perchlorate was also measured at the same time points and no peaks in absorbance at any of the wavelengths were observed. Using the modified Beer-Lambert law \( c = \frac{A l}{\varepsilon} \); \( c \) = concentration, \( A \) = absorbance, \( l \) = distance travelled by light, \( \varepsilon \) = molar absorptivity), the absorption was used to calculate the concentrations over irradiation time for hypochlorite, chlorite and chlorine dioxide (fig. 5.5). The concentrations showed increases in hypochlorite and chlorite within the range of \( 10^{-3} \) mol/L, but no observable increase for chlorine dioxide.
Figure 5.4  UV spectrophotometric absorbance of irradiated perchlorate, 250 - 400 nm: 6 wt% Mg(ClO$_4$)$_2$ irradiated with UVC for 300 min. UV-irradiated perchlorate (λ = 254 nm) in sterile, distilled water.

Figure 5.5  Perchlorate photoproduct concentration. UV-irradiated 6 wt% Mg(ClO$_4$)$_2$ (λ = 254 nm) in sterile, distilled water; concentrations over irradiation time at 260, 290 and 360 nm.
Figure 5.6  Effects of low concentrations of UVC-irradiated Mg(ClO$_4$)$_2$ on cell viability, 60 seconds exposure: UV = UVC irradiated control; ClO$_4^-$ = Mg(ClO$_4$)$_2$ at given wt%. p < 0.05 was considered statistically significant (*); error bars are + s.e. (n = 3).

Figure 5.7  Influence of increased perchlorate concentration on bacteriocidal effects under UV irradiation: Mg(ClO$_4$)$_2$ at representative measured Martian concentration (0.6 wt %), 1, 2.5 and 5 wt% (30 & 60 s); UV = UVC irradiated control (30 & 60 s). p < 0.05 was considered statistically significant (*) between and among groups; error bars are + s.e. (n = 3).
To determine whether altering the concentration of perchlorate affected the loss of cell viability when irradiated, the 0.6 wt% perchlorate solution was serially diluted to yield 0.06 wt% and 0.006 wt% solutions, which were irradiated in the presence of cells. Figure 5.6 shows that at 0.06 wt% and 0.006 wt% there were no statistically significant differences in cell viability compared to the UV-irradiated controls.

The effects of perchlorate were also investigated at higher concentrations than those measured in the Martian surface regolith (fig. 5.7). Although the regolith contains a concentration of 0.4 - 0.6 wt% [186], the spectral detection of putative perchlorate brines suggests that, in some local regions on Mars, the concentrations of this chemical could be much higher. At a perchlorate concentration of 1 wt%, viability dropped over an order of magnitude after 30 seconds irradiation compared to results at 0.6 wt%. A complete loss of viability was observed after 60 seconds exposure. An increase of perchlorate concentration to 5 wt% resulted in complete loss of viability after only 30 seconds of irradiation.

### 5.4.3 Interactions of other Martian soil components

After simulating the physical effects of the Martian environment on perchlorate activity, additional Martian soil components and their potential interactions were also investigated. Experiments were conducted to study whether other components of the Martian surface could affect the previously observed perchlorate reactions.

Sulphate is an abundant component of the Martian regolith with 30 wt% of sulphate within sediments having been reported [193]. The experiment using perchlorate at 0.6 wt% was repeated, but with the addition of sulphate at the estimated Martian concentration of 30 wt% (fig. 5.8). The results show that there was no significant effect of sulphate on the loss of viability of the cells in the presence of UV-irradiated perchlorate, nor did irradiated sulphate on its own significantly differ from the UV-irradiated control in terms of effects on viability.

Two additional forms of perchlorate were examined that have been detected on Mars (fig. 5.8). Sodium perchlorate (NaClO$_4$) was detected in the reoccurring slope lineae by Ojha L. et al. [87] and calcium perchlorate (Ca(ClO$_4$)$_2$) is thought to be the best candidate for the oxychlorine compounds found in Rocknest [186]. The perchlorates were both irradiated with UVC at a concentration of 0.6 wt%
(a) Effects of UVC-irradiated sodium/calcium perchlorate and sulphate on *B. subtilis* viability. Vertical grey line indicates separate experiment with different control.

(b) Comparison of normalised UVC-irradiated Ca(ClO₄)₂, NaClO₄ and Mg(ClO₄)₂. Mg(ClO₄)₂ data from fig. 5.7.

**Figure 5.8** Effects of UVC-irradiated sodium/calcium perchlorate and sulphate on *B. subtilis* viability: UV = UVC irradiated control at given distance from light source; Ca = 0.6 wt% Ca(ClO₄)₂ at given distance from light source; Na = 0.6 wt% NaClO₄ at given distance from light source; Perchl. and Mg = 0.6 wt % Mg(ClO₄)₂; Sulph. = 30 wt% MgSO₄. Letters shared in common between or among the groups indicate no significant difference (p > 0.05); error bars are ± s.e. (n = 3).
for comparison with the magnesium perchlorate. The calcium and sodium perchlorates showed a significantly lower cell count than the UV-irradiated controls after 30 seconds. The samples treated with calcium perchlorate were completely sterilised and the samples treated with sodium perchlorate showed a 15-fold drop in viability compared to the controls; all samples were sterilised after 60 seconds UV radiation exposure. To get a better resolution of the effect of the perchlorates, they were additionally irradiated in the same set up at a four times greater distance from the light source (16 times less irradiance). These results showed no significant difference in viability in any samples in the first 30 seconds of irradiation. However, after 60 seconds both calcium and sodium perchlorate-treated samples showed significantly lower cell counts than the UV-irradiated controls (1.9 and 1.7-fold, respectively).

The influence of two other components of the Martian surface environment was additionally examined, namely iron oxides and the oxidant hydrogen peroxide. Experiments were carried out to determine whether these substances would act in synergy with irradiated perchlorates to contribute to the hostile environment on the surface of Mars. Figure 5.9 shows the effects of the individual components, effects of combinations of two components and the combined effect of all three components under UV irradiation. Experiments were conducted with the iron oxide, hematite, with a grain diameter of 5 μm (Sigma-Aldrich), which was added to the liquid system or rock analogue system at a concentration of 1 g/L. When hydrogen peroxide was used it was added to a final concentration of 10 mM. Mg(ClO₄)₂ was added to a concentration of 0.6 wt%, as in previous experiments.

Firstly, the individual components, hematite, hydrogen peroxide and perchlorate, were added to M9 containing *Bacillus subtilis* cells and irradiated under the monochromatic UVC source for the indicated length of time (fig. 5.9). UV-irradiated controls containing cells in M9 served as a control. Samples in the presence of hematite showed significantly higher cell viability after 60 seconds exposure than cells in the UV controls after the same length of time. Samples individually treated with hydrogen peroxide or perchlorate showed a significant drop in viability in comparison to UV irradiated controls after 60 seconds (1.9-fold and 1.6-fold, respectively).

Secondly, the individual components were paired as follows and irradiated for the indicated length of time: hematite and hydrogen peroxide; hematite and perchlorate; hydrogen peroxide and perchlorate (fig. 5.9). The combination of the iron oxide and hydrogen peroxide in the presence of UV radiation caused
Figure 5.9 Perchlorate-induced bacteriocidal effects in the presence of other components of the Martian surface (hematite and hydrogen peroxide) after 30 and 60 seconds UV exposure: UV = UV irradiated control; H = 1 g/L hematite; HP = 10 mM hydrogen peroxide; ClO$_4^-$ = 0.6 wt% Mg(ClO$_4^-$)$_2$. Letters shared in common between or among the groups indicate no significant difference (p > 0.05); error bars are ± s.e. (n = 3). Vertical grey lines serve as visual separation of single, double and triple combinations.
a significantly greater loss in viability (2.7-fold) as well as combined hydrogen peroxide and perchlorate (2.9-fold) compared to the control than any of the individual components. The iron oxide and perchlorate combination showed no significant difference in viability when compared to perchlorate alone. Cells treated with perchlorate and hydrogen peroxide showed a 2.9-fold loss in viability compared to the control. This combination did not significantly differ from cell viability after irradiation in the presence of hydrogen peroxide and hematite.

Lastly however, when combined, all three components were shown to result in the largest drop in viability (fig. 5.9). After 60 seconds of UV radiation exposure cell viability was reduced to 0.21%, a 10.9-fold reduction compared to the control, which was significantly lower than all other combinations examined.

5.5 Discussion

Perchlorate, although stable at room temperature, is a powerful oxidant when activated, for instance at high temperatures [93]. Oxidants were hypothesised to be on the surface of Mars and responsible for the lack of organics found by the Viking missions [182, 183]. Perchlorate was not initially suspected as a candidate, as it was thought that the majority of chlorine would occur as chloride ions [194]. Its presence was confirmed by NASA’s Phoenix Lander [94] and recently spectrally identified as a component of brine seeps on the Martian surface by the Mars Reconnaissance Orbiter in late 2015 [87]. Although perchlorate can be used as an electron acceptor for microbial growth, its potentially deleterious effects have been little explored.

The work presented in this chapter demonstrates that when magnesium perchlorate, at concentrations relevant to the Martian surface, is irradiated under short-wave UVC radiation encountered on the Martian surface, it becomes bacteriocidal. This effect is observed both in liquid culture and in a rock analogue system that replicates a micro-environment within rocks. The effect is less pronounced within the rock analogue system, which is likely caused by screening within the rock. Screening reduces the penetration of UV radiation compared to the liquid system. The bacteriocidal effect is also replicated when using other forms of perchlorate found in the Martian regolith: calcium and sodium perchlorate. Both perchlorates significantly reduce viability of cells when irradiated in comparison to controls. Bacterial samples in the presence of
perchlorate at Martian concentrations but in the absence of UV radiation show no loss of viability, which is consistent with the findings by Nicholson et al. [195], which indicate no growth inhibition of Bacillus subtilis 168 and Bacillus pumilus SAFR-032 when in the presence of perchlorate without UV exposure.

The mechanism of perchlorate’s effect on cells is still not verified, but may involve its reduction to other oxychlorine species such as chlorate (ClO$_3^-$), chlorite (ClO$_2^-$) and hypochlorite (ClO$^-$). During irradiation, an increase in absorption at the expected maxima of hypochlorite (290 nm) and chlorite (260 nm) is observed. Similar photoproducts have been previously observed of perchlorate irradiated with ionising radiation [90, 93]. All of the above-listed chlorine species have been linked to DNA or membrane damage [196–198]. Although it is unlikely for ions to diffuse across bacterial membranes, if protonated to their non-charged form (e.g. hypochloric acid HOCl) they would be able to reach the cell’s interior. Proposed mechanisms for the radiolysis of perchlorate show the generation of atomic oxygen in addition to reduced oxychlorine species [88, 92]. Atomic oxygen is highly reactive and could result in a dual attack on organics, consisting of chlorination by oxychlorine species and degradation by oxygen. If this is indeed the mechanism by which cell death is increased, it may have further implications for the scope of habitability on Mars. Although perchlorate appears to be deleterious when irradiated, the argument could be made that any perchlorate-containing potential habitat sheltered from irradiation may be habitable. However, if the mechanism of the bacteriocidal effect is through secondary products, their diffusion through the Martian soil may broaden the range of toxicity, e.g., to the subsurface.

The interpretation of the chemical nature of this bacteriocidal effect is supported by carrying out the experiment at 4°C. The resulting loss of viability is over ten times lower than at 25°C, suggesting that low temperatures lower the rate of the chemical reaction or the diffusion of products and thereby reduce the rate of bacteriocidal effects. Nevertheless, the effect is still observable, consistent with the findings by Turner et al. [92]. They argue that oxygen production is the main reason for organic degradation and observed a more pronounced time delay in oxygen production as the temperature dropped. Although the average surface temperature on Mars is approximately 218 K (−55°C), the Mars Exploration Rover Opportunity measured a daily maximum of 295 K (21.85°C) [162]. Therefore, a range of reaction rates varying with latitude, season and time of day could be expected. By lowering the perchlorate concentration by one order
of magnitude to below that found at the Martian surface, the loss of viability is reduced to values not statistically significant from UV irradiation alone, showing that under conditions where perchlorates are diluted, the bacteriocidal effect is mitigated. By contrast, any environment that concentrates perchlorates, such as in putative Martian brines [185], will be uninhabitable on account of the bacteriocidal properties of irradiated perchlorates measured in this work. These properties suggest that the mere presence of liquid water seeps, thought to be good locations to search for life, does not imply environments fit for life.

Interactions of perchlorate with other major Martian soil components were investigated. Sulphate concentrations vary extensively across the Martian surface. Spectrometers onboard NASA’s Mars Explorations Rovers measured levels of sulphate at the Meridiani Planum with concentrations of 20 - 40 wt% [199]. The effects of sulphate at 30 wt% were investigated with no observable effect on the perchlorate-induced loss of cell viability under UV irradiation. However, other regolith components do have bacteriocidal properties. Mars has approximately 18 wt% iron oxides in their ferrous Fe$^{2+}$ and ferric Fe$^{3+}$ oxidation states combined [200], which can participate in photochemical reactions.

A higher loss of cell viability is observed when hematite and hydrogen peroxide are combined than when they are added to cells individually under UV irradiation. These results can be explained by the Photo-Fenton reaction. The standard Fenton reaction consists of hydrogen peroxide reacting with catalytic ferrous iron, which results in its oxidation to ferric iron and the production of hydroxyl radicals [62]. The Photo-Fenton reaction is a more efficient variation as it utilises UV light to catalyse the recycling of the iron (in dissolved or in oxide form) [145, 146]. The maximum bacteriocidal effect at neutral pH is empirically established to require 1 g/L of iron oxide in an aqueous environment and a hydrogen peroxide concentration of 10 mM [147]. When hydrogen peroxide, hematite and perchlorates are combined, which might represent a combination of compounds in the Martian soil, the greatest loss of cell viability is achieved. This observation is attributed to the combined effect of UV-irradiated perchlorate-induced cell killing with Photo-Fenton-induced killing by iron oxides and hydrogen peroxides. Research has shown that Photo-Fenton-like reactions under oxic and anoxic conditions can be used for the reduction of perchlorate (i.e., for its removal from groundwater). However, this can only occur in the presence of a radical scavenger such as methanol, as the produced radicals compete with the perchlorate for the electron generated by iron oxidation [170].
Although the toxic effects of oxidants on the Martian surface have been suspected for some time, these observations show that the surface of present-day Mars is highly deleterious to cells, caused by a toxic cocktail of oxidants, iron oxides, perchlorates and UV irradiation. There has been recent research into the use of perchlorates as a potential energy source for bacteria on Mars [191, 192] and suggestions [147] that such life may have been detected in the Viking Labeled Release experimental results. However, these results demonstrate the bacteriocidal effects of UV-irradiated perchlorates, which provide yet further evidence that the surface of Mars is lethal to vegetative cells, and point to much of the near-surface regions being uninhabitable. The enhancement of the bacteriocidal properties of perchlorates by UV-irradiation suggests that these aqueous environments are even more deleterious to potential contaminants from spacecraft, and potentially less habitable, than was previously thought. These data have implications for planetary protection, specifically regarding concerns about the forward contamination of Mars in both robotic and human exploration.

5.5.1 Limitations

Despite the significant bacteriocidal effect that irradiated perchlorate has shown in the experiments, there are limitations to take into consideration. The lack of understanding of the destructive mechanism limits the ability to predict the scope of damage to the Martian environment. Previous accounts [90, 92, 93] of reduced oxychlorine species and oxygen as products of perchlorate radiolysis fit the presented observations, as does the reported DNA and membrane damage they can cause [196–198]. However, the fact that perchlorate-reducing bacteria also generate the same oxychlorine species as intermediate products (fig. 5.10) and show no toxic effects highlights the need to properly investigate the cause of the observed cell death in the experiments. One possible explanation for the lack of damage in perchlorate-reducing species is the rapid further reduction of these intermediate products to Cl$^-$ and H$_2$O before any significant biological damage can take place.
5.5.2 Future work

The experiments in this chapter focused on vegetative cells of the model organism *B. subtilis*, which is not an extremophile although its spores are known to be highly resistant to radiation (as discussed in chapter 4). Future experiments using various model organisms, including extremophiles and perchlorate-metabolising bacteria, may shed light on the bacteriocidal mechanism and give insight into the scope of its effects. Further research on the formation, distribution and degradation pathway of perchlorates on Mars is also necessary to understand and predict its effect on the surrounding environment. This also requires more accurate radiation modelling with regard to the sub-surface radiation range to enable a depth-profile of the reactions such irradiation would enable and subsequent damage to organics. Perchlorate will also be a health risk to future human landing parties on the Martian surface. Research dedicated to the removal of perchlorate from the immediate environment such as from drinking water (e.g. using zero-valent iron and UV [170]) will also be valuable with the beginning of manned missions to, and prolonged settlement on the planet.
5.6 Conclusions

Perchlorates are known to be powerful oxidants, yet are stable at ambient temperatures. However, this work shows that, when irradiated with non-ionising UV at room temperature, they become activated and significantly more bacteriocidal than the UV light on its own. This effect was reproduced under a variety of simulated Martian environmental conditions. In addition, they act in synergy with other Martian regolith components to enhance the inhospitable nature of the surface layers. These findings highlight the need for a thorough understanding of the photochemical environment and the thereby-induced reactions to be able to more accurately predict the overall effect on habitability. This is relevant not only for evaluating the likelihood of extant Martian life and biosignatures but also for the prospect of forward contamination by non-sterile scientific equipment and instruments.
Chapter 6

Survival of cyanobacteria after extended exposure to space and simulated Martian environments

6.1 Introduction

Missions carrying biological and chemical substances to the International Space Station, to be exposed to the extreme conditions of low Earth orbit outside the station, have become routine in the past few decades. Although many space-like conditions can be simulated on the Earth, factors such as microgravity, the full solar-spectrum and ionising radiation are practically and economically difficult to simulate. The European Space Agency’s EXPOSE facility [202], located on the outside of the space station, provides a platform for scientists to expose samples to the multiple extremes of space. These conditions can also be used to simulate exposure to non-terrestrial environments such as Mars. This chapter presents and discusses the various post-flight analyses that were performed on the Edinburgh “BIOlogy Mars EXperiment” (BIOMEX) and “Biofilm Organisms Surfing Space” (BOSS) experiments that were integrated in the EXPOSE(-R2) facility. Both experiments were subjected to space and simulated Mars environments for 531 days before returning to Earth. Their objectives were to test the hypothesis that a Martian soil simulant could confer sufficient protection to a bacterial culture to enable its survival, as well as to investigate the role biofilms play in providing a shield for bacteria, thus making survival in otherwise lethal conditions such
as low Earth orbit or simulated Martian conditions possible. In addition to the hypothesised protective characteristics of regolith and self-shielding by biofilms, the ability of an organism to protect and maintain viability of a co-cultured species was tested when both organisms were exposed to these extreme conditions. Post-flight analysis of the experiments confirmed both the protective property of the Martian soil simulant as well as biofilms, enabling the samples to be cultured after 1.5 years of exposure to low Earth orbit or simulated Martian conditions and to retain a detectable biomarker signal. Additionally, the experiments demonstrated the ability of the bacteria to provide protection from the extreme conditions for the additional, co-cultured species. This has significant implications for planetary protection as well as understanding the limitations of extreme radiation exposure on life.

### 6.2 Background

The low Earth orbit (LEO) environment consists of multiple extreme conditions that pose many challenges to life (see section 2.3.3). It also allows for exposure to environmental factors that are unavailable or not faithfully reproducible on Earth, such as the full solar spectrum, high-energy particles and microgravity. LEO extends from 160 km to 2000 km above the surface of the Earth and is still within the Earth’s upper atmosphere and magnetosphere. Whilst the magnetosphere is able to deflect some of high-energy radiation found in space, the LEO radiation environment is still more hazardous than that of the Earth’s surface, especially due to galactic cosmic rays (GCR) and shortwave UV [60]. Temperatures can fluctuate between $-120^\circ\text{C}$ in shaded environments and $+120^\circ\text{C}$ in full sunlight [203], while acceleration due to gravity is $1 \times 10^{-6} \text{ g}$ [204]. The atmospheric pressure of the upper atmosphere above 100 km drops to $3.2 \times 10^2 \text{ Pa}$ [205], essentially a vacuum. These LEO conditions are practically irreproducible on Earth and have therefore been exploited outside the ISS to test the response and degradation of various organisms and substances including prokaryotes, eukaryotes and chemical compounds [206–208]. The European Space Agency’s (ESA) EXPOSE facility was first installed on the International Space Station (ISS) in 2008 with the goal of providing a platform for European scientists to expose samples to LEO conditions in situ and condition combinations of the upper atmosphere that are often outside the scope of laboratory simulations. The platform is attached to the Russian Zvezda module, which is accessed by
astronauts performing space walks and has hosted three consecutive experiment payloads to date: EXPOSE-E, EXPOSE-R and EXPOSE-R2 \[209\].

Prof. Charles Cockell and his lab in the University of Edinburgh had two experiments on board the EXPOSE-R2 module for a total of 531 days exposed on the outside of the ISS, launched July 23\textsuperscript{rd} 2014 and returned to Earth on June 18\textsuperscript{th} 2016. The two experiments were a part of the BIOMEX and BOSS experimental set up (fig. 6.1), with principal investigators Jean-Pierre de Vera and Petra Rettberg respectively, based at the German Space Agency.

![Figure 6.1](image.png)

**Figure 6.1** Experimental layout of BOSS and BIOMEX. Red indicates BIOMEX biofilm samples with added P-Mars soil simulant; black indicates biofilm samples used in BIOMEX and BOSS without P-Mars; blue indicates BOSS planktonic samples without P-Mars. Colour-coding of experimental conditions is retained throughout the chapter.

The focus of the Edinburgh BIOMEX experiments was to test the protective capacity of an artificial, Phyllosilicatic Mars Regolith Simulant (P-MRS), referred to as P-Mars in this work, with regard to its capacity to shield cyanobacteria biofilms. The precise P-Mars composition is described in Böttger \textit{et al.} \[210\]. Previous research has shown that microorganisms are capable of being revived after various degrees of exposure to space conditions, such as microgravity and radiation \[211 \text{ 212}\], and that shallow layers of regolith can provide extensive shielding from the space radiation environment \[213\]. This is additionally supported by data from chapter 4 (fig. 4.3). Understanding the protective properties of Mars simulant soil will inform on its potential for the preservation of life and biomarkers such as pigments. The post-flight assessments of exposed cyanobacteria will additionally provide insight into biomarker detectability by techniques such as Raman spectroscopy. A variety of techniques capable of biomarker detection are currently integrated into instruments on board the joint Russian-European Mars rover (ExoMars) set to launch in 2020, as well as NASA’s
The main objective of the Edinburgh BOSS experiments was to test the protective characteristics of bacteria in a biofilm, as opposed to planktonic cells under the multiple extremes of LEO. Most naturally occurring microorganisms form biofilms, which provide a barrier between the culture and toxic substances as well as damaging environments, for example high-UV environments on Earth including the Atacama desert and Antarctica. The International Union of Pure and Applied Chemistry’s (IUPAC) definition of a biofilm is an “Aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substances (EPSs) adhere to each other and/or to a surface”. However, the *Gloeocapsa* films are not strictly biofilms as defined above. Rather, they can be described as an amorphous cellular mass (ACM), a more primordial version of a biofilm with less well-defined structures. The main objective of BOSS aligns itself with the objective of BIOMEX, in that it tests the limits of bacterial survival. In the BOSS experiment, biofilm and planktonic bacterial cultures are compared after exposure to LEO conditions, to inform on life and biomarker detection probability after similarly extreme exposure to an array of harsh conditions. While the BIOMEX and BOSS experimental set ups were aimed at investigating whether biogenic and abiogenic structures could provide protection to a single species, they were additionally used to test the ability of an ACM-forming bacteria to shield a less resistant co-species. The potential protection of another species by a more robust one would be an additional concern for planetary protection.

### 6.3 Methods

#### 6.3.1 Model organism selection

The model organism used in both Edinburgh BIOMEX and BOSS experiments was the endolithic cyanobacteria species *Gloeocapsa*. The bacterium is surrounded by a concentric gelatinous sheath (ACM), which can also envelop multicellular groups of bacteria, especially recently divided pairs (fig. 6.2 d). The gelatinous sheath of *Gloeocapsa* has previously been shown to provide effective protection against UV. The cyanobacterium *Chroococcidiopsis*, related to *Gloeocapsa*, similarly possesses a gelatinous sheath and has been shown to have
a heightened tolerance for radiation exposure [220, 221] and is also included in various BOSS/BIOMEX experiments by other investigator teams [222].

The *Gloeocapsa* strain used in BIOMEX and BOSS was found in an environmental sample taken from the upper greensand layer of the limestone cliffs in Devon, UK, and was isolated by exposure of the environmental sample to the LEO environment for 10 days [136]. It was used in the previous EXPOSE-E project, where it survived 548 days exposed to LEO but shielded from UV exposure [223]. A co-cultured bacterial species was present in all BIOMEX and BOSS cyanobacterial cultures to test the inter-species protection hypothesis. The co-cultured species used in EXPOSE-R2 was also isolated from the same cell culture as *Gloeocapsa* during the EXPOSE-E mission and was identified as an α-proteobacteria isolate related to *Geminicoccus roseus* [137]. It is described as a diplococcoid bacterium containing carotenoids and low amounts of bacteriochlorophyll a, giving its colonies a pink colouration.

As shown in fig. 6.2 (a), samples for the BIOMEX and BOSS experiments were grown on commercially produced, porous, sintered, silica discs with 10 mm diameter, 3 mm thickness and a pore size of 100 - 160 µm (Scientific Glass, UK) in liquid BG-11 media before being desiccated and sent to the German Space Agency for integration into the EXPOSE-R2 facility and the ground control replicate.

All pre-flight experiments were performed by Casey Bryce, University of Edinburgh.
Figure 6.2  Model organism *Gloeocapsa* on sintered discs used in the ESPOSE-R2 experimental set up (a & b) and in liquid media (c & d). Each bacterium is ~ 6 μm in size but can form larger aggregates (b & c) many hundred μm in diameter.

### 6.3.2 EXPOSE-R2 platform

The EXPOSE-R2 platform is a box-shaped facility with the dimensions 480 mm × 390 mm area, 140 mm height and an approximate mass of 44 kg (figs. [6.3](#) A, [6.4](#) [6.5](#)). It is equipped with UV sensors (OEC GmbH, Germany), a radiometer (Dexter 6M Thin Film Based Thermopile Detector) and temperature sensors to enable the temperature of the electronics to be kept between −25°C and 52°C. Each sample compartment has two layers for samples (fig. [6.3](#) B), with the upper sample carrier containing samples that are subjected to the full range
of environmental factors including UV radiation and the lower sample carrier containing samples that are exposed to the same factors with the exception of UV. The filter frame windows on top of each sample tray allow for wavelength exposure of light-exposed samples to be controlled [209].

Figure 6.3 View of whole EXPOSE-R2 platform (A); positioning of exposed (light-exposed) and dark (dark control) cells of BOSS and BIOMEX (B) [225].

The upper (i.e. light-exposed) samples and lower (i.e. dark control) samples in the BIOMEX and BOSS EXPOSE trays were exposed to two sets of conditions: one “space” (LEO) condition and one simulated “Mars” condition. The full condition parameters are summarised below.

**Space conditions:**

- No long-pass cut-off filters
- MgF$_2$ neutral density filters 0.1% (> 120 nm)
- Pressure = Vacuum
- N$_2$ gas
- Extreme temperature fluctuations
- Microgravity

**Simulated Mars conditions:**

- > 200 nm cut-off filters
• Quartz neutral density filters 0.1% (> 200 nm)

• Pressure = 1000 Pa

• N₂ gas

• Extreme temperature fluctuations

• Microgravity

Ground controls were housed in identical hardware in the Planetary and Space Simulation Facilities (PSI) at the Cologne German Space Agency site, where the controls were exposed to as similar conditions as technically feasible (see ground control conditions list). Ionising radiation such as solar UV below 200 nm and high-energy particles could not be controlled for on the ground, as well as microgravity.

**Ground control conditions:**

• Solar UV wavelength = > 200 nm

• Pressure = Vacuum or 1000 Pa

• N₂ gas

• Extreme temperature fluctuations

Samples were exposed to their respective conditions for 531 days. This does not include the first 62 days of the mission, in which samples were protected from solar irradiation during a period of outgassing. The calculated mean fluence of the wavelength range 200 - 400 nm (biologically active) was 536 ± 116 MJm². Post-flight data confirmed the temperature range to have been between −20.9°C and 57.98°C. The atmospheric pressure outside the Zvezda module was not directly measured by equipment on board the EXPOSE-R2 platform but was confirmed by Russian agency RSC Energia to vary between 1.33 × 10³ and 1.33 × 10⁴ Pa [209].

As mentioned in section 2.3.3 there are three main ionising radiation sources contributing to the radiation environment outside the ISS: the Inner and Outer Radiation Belt (South Atlantic Anomaly protons and electrons, respectively), galactic cosmic rays and solar particle events. Maximum doses from these sources
were determined to be 844 $\mu$Gy/d, 82 $\mu$Gy/d, and 2960 $\mu$Gy/d, respectively [209, 226]. Ionising radiation caused by a solar energetic particle event on June 22$^{nd}$ 2015 was measured by the R3DR2 instrument on board EXPOSE-R2. The increased resulting dose from the event was determined to be $> 5000 \mu$Gy/h [227].

The BOSS and BIOMEX samples were stored on board the ISS before the return to Earth for 136 days. After arriving they were transported to the German Space Agency Cologne site where the samples were confirmed to have not exceeded their shock and temperature limits and were steriley prepared and sent to the respective investigators.

**Figure 6.4** EXPOSE-R2 facility on board the ISS (left) and mounted on the Russian Zvezda module (circled) on the outside of the ISS [228].

**Figure 6.5** Previous EXPOSE-R facility on the outside of the ISS [229].
Table 6.1  BIOMEX & BOSS sample nomenclature:
Table colouring reflects layout shown in fig. 6.1. Red indicates BIOMEX biofilm samples with added P-Mars soil simulant; black indicates biofilm samples used in BIOMEX and BOSS without P-Mars; blue indicates BOSS planktonic samples without P-Mars; (D) denotes duplicate samples; Light and dark denotes light-exposed or dark control samples, respectively.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Exposed conditions</th>
<th>Light exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1-t-03</td>
<td>Space + P-Mars</td>
<td>Light</td>
</tr>
<tr>
<td>(D) 1-1-t-07</td>
<td>Space + P-Mars</td>
<td>Light</td>
</tr>
<tr>
<td>1-1-b-03</td>
<td>Space + P-Mars</td>
<td>Dark</td>
</tr>
<tr>
<td>(D) 1-1-b-07</td>
<td>Space + P-Mars</td>
<td>Dark</td>
</tr>
<tr>
<td>2-1-t-03</td>
<td>Mars + P-Mars</td>
<td>Light</td>
</tr>
<tr>
<td>(D) 2-1-t-07</td>
<td>Mars + P-Mars</td>
<td>Light</td>
</tr>
<tr>
<td>2-1-b-03</td>
<td>Mars + P-Mars</td>
<td>Dark</td>
</tr>
<tr>
<td>(D) 2-1-b-07</td>
<td>Mars + P-Mars</td>
<td>Dark</td>
</tr>
<tr>
<td>1-3-t-11</td>
<td>Space</td>
<td>Light</td>
</tr>
<tr>
<td>(D) 1-3-t-12</td>
<td>Space</td>
<td>Light</td>
</tr>
<tr>
<td>1-3-b-11</td>
<td>Space</td>
<td>Dark</td>
</tr>
<tr>
<td>(D) 1-3-b-12</td>
<td>Space</td>
<td>Dark</td>
</tr>
<tr>
<td>2-3-t-11</td>
<td>Mars</td>
<td>Light</td>
</tr>
<tr>
<td>(D) 2-3-t-12</td>
<td>Mars</td>
<td>Light</td>
</tr>
<tr>
<td>2-3-b-11</td>
<td>Mars</td>
<td>Dark</td>
</tr>
<tr>
<td>(D) 2-3-b-12</td>
<td>Mars</td>
<td>Dark</td>
</tr>
<tr>
<td>1-3-t-15</td>
<td>Space</td>
<td>Light</td>
</tr>
<tr>
<td>(D) 1-3-t-16</td>
<td>Space</td>
<td>Light</td>
</tr>
<tr>
<td>1-3-b-15</td>
<td>Space</td>
<td>Dark</td>
</tr>
<tr>
<td>(D) 1-3-b-16</td>
<td>Space</td>
<td>Dark</td>
</tr>
<tr>
<td>2-3-t-15</td>
<td>Mars</td>
<td>Light</td>
</tr>
<tr>
<td>(D) 2-3-t-16</td>
<td>Mars</td>
<td>Light</td>
</tr>
<tr>
<td>2-3-b-15</td>
<td>Mars</td>
<td>Dark</td>
</tr>
<tr>
<td>(D) 2-3-b-16</td>
<td>Mars</td>
<td>Dark</td>
</tr>
</tbody>
</table>

Table 6.1 gives an overview of the experimental sample labels and respective conditions: 1-1-t-03 to 2-1-b-07 contained biofilms with added P-Mars soil simulant (in red), 1-3-t-11 to 2-3-b-12 contained only biofilms (in black), 1-3-t-15 to 2-3-b-16 contained planktonic cells, also without added P-Mars (in blue).
Henceforth, “light-exposed” will denote samples exposed to the incident solar radiation in the upper sample carrier, and “dark controls” will denote the covered samples from the lower sample carrier.

The following post-flight analysis techniques were performed on the Edinburgh BIOMEX & BOSS samples:

- Liquid culturing
- Agar plate culturing
- Bright field and fluorescent microscopy
- Transmission electron microscopy
- Scanning electron microscopy
- Raman spectroscopy

Upon arrival at the Edinburgh lab, all samples were stored at $-80^\circ$C until further analysis. For the various analysis techniques, the sintered discs were split into approximately $\frac{1}{3}$ sections. One section for liquid cultivation, one for agar plate cultivation and an extra section kept at $-80^\circ$C for further assays.

### 6.3.3 Post-flight culturing

In order to establish whether samples were able to be revived, cells were cultured in liquid media and on agar plates. This was a plus/minus assay, i.e. to determine whether the cells were alive or dead, not to establish the extent of growth if viable. For liquid cultivation, $\frac{1}{3}$ of one of the duplicates was placed in BG-11 media (Sigma Aldrich, 73816) with aerobic bottle head space and cultured at room temperature $25^\circ$C for three months. For agar plate cultivation, $\frac{1}{3}$ of one of the duplicate disc sections was gently ground up with mortar and pestle in sterile water and then spread onto BG-11 agar plates. These were also incubated at $25^\circ$C for three months. Both positive and negative liquid and plate controls were established, using *Chroococcidiopsis* as positive controls. As *Gloeocapsa* cells form aggregates, single colony counting would be imprecise and not representative of actual cell numbers. Growth assessment was done relative to positive controls, and ground controls subjected to the corresponding space conditions at the DLR (German Space Agency).
6.3.4 Staining

Live/Dead stain (SYTO9 & propidium iodide)

In order to determine the overall viability of samples in addition to culturing, the LIVE/DEAD® BacLight™ Bacterial Viability kit was used (Thermo Fisher Scientific, catalog number: L7012), which indicates cell viability as a function of membrane integrity. Five microlitres of SYTO 9 dye (1.67 mM) and Propidium iodide (PI, 1.67 mM) were combined. One microlitre of this solution is added to H₂O to make a 1:10 working stock solution. Five microlitres of the working stock solution is added per 200 µl of sample. After incubation for 20 minutes at room temperature and protected from light, 10 µl per sample was trapped on a microscope slide and viewed with a fluorescent microscope. SYTO9 has an excitation wavelength of 485 nm and an emission wavelength of 498 nm (source: LIVE/DEAD® BacLight™ Bacterial Viability kit online protocol [230]).

Fluorescein diacetate (FDA)

To further establish the extent of damage to cells, their enzymatic activity was measured using Fluorescein Diacetate (FDA) supplied by Thermo Fisher Scientific (catalog number: F1303). Active enzymes hydrolyse the dye, which is indicated by a change in colour. Fifty milligrams of FDA was added to 10 ml of acetone, of which 1 µl was added per 200 µl of sample and incubated for 20 minutes at room temperature. Ten microlitres per sample were trapped on a microscope slide and viewed with a fluorescent microscope at an excitation wavelength of 485 nm.

DiBAC₄(3)

An additional assay to establish cell damage is the measurement of membrane potential, which can be done using DiBAC₄(3) (Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol). If a cell is depolarised the dye can enter and bind to proteins, which is indicated by a change in colour. DiBAC₄(3) was supplied by Thermo Fisher Scientific (catalog number: B438). One microlitre of the solution was added per 100 µl of sample and incubated at room temperature for 20 minutes. The dye has an excitation maximum of 490 nm and emission
maxima of 516 nm (source: Thermo Fisher Scientific online protocol [231]).

6.3.5 TEM preparation

Transition electron microscopy (TEM) allows for high magnification of sample cross sections. Samples were centrifuged at 10,000 × g/min for five minutes with two washing steps using PBS. The supernatant was discarded after each wash and fixed after the second wash using 3% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.3). The subsequent preparation was performed by Stephen Mitchell (University of Edinburgh). After two hours of fixation, samples were washed in three 10 minute changes of 0.1M sodium cacodylate. Specimens were then post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate for 45 minutes, then washed in three 10 minute changes of 0.1M sodium cacodylate buffer. These samples were then dehydrated in 50%, 70%, 90% and 3× 100% ethanol for 15 minutes each, then in two 10-minute changes in propylene oxide. Samples were then embedded in TAAB 812 resin. One micrometre thick sections were cut on a Leica Ultracut ultramicrotome, stained with Toluidine Blue, and viewed in a light microscope to select suitable areas for investigation. Ultra-thin sections, 60 nm thick were cut from selected areas, stained in uranyl acetate and lead citrate.

I acknowledge the support of the Wellcome Trust Multi User Equipment Grant (WT104915MA) for use of the TEM.

6.3.6 SEM preparation

Scanning electron microscopy (SEM) allows for high magnification of sample surfaces. Sample preparation was performed by Stephen Mitchell (University of Edinburgh) excluding the initial PBS washing steps. SEM sample preparation is identical to the TEM sample preparation up until and including dehydration using 50%, 70%, 90% and 3× 100% ethanol.

Each dehydration was followed by critical point drying using liquid carbon dioxide. After mounting on aluminium stubs with carbon tabs attached, the specimens were sputter coated with 20 nm gold palladium.
6.3.7 Raman spectroscopy

Raman signatures were measured with the help of Peter Chung using the Renishaw inVia confocal Raman microscope located in ISAAC (Imaging Spectroscopy and Analysis Centre), at the School of Geographical and Earth Sciences (GES), University of Glasgow. Spectra were analysed using the WiRE Raman analysis software version 4.4.

6.4 Results

The following post-flight results describe the viability and extent of damage of BIOMEX and BOSS samples in comparison to the various conditions and respective ground controls. Because of significant discrepancies between the ground control and ISS samples, which were confirmed by other investigator groups, the sample comparison in this chapter will focus on the ISS dark controls and biofilm samples serving as BIOMEX and BOSS controls. Due to the experimental design used in BIOMEX and BOSS, samples were only prepared in duplicate. This restricted the amount of each sample available for use in analysis and from which conclusions could be drawn (further discussed in section 6.5.1). The presented results are qualitative and not quantitative; pictures featured in this chapter were chosen to be representative of the effects observed in the sample as a whole.

6.4.1 Growth assays

To establish whether Gloeocapsa cultures exposed to the various space conditions were still viable, the samples were grown both on agar and in liquid growth media. They were cultured for an initial period of three months (tab. 6.2) with their growth progress being additionally documented at nine months (tab. 6.3).
Table 6.2  BIOMEX/BOSS sample growth three months. Red indicates BIOMEX biofilm samples with added P-Mars soil simulant; black indicates biofilm samples used in BIOMEX and BOSS; blue indicates BOSS planktonic samples. “t” and “b” denote top (light-exposed) and bottom (dark control) samples, respectively.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Condition</th>
<th>Liquid culture\ground control</th>
<th>Agar culture\ground control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1-t-03</td>
<td>Space</td>
<td>Growth\Growth</td>
<td>Growth\Growth</td>
</tr>
<tr>
<td>1-1-t-07</td>
<td>Space</td>
<td>No Growth\No Growth</td>
<td>Growth\No Growth</td>
</tr>
<tr>
<td>1-1-b-03</td>
<td>Space</td>
<td>Growth\Growth</td>
<td>No Growth\Growth</td>
</tr>
<tr>
<td>1-1-b-07</td>
<td>Space</td>
<td>Growth\Growth</td>
<td>No Growth\Growth</td>
</tr>
<tr>
<td>2-1-t-03</td>
<td>Mars</td>
<td>Growth\Growth</td>
<td>No Growth\No Growth</td>
</tr>
<tr>
<td>2-1-t-07</td>
<td>Mars</td>
<td>Growth\No Growth</td>
<td>Growth\Growth</td>
</tr>
<tr>
<td>2-1-b-03</td>
<td>Mars</td>
<td>Growth\Growth</td>
<td>Growth\Growth</td>
</tr>
<tr>
<td>2-1-b-07</td>
<td>Mars</td>
<td>Growth\Growth</td>
<td>Growth\Growth</td>
</tr>
<tr>
<td>1-3-t-11</td>
<td>Space</td>
<td>No Growth\Growth</td>
<td>No Growth\Growth</td>
</tr>
<tr>
<td>1-3-t-12</td>
<td>Space</td>
<td>No Growth\Growth</td>
<td>No Growth\Growth</td>
</tr>
<tr>
<td>1-3-b-11</td>
<td>Space</td>
<td>Growth\Growth</td>
<td>No Growth\Growth</td>
</tr>
<tr>
<td>1-3-b-12</td>
<td>Space</td>
<td>No Growth\Growth</td>
<td>No Growth\Growth</td>
</tr>
<tr>
<td>2-3-t-11</td>
<td>Mars</td>
<td>No Growth\No Growth</td>
<td>Growth\Growth</td>
</tr>
<tr>
<td>2-3-t-12</td>
<td>Mars</td>
<td>No Growth\No Growth</td>
<td>Growth\No Growth</td>
</tr>
<tr>
<td>2-3-b-11</td>
<td>Mars</td>
<td>Growth\Growth</td>
<td>Growth\Growth</td>
</tr>
<tr>
<td>2-3-b-12</td>
<td>Mars</td>
<td>Growth\Growth</td>
<td>Growth\Growth</td>
</tr>
<tr>
<td>1-3-t-15</td>
<td>Space</td>
<td>No Growth\No Growth</td>
<td>No Growth\No Growth</td>
</tr>
<tr>
<td>1-3-t-16</td>
<td>Space</td>
<td>No Growth\No Growth</td>
<td>No Growth\Growth</td>
</tr>
<tr>
<td>1-3-b-15</td>
<td>Space</td>
<td>No Growth\No Growth</td>
<td>No Growth\No Growth</td>
</tr>
<tr>
<td>1-3-b-16</td>
<td>Space</td>
<td>No Growth\No Growth</td>
<td>No Growth\No Growth</td>
</tr>
<tr>
<td>2-3-t-15</td>
<td>Mars</td>
<td>No Growth\No Growth</td>
<td>No Growth\Growth</td>
</tr>
<tr>
<td>2-3-t-16</td>
<td>Mars</td>
<td>Growth\No Growth</td>
<td>No Growth\No Growth</td>
</tr>
<tr>
<td>2-3-b-15</td>
<td>Mars</td>
<td>No Growth\No Growth</td>
<td>Growth\No Growth</td>
</tr>
<tr>
<td>2-3-b-16</td>
<td>Mars</td>
<td>Growth\No Growth</td>
<td>No Growth\No Growth</td>
</tr>
</tbody>
</table>
Table 6.3  BIOMEX/BOSS sample growth nine months. Red indicates BIOMEX biofilm samples with added P-Mars soil simulant; black indicates biofilm samples used in BIOMEX and BOSS; blue indicates BOSS planktonic samples. “t” and “b” denote top (light-exposed) and bottom (dark control) samples, respectively.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Condition</th>
<th>Liquid culture</th>
<th>Agar culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ground control</td>
<td>ground control</td>
</tr>
<tr>
<td>1-1-t-03</td>
<td>Space</td>
<td>No Growth</td>
<td>Growth \ No Growth</td>
</tr>
<tr>
<td>1-1-t-07</td>
<td>Space</td>
<td>No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
<tr>
<td>1-1-b-03</td>
<td>Space</td>
<td>No Growth \ Growth</td>
<td>Growth</td>
</tr>
<tr>
<td>1-1-b-07</td>
<td>Space</td>
<td>No Growth \ Growth</td>
<td>No Growth \ Growth</td>
</tr>
<tr>
<td>2-1-t-03</td>
<td>Mars</td>
<td>Growth \ Growth</td>
<td>Growth \ Growth</td>
</tr>
<tr>
<td>2-1-t-07</td>
<td>Mars</td>
<td>Growth \ No Growth \ No Growth</td>
<td>No Growth \ Growth</td>
</tr>
<tr>
<td>2-1-b-03</td>
<td>Mars</td>
<td>Growth \ Growth</td>
<td>Growth \ No Growth</td>
</tr>
<tr>
<td>2-1-b-07</td>
<td>Mars</td>
<td>Growth \ Growth</td>
<td>Growth \ Growth</td>
</tr>
<tr>
<td>1-3-t-11</td>
<td>Space</td>
<td>No Growth \ No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
<tr>
<td>1-3-t-12</td>
<td>Space</td>
<td>No Growth \ No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
<tr>
<td>1-3-b-11</td>
<td>Space</td>
<td>No Growth \ No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
<tr>
<td>1-3-b-12</td>
<td>Space</td>
<td>No Growth \ No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
<tr>
<td>2-3-t-11</td>
<td>Mars</td>
<td>No Growth \ No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
<tr>
<td>2-3-t-12</td>
<td>Mars</td>
<td>No Growth \ No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
<tr>
<td>2-3-b-11</td>
<td>Mars</td>
<td>Growth \ Growth</td>
<td>Growth \ Growth</td>
</tr>
<tr>
<td>2-3-b-12</td>
<td>Mars</td>
<td>Growth \ Growth</td>
<td>Growth \ Growth</td>
</tr>
<tr>
<td>1-3-t-15</td>
<td>Space</td>
<td>No Growth \ No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
<tr>
<td>1-3-t-16</td>
<td>Space</td>
<td>No Growth \ No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
<tr>
<td>1-3-b-15</td>
<td>Space</td>
<td>No Growth \ No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
<tr>
<td>1-3-b-16</td>
<td>Space</td>
<td>No Growth \ No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
<tr>
<td>2-3-t-15</td>
<td>Mars</td>
<td>No Growth \ No Growth</td>
<td>No Growth \ Growth</td>
</tr>
<tr>
<td>2-3-t-16</td>
<td>Mars</td>
<td>No Growth \ No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
<tr>
<td>2-3-b-15</td>
<td>Mars</td>
<td>No Growth \ No Growth</td>
<td>Growth \ No Growth</td>
</tr>
<tr>
<td>2-3-b-16</td>
<td>Mars</td>
<td>Growth \ No Growth</td>
<td>No Growth \ No Growth</td>
</tr>
</tbody>
</table>

As shown in table 6.2, all biofilm samples in the presence of P-Mars soil simulant (shown in red) were able to be revived in liquid culture after an initial three-month incubation period (except from sample 1-1-t-07). This was also supported by the ground controls, except in the case of 2-1-t-07, where there was no growth in
the ground control. Fewer samples could be revived on agar, with three showing no growth. Unlike in the liquid cultures, this did not include 1-1-t-07, which was able to grow on agar. There was more discrepancy between agar-grown samples and ground controls than between the liquid-grown samples and ground controls, with three out of four agar ground controls’ growth diverging from that of the ISS samples’ under space conditions. Importantly, between liquid and agar plate culturing, cells from all biofilm + P-Mars conditions were able to be cultured after exposure to the space or simulated Martian environments for 1.5 years. With regard to the biofilms exposed to the same set of conditions but without the addition of P-Mars soil simulant (shown in black), none of the samples - with the exception of 1-3-b-11 - were able to be cultured in liquid or on agar with the exception of the duplicate dark controls under simulated Martian conditions. Additionally, three of the eight liquid-cultured controls and half of the agar-cultured controls diverged from their respective samples’ viability. Cultured planktonic cells (shown in blue), i.e. not in a biofilm, presented the fewest viable samples. None of the samples from the ISS that were exposed to space conditions retained viability, nor their respective ground controls with the exception of 1-3-t-16. Samples 2-3-t-16 and 2-3-b-16, both exposed to the less harsh simulated Martian conditions showed growth in the liquid cultures, as well as 2-3-b-15 on the agar plate. None of their ground controls were culturable. The reduced viability after the space environment exposure is similarly reflected in the biofilm samples (shown in black). However, five out of eight of the biofilm samples still showed viability after simulated Martian condition exposure whilst only three out of eight planktonic samples were still viable after exposure to the same condition.

The nine-month growth table shown in tab. 6.3 indicates that in both the liquid and agar cultures, biofilm samples that had shown growth after three months appeared to have lost viability (i.e. no visible cell colonies). All biofilm samples that had been exposed to space conditions and cultured in liquid media had died after nine months of culturing, whilst all biofilm + P-Mars cultures and all dark control biofilm samples without P-Mars exposed to simulated Martian conditions survived. The nine-month agar cultures revealed three samples that were previously cultivable that had died after the further six months and a further two (1-1-b-03 and 2-1-t-03) showed growth after previously not appearing to be revivable. There also appeared to be more discrepancy between samples and ground controls in the liquid samples, with ground controls thriving where samples showed no growth and one case of ground control death (2-1-t-07). The number of sample-ground control discrepancies in agar cultures remained
the same. However, two out of three conditions were different from the ones in the three-month growth table. Three out of four biofilm space samples on agar showed no growth after nine months, whereas only one out of four Mars simulation samples appeared to have died. In contrast, all biofilm samples save 2-3-b-11 & 12 showed no sign of growth after nine months in accordance with their respective ground controls. Fewer planktonic samples were still viable than at the three-month time point. Only one sample in liquid culture (2-3-b-16) and one on agar (2-3-b-15), both dark control Mars simulation samples, still showed signs of viability. All planktonic ground controls except that of 2-3-t-15 were not culturable.

In conclusion, the growth assay showed that the dark control biofilm samples with and without added P-Mars under simulated Martian conditions were able to produce the highest number of viable samples, whilst the planktonic light-exposed and dark control cells under space conditions produced no reculturable samples. Biofilm samples without P-Mars showed the most viable samples under dark control simulated Martian conditions, with no light samples surviving the exposure to space conditions.

6.4.2 Fluorescent microscopy

In order to assess the nature of damage to cells under the various conditions, multiple fluorescent stains were applied and analysed under the microscope in addition to autofluorescence and bright field microscopy. Bright field images of samples show the clumped colonies of cells and the distinctive green colouring of the cyanobacteria as seen in fig. 6.2. However the retention of pigmentation is not indicative of undamaged cells. Internal damage may still have been sustained, which can be visualised by the use of stains. Samples used for microscopy had been stored at $-80^\circ$C leading up to analysis. The following staining techniques were used to reveal different characteristics of potential cell damage:

- Live/Dead stain with SYTO9 & propidium iodide (PI)
- Fluorescein diacetate (FDA)
- DiBAC$_4$(3)
Live/Dead

The SYTO9 PI live dead stain is a two-component stain, which indicates cell viability as a function of membrane integrity. SYTO9 is green fluorescing and permeates all cell membranes and stains nucleic acids. PI on the other hand cannot permeate cell membranes except if they are ruptured and stains the nucleic acids red. Below are selected representative live/dead images from various conditions.

Figure 6.6 shows biofilm samples with intact membranes fluorescing green to a lesser (a) and greater (b) extent, which corresponds to the cells being subjected to incoming light with no cut-off filters in the space condition (a), and cut-off filters at 200 nm for the simulated Martian condition (b). Both samples had been exposed in the presence of P-Mars. This apparent retention of viability corresponds to the three-month growth assay data, which found viable cells from these conditions. The samples exposed to space and simulated Martian conditions without added P-Mars (c,d) showed enhanced membrane damage shown by a dull green fluorescence, which correspond to the diminished growth in cultured samples after three months. Similar to the samples in (a) and (b) with P-Mars, the sample exposed to space conditions (c) appears to have sustained more damage than the Mars simulation sample (d).

The planktonic cells from BOSS exposed to the space and simulated Mars environments (e,f) also showed fewer live cells than the BIOMEX samples exposed in the presence of P-Mars and continues the trend of the greater ability of P-Mars to protect cells that are exposed the simulated Martian environment than those exposed to the space environment. Despite the culture data showing reduced viability of planktonic BOSS samples in comparison to the biofilm samples, the live/dead stains reveal sections of live, bright green fluorescing cells, especially in the Mars simulation sample (f).
Figure 6.6  Live/Dead stain showing intact membrane integrity in green of light-exposed biofilm cells under space (a) and simulated Martian (b) conditions in the presence of P-Mars; Biofilm samples without added P-Mars after space (c) and simulated Martian (d) environment exposure; Planktonic cells without P-Mars under space (e) and simulated Martian (f) conditions.
**Fluorescein diacetate (FDA)**

FDA is itself non-fluorescent. However, when accumulated and hydrolysed by live cells it becomes fluorescent; dead cells cannot hydrolyse FDA and do not fluoresce. This stain can thereby indicate whether a cell is enzymatically active (green) or not. This gives an extra dimension of information regarding cell damage as the live/dead stain on its own cannot show us whether a ‘live’ cell is metabolically active.

In line with the live/dead stain results, fig. 6.7 shows the P-Mars containing space-exposed sample fluorescing slightly in the centre (a), indicating possible enzymatic activity. The P-Mars sample exposed to simulated Martian conditions showed strong fluorescence (b). Both samples exposed to space and simulated Martian conditions without added P-Mars showed no signs of enzymatic activity (c,d).

1-3-t-15 containing light-exposed planktonic cells exposed to space conditions shows very weak signs of enzymatic activity in the top left corner of the photo (e), whilst dark control cells exposed to the simulated Mars environment showed greater activity along the periphery of the cell, indicating protection offered by the surrounding cells that showed no enzymatic activity (f).
Figure 6.7 FDA stain showing metabolic activity in green of light-exposed biofilm cells under space (a) and simulated Martian (b) conditions with added P-Mars and without (c,d); Planktonic cells exposed to space (e) and simulated Martian (f) conditions.
**DiBAC$_4$(3) and autofluorescence**

Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol, or DiBAC$_4$(3) for short, is a potential-sensitive molecule that can enter depolarised cells and bind to proteins or the membrane staining them green. Thus, green staining indicates the loss of potential. The DiBAC$_4$(3) images are shown in conjunction with the autofluorescence of the cyanobacteria.

One key observation was the lack of membrane potential (green) accompanying a lack of autofluorescence in the samples (fig. 6.8 & 6.9), suggesting damage as the loss of membrane potential could be coupled with further cell damage. Cells subjected to space and simulated Martian conditions in the presence of P-Mars showed some loss of potential, but intact autofluorescence (fig. 6.8). In contrast, the sample that lacked P-Mars showed a complete loss of autofluorescence in certain parts of the culture when exposed to space conditions, accompanied by green fluorescence indicating loss of membrane potential (fig. 6.9). However, Mars simulation samples were still able to retain their autofluorescence and not suffer substantial membrane potential loss in the absence of P-Mars. Planktonic samples in fig. 6.10 showed no extensive signs of fluorescence, indicating an intact membrane potential and retention of autofluorescence after exposure to both space (a,b) and simulated Mars (c,d) conditions.
Figure 6.8  DiBAC$_4$(3) stain showing lack of membrane potential in green (a) and natural autofluorescence in red (b) of light-exposed biofilms under space (a,b) and simulated Martian (c,d) conditions with added P-Mars.
Figure 6.9  DiBAC$_4$(3) stain showing lack of membrane potential in green (a) and natural autofluorescence in red (b) of light-exposed biofilms under space (a,b) and simulated Martian (c,d) conditions without added P-Mars.
Figure 6.10  DiBAC$_4$(3) stain showing lack of membrane potential in green (a) and natural autofluorescence in red (b) of light-exposed planktonic cells under space (a,b) and simulated Martian (c,d) conditions without added P-Mars.
6.4.3 Transmission electron microscopy

Transmission electron microscopy (TEM) allows for high magnification and resolution of ultra-thin fixed sections of cells and their interior. TEM was performed on select samples to further visualise cell integrity (fig. 6.11).

The samples with added P-Mars (a,b) show what appears to be the loss of the gelatinous sheath (dark grey) in samples exposed to space conditions but appear to remain largely intact. The sheath of Mars simulation samples is still visible around the cells (a,b; white arrows). In contrast, the samples exposed to space and simulated Martian conditions without the addition of P-Mars (c,d) show increased cell lysis and lack of sheath (white arrow). Similarly, the planktonic cells exposed to both space and simulated Mars environments (e,f) show the dark sheath detached from the cell and cell lysis. In addition, there was evidence of a smaller bacteria species (dark spots in c,d,e & f; blue arrows) that was sometimes found within the lysed cyanobacterial cell, such as in (f) (blue arrow). The additional bacteria will be further investigated in section 6.4.6.
Figure 6.11  TEM of ultra-thin sections of representative cell cultures under space conditions with P-Mars (a), simulated Martian condition with P-Mars (b) and without P-Mars respectively (c,d); Planktonic cells shown after space (e) and simulated Mars (f) environment exposure.
6.4.4 Scanning electron microscopy

In addition to TEM microscopy, SEM microscopy was performed, which enables high-resolution 3D images of surface topography. SEM was performed on one sample (2-1-b-3, a BIOMEX dark control biofilm sample exposed to the simulated Mars environment) to visualise general structural damage to the cells and in the hope of capturing evidence of the smaller bacteria seen in the TEM photos in fig. 6.11. Figure 6.12 (b) and (c) clearly show a significant rupturing of the cyanobacterial membrane (arrows). Picture (c) additionally shows an example of a puncturing of the cell membrane with the cell maintaining most of its structural integrity. Additionally, picture (c) shows a variability of cyanobacterial surface texture of either smooth or “framboidal”. Picture (d) shows surface images of the suspected co-cultured rod-shaped bacteria (arrows), showing a cyanobacteria-bacteria size ratio which is in accordance with the size ratio shown in the TEM images.
Figure 6.12 SEM of BIOMEX sample 2-1-b-3.
6.4.5 Raman spectroscopy

Raman spectroscopy was used to assess whether, and to what extent, the cyanobacteria’s carotenoids had been damaged. Characteristic Raman scattering allows for the identification of chemical compounds and crystal structure. Raman analysis of biological samples can result in very noisy signals. However, carotenoids lend themselves well to this analysis technique as they have a very distinct signal pattern consisting of three main peaks at $\sim 1000$, $1150$ and $1500$ cm$^{-1}$ (corresponding to a C—CH, C—C and C=C chemical bond, respectively) when excited at 514 nm (ground control, fig. 6.13) and are considered key biomarkers for life.

![Figure 6.13](image)

**Figure 6.13** Raman spectra (excitation at 514 nm) of ground control showing three characteristic carotenoid peaks at 1004, 1156 and 1518 cm$^{-1}$.

Peak heights indicate substance concentration (in this case carotenoid) and were used to demonstrate the state of preservation or damage to the cell. Peak heights are shown in table 6.4. When comparing the two light-exposed biofilm samples (1-1-t-3 & 2-1-t-7, fig. 6.14) that had the added P-Mars and were exposed to a space and simulated Martian environment respectively, it can be seen that the latter has much higher peaks. Radiation is a major threat to biomarkers as it degrades organic matter. It is therefore not surprising that the samples exposed with cut-off filters were less degraded than those exposed to unfiltered radiation in space. When comparing space-exposed biofilm samples with and without P-Mars (1-1-t-3 & 1-3-t-11) there is little difference between the peak heights. However, the Mars simulation samples in the presence of P-Mars (2-1-t-7) showed an increased peak height in comparison to the sample not containing P-Mars (2-3-t-11). When comparing samples that had been exposed to space and Martian conditions without added P-Mars (1-3-t-11 & 2-3-t-11), the latter showed a higher intensity and clearer Raman signal than the former. However,
the Mars simulation samples with P-Mars (red in table) showed much greater retention of signal than that without added P-Mars (black in table). When the intensities are overlaid (fig. 6.15) we get a clearer picture of signal clarity as well as peak height. The clear peak of 2-1-t-7 (light-exposed biofilm with P-Mars under simulated Martian conditions, in red) corresponds to the low damage the fluorescent stains showed in comparison to the other conditions. A dark control sample from the same condition (2-b-1-7) was added for an additional comparison between light-exposed and dark sample signal retention.

The planktonic cell samples (1-3-t-15 & 2-3-t-15, blue in table 6.4) showed the sample exposed to the space condition (1-3-t-15) having higher peaks than the Mars simulation sample (2-3-t-15) – a reversal of what both biofilm samples demonstrated. Additionally, when compared to both biofilm samples (red & black in table) exposed to space conditions, the peak heights are comparable but generally higher than both biofilm carotenoid peaks. Yet, when compared to biofilm peaks under simulated Martian conditions, the planktonic cells’ carotenoid peaks are consistently lower than those of the biofilms. Moreover, in context of the other conditions (fig. 6.15), the planktonic samples (1-3-t-15 & 2-3-t-15, in blue) show the deterioration of signal clarity in spite of relatively tall peak height of 1-3-t-15, as seen in table 6.4. Figure 6.15 (a) shows the Raman spectra of the samples without a baseline correction, while (b) shows the peaks with the baseline subtracted for comparison. The baseline was removed by subtracting a quadratic function from the data, where $x$ is the Raman shift [cm$^{-1}$].

$$\text{baseline}(x) = a_0 + a_1x + a_2x^2$$

An example of the baseline is shown in fig. 6.15 (a) by the dashed line. The coefficients $a_0$, $a_1$ and $a_2$ were tuned to give a flat baseline for all spectra shown in fig. 6.15 (b).
Table 6.4  Raman peak heights of three characteristic carotenoid peaks at \( \sim 1000, 1150 \) and 1500 cm\(^{-1}\) from the various experimental conditions. Red indicates BIOMEX biofilm samples with added P-Mars soil simulant; black indicates biofilm samples used in BIOMEX and BOSS; blue indicates BOSS planktonic samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1-t-3</td>
<td>5815.7</td>
<td>23442.2</td>
<td>27852.9</td>
</tr>
<tr>
<td>2-1-t-7</td>
<td>18959.6</td>
<td>76591.1</td>
<td>92383.3</td>
</tr>
<tr>
<td>1-3-t-11</td>
<td>5889.2</td>
<td>22713.3</td>
<td>27660.6</td>
</tr>
<tr>
<td>2-3-t-11</td>
<td>10532.1</td>
<td>43016.4</td>
<td>56408.5</td>
</tr>
<tr>
<td>1-3-t-15</td>
<td>6132.8</td>
<td>26636.9</td>
<td>36880.2</td>
</tr>
<tr>
<td>2-3-t-15</td>
<td>3105.5</td>
<td>14076.9</td>
<td>18311.2</td>
</tr>
</tbody>
</table>
Figure 6.14  Raman spectra (excitation at 514 nm) of representative biofilm cell cultures under space conditions with P-Mars (a), simulated Martian condition with P-Mars (b) and without P-Mars respectively (c,d). Planktonic cells are shown under space (e) and simulated Martian (f) conditions without added P-Mars.
(a) Raman spectra without baseline correction. Example of baseline for 1-3-t-15 shown in black dashed line.

(b) Raman spectra with baseline correction.

Figure 6.15 Overlay of sample Raman spectra with relative intensity shown on the y-axis [arbitrary units].
6.4.6 Bacterial co-culture

Evidence of an additional bacterial species was observed in fig. 6.11, most likely to be the co-cultured, pigmented α-proteobacteria species. The secondary species was added to the BIOMEX and BOSS Gloecapsa samples to test the ability of the cyanobacteria to confer protection to an additional species.

Pink-coloured colonies believed to be the proteobacteria were observed on the agar plate cultures of the EXPOSE-R2 cyanobacteria after three months of culturing. However, the co-culture was unable to be isolated under lab conditions such as in BG-11 or nutrient broth media at room temperature or at 38°C. The bacteria were observed to be fluorescing green (alive) in the Live/Dead stains amongst the larger green or red (dead) cyanobacteria in both BOSS and BIOMEX samples (fig. 6.16 (a - d)). Figure 6.16 (a - d) shows live (green) cyanobacteria cells but also much smaller live cells within and around the cyanobacterial structures. Figure 6.16 (c) & (d) shows a “jet” of these smaller bacteria erupting from the larger cyanobacteria aggregate after slight pressure was applied to the sample under the microscope slide. At higher magnification, these smaller cells were clearly distinct from the cyanobacteria aggregates, as they showed no sign of sheath or aggregates that even single cyanobacteria demonstrate [218]. They were also detectable in the TEM pictures in fig. 6.11 and in fig. 6.16 (e & f, arrows). Coccoidal shapes corresponding to the smaller bacteria size are also visible in the SEM image in fig. 6.12 (d). In the TEM pictures, multiple smaller bacteria were often observed to be located within the ruptured larger cyanobacteria cells.
Figure 6.16  Evidence of co-cultured bacteria in live/dead stains and TEM pictures of both BIOMEX and BOSS samples.
6.5 Discussion

Space conditions and other extreme environments, such as the surface of Mars, can be simulated to a certain extent on the surface of the Earth. However, short of sending samples to other planetary bodies, sending samples into low Earth orbit (LEO) provides a practical way of obtaining accurate information on bacterial viability after exposure to similarly extreme conditions. LEO enables exposure to environmental factors generally outside the scope of simulation such as galactic cosmic rays (GCR), shortwave UV (< 200 nm) and microgravity. The European Space Agency’s (ESA) EXPOSE platform provides an opportunity for scientists to subject a variety of taxa and molecules to these extreme environments with objectives varying from endurance testing of materials to probing the preservation of biomarkers. Cyanobacteria samples provided by Prof. Charles Cockell’s group at the University of Edinburgh were sent to LEO and exposed for 1.5 years with the goal of determining the ability of Mars soil simulant (BIOMEX experiment) as well as biofilms (BOSS experiment) to protect cyanobacterial cultures from space and a simulated Martian environment. A secondary bacteria co-culture was also present in the BOSS and BIOMEX samples, to investigate ability of the cyanobacteria species to protect the viability of the secondary species.

BIOMEX

The BIOMEX experiment hypothesis is that regolith, in this case Mars simulant regolith, could confer protection for life and by extension biomarkers from the lethal conditions of space and a simulated Martian environment. Data from the BIOMEX experiment show a consistent picture of (light-exposed and dark control) biofilms in the presence of P-Mars having a higher number of viable post-flight samples, less severe structural damage and clear carotenoid signatures in comparison to samples in the absence of P-Mars. The Mars simulation samples show, unsurprisingly, the least damage to cells when compared to cells exposed to space conditions. With high-energy radiation being a major limiting factor of the space and simulated Mars conditions, the > 200 nm cut-off filter in the simulated Mars environment reduces the expected damage to organics in the samples. The mechanism of protection afforded by the P-Mars soil simulant may be two-fold. Not only do the mineral particles provide a reflective shield to cells, but the presence of iron oxide in the simulant, which is known to absorb in
the UV, may allow for an additional adsorption of certain harmful wavelengths \[150\]. Although light-exposed samples without P-Mars are mostly unviable and suffered various kinds of cell damage, dark control cells under simulated Martian conditions are still culturable without the presence of P-Mars. This demonstrates the effectiveness of a small amount of surface P-Mars or similar substrates to offer protection to cells, not only under simulated Martian conditions, but also under the harsher conditions of space.

In spite of the retained viability of P-Mars samples, results from the three-month growth assay did however not correspond to those of the nine-month assay with most of the samples showing no viability after nine months in culture. The drop in viability is unlikely to result from their exposure to space or simulated Martian conditions, as even samples that were previously viable become unculturable after nine months. A lack of nutrients or other stress factors may have caused the general lower viability than at the three-month culturing time point. Discrepancies between ISS and ground control growth data resulting in ground control growth and ISS lack of growth can be explained by the lack of highly-limiting ionising radiation (GCR and shortwave UV) in the ground control exposure. This was also the case for the BOSS ground controls.

Bacterial stains reveal cells with added P-Mars retain intact membranes, membrane potential, autofluorescence and enzymatic activity. Although cells exposed to the space and simulated Martian conditions without P-Mars show some signs of intact membranes (sample 2-3-t-12), there is no sign of enzymatic activity and a complete loss of membrane potential coupled with loss of autofluorescence in whole sections of the colonies. TEM images additionally highlight the increased damage that samples with no added P-Mars sustained, causing cell lysis and/or loss of the glutinous sheath that envelops healthy cells. Whilst cells with P-Mars exposed to space conditions also display the loss of sheath and lysis but to a lesser extent, Mars simulation samples appear to be mostly intact in comparison.

Raman spectroscopy was used to evaluate the presence and intensity of the cyanobacteria’s carotenoid signal, with a deteriorated signal corresponding to damage of the carotenoid and by extension the cell. Raman spectral analysis confirms the enhanced damage of samples exposed to both simulated Martian and space conditions in the absence of P-Mars in comparison to those with P-Mars. The best signal retention is seen in the sample exposed to simulated Martian conditions and with added P-Mars, indicating the potential for carotenoid
preservation on Mars under similar conditions. This is not only important for biomarker detection, but also from a planetary protection standpoint as it gives an indication of survival potential of contaminant organisms.

BOSS

The BOSS experiments were carried out to test the hypothesis that biofilms provide additional protection to a culture over single, planktonic cells. Whilst biofilm samples still sustained cellular damage and did not produce many reculturable samples, the Raman signal retention was much greater than that of planktonic cells. The protection by biofilms is supported by growth results from the planktonic samples. Planktonic cells show the lowest viability with only two samples in total being culturable and both under the less harsh, simulated Martian conditions. Although planktonic cells may show heightened viability if protected by an additional substrate, whilst not in a biofilm they are most exposed to the multiple extreme conditions of space and Mars-like environments. This is shown in fig. 6.11 (e & f) where planktonic cells show the loss of the gelatinous sheath and ruptured cells to a greater extent than in biofilm samples.

Interestingly, the planktonic stain results show more intact cells, despite the low viability of the growth assays. Live/dead stains show small patches of live cells, especially in the Mars simulation sample, as well as FDA results showing small areas of enzymatic activity. The planktonic DiBAC\(_4\)(3)-stained samples show the highest intact membrane potential couples with preserved autofluorescence out of all the conditions. In spite of the apparent preservation of cell integrity in the fluorescent microscopy pictures, the planktonic Raman spectra show the most deteriorated carotenoid signal out of all sample conditions. The planktonic spectra of the space-exposed samples are comparable in peak height to both biofilm samples, despite the lack of culturable cells from exposed to the space environment, which was initially surprising. However, when planktonic spectra from the simulated Mars conditions are compared to biofilm spectra exposed to simulated Mars conditions there is a clear hierarchy with biofilm samples having the clearest peak signatures, followed by planktonic samples. Moreover, when all spectra are overlaid and compared by signal clarity, the spectra of the planktonic cells were diminished in comparison to other conditions. The clearest signal is seen in the biofilm sample containing P-Mars and exposed to simulated Martian conditions, which are the least harsh and fits the rest of the results for this
condition, which is followed by the signal of non-P-Mars containing biofilms.

Discrepancies and unexpected results of the BOSS experiment’s planktonic samples may be due to the incomplete separation of cells from their aggregated form during the pre-flight preparation. *Gloeocapsa* cells naturally form aggregates, with daughter cells often remaining within the parental cell’s gelatinous sheath after mitosis [218]. Therefore, assuming the pre-flight separation was successful, any consequent growth may have resulted in the reformation of aggregates. Additionally, cells may have re-aggregated due to the desiccation process and clustered within the disc pores. This would explain the damage still caused by the absence of an intact biofilm, yet the retention of smaller aggregates would allow for a subset of cells to be cultured. Additionally, the smaller clumps of cells would be able to fit into smaller pores on the sintered discs before being desiccated. This would result in the “planktonic” cells being protected by the small aggregates as well as the discs themselves. Although it is shown to not be substantial enough protection to allow for a high number of culturable samples, it would explain the partially preserved membrane integrity and enzymatic activity. Evidence of these smaller aggregates persisting in alleged planktonic cell samples can be seen in the fluorescent stain microscopic pictures (figs. 6.6-6.10).

The *Gloeocapsa* species is of particular interest with regard to its biofilm. As stated in section 6.3.3, *Gloeocapsa* does not produce a “true” biofilm, rather an amorphous cellular mass that lacks in structure in comparison to a true biofilm. There have been previous studies showing the protective capacity of biofilms [214–216]. However, the BOSS results demonstrate that the *Gloeocapsa* proto-biofilm is also able to confer protection. This is interesting with regard to understanding how early bacteria colonies may have survived the climate of the early Earth.

**Inter-bacterial protection**

Whilst the focus of BIOMEX and BOSS was the protection from abiogenic and biogenic substances for one species, the additional hypothesis tested with these experiments was that of inter-species protection. The post-flight growth assays showed the first signs of the co-cultured, pink proteobacteria having survived LEO exposure. Although it was not observable on all agar plates (i.e. duplicates), it was observed for each condition. This is surprising in the case of the planktonic samples, as it would be expected that they provide little additional protection when compared to biofilm protective properties [214, 216]. However, as discussed
in the previous BOSS section, it is unlikely that the BOSS samples were truly planktonic but instead remained in smaller aggregates.

More compelling evidence of the cyanobacteria providing protection for the secondary bacteria is seen in the bacteria live/dead stain pictures (fig. 6.16 a-d). TEM pictures (fig. 6.11, fig. 6.16 e & f) shed more detailed light on the exact location of the co-culture. The proteobacteria was often observed to be near or in ruptured cyanobacteria cells, suggesting that they may actively contribute to the cyanobacteria’s damage. Conversely, they may have drifted into already damaged/ruptured cells and become stuck, but nonetheless shielded. Although the relationship between the two cultures is yet uncharacterised, the cyanobacteria structures clearly provide protection for the smaller bacteria, allowing them to survive 1.5 years in space & simulated Martian environments. This has significant implications for planetary protection. If an organism is determined to not be able to survive space or specific planetary conditions, and thereby not deemed a threat to planetary protection protocols, it may still be harbouring one or multiple additional species that were protected from extreme conditions by the known organism. Most bacterial cultures naturally exist in a multi-species environment as opposed to monocultures often grown in the labs, which may account for the vast amount of “unculturable” bacterial and archaean species to date [232]. As the likelihood of co-culture species’ presence is high, planetary protection protocols may have to be adjusted to take more realistic, multi-species contamination and respective protection into consideration.

The results of the BIOMEX, BOSS and co-culture experiments provide insight into viability in extreme, extra-terrestrial conditions. Firstly, they offer an understanding of what possible biomarkers of extinct or extant life we might hope to detect on Martian planetary exploration missions. Appropriate instrumentation design and testing to establish upper bounds of detectability can already influence upcoming Mars rovers such as ESA’s ExoMars rover or NASA’s 2020 Mars rover that are equipped with spectroscopic biomarker detectors. The data indicate that the preservation of carotenoids, especially if covered by Martian regolith (P-Mars-like), is likely and may render a detectible signal. Furthermore, the results of this chapter help us comprehend the general limits to life with regard to exposure to extreme radiation conditions and the probability of conserved viability. This also lends credence to the possibility of (litho-)panspermia, a hypothesis describing the propagation of organisms between planetary bodies via impact-expelled rocks, originally proposed by Lord Kelvin [233]. The model
organism *Gloeocapsa* is not evolved to endure the radiation environment, vacuum or temperature range of LEO, yet it was able to retain its culturability once returned to less hostile conditions. Moreover, it is able to provide protection to a secondary co-culture which is unlikely to have survived in monoculture form in space or Martian conditions. Most planetary protection-centric identification of contaminants has focused on single species [234, 235] or that has not considered shielding by co-cultured species. These data demonstrate that a more realistic and holistic analysis of contaminants and potential co-contaminants is necessary for an efficient implementation of planetary protection.

### 6.5.1 Limitations

Although sending samples to LEO allows for a more realistic exposure to space and simulated non-terrestrial environments such as Mars, space missions bring with them a set of stringent regulations that may impede experimental quality. One main issue with the BOSS/BIOMEX experimental set up was that it only accommodated duplicate samples. A future set up would benefit from forfeiting the number of conditions to allow for a greater number of replicates. A further limitation was the irreproducible factors of ionising radiation and microgravity conditions in the ground control samples. Ionising radiation is deemed a major limiting factor to viability [35]. Therefore, ground controls not exposed to a similar dose would be unlikely to highlight any additional damage caused to ISS samples solely by space flight, re-entry and other additional factors. Another limitation caused by having an ISS experiment is that the return of the samples is dictated by the re-entry schedule of the Soyuz capsule. BIOMEX and BOSS samples were stored inside the ISS for an additional 136 days before being returned to Earth. ISS-internal conditions are less harsh that outside. However, samples are still subjected microgravity and heightened radiation exposure. Conversely, the ISS-internal conditions may allow for cellular repair and recovery, which may alter initial results. A solution would be to fix the cells after an allocated exposure time, to constrain and preserve damage from exposure to the intended environment.
Additional limitations were listed in the post-flight report by Rabbow et al. [209]:

- UV sensors were unable to be re-calibrated. UV sensor data from the beginning of the mission were recorded. However, continuous UV fluence data is necessary for accurate evaluation of sample damage.

- ISS shadowing, i.e. shadows cast by structures on the ISS causing spatial gradients of radiation dose and temperature in samples.

- Pressure data was not measured by EXPOSE-R2 instruments, but was reliant on information from a secondary source.

A limitation more specific to the BOSS sample involves the selection of the model organism for the biofilm versus planktonic experiment. As *Gloeocapsa* cells clump even in their smallest units, such as after cell division, is difficult to estimate how “de-aggregated” the supposedly planktonic cell samples for BOSS were. Post-flight microscopy revealed clumping that was comparable to biofilm samples and may have skewed results.

### 6.5.2 Future work

The most surprising result of the post-flight analysis was the survival of the co-cultured bacteria, thought to be *Geminicoccus roseus* [223]. Future work focussing on isolating and sequencing the bacteria would enable conformation of its identity. Furthermore, ground experiments testing its viability and resistance to extreme conditions such as UV exposure without the protection of *Gloeocapsa* would reveal the extent of protection provided by the cyanobacteria and how necessary it is to the smaller bacteria’s survival in space-like conditions. Additionally, TEM images showing the co-cultured bacteria within ruptured cells of the cyanobacteria cells warrants further investigation. If it is possible to isolate cyanobacteria and smaller bacteria from each other, one could compare cell damage and cell rupturing between the cyanobacteria monoculture and when co-cultured with the additional bacteria.
6.6 Conclusions

To test the shielding properties of Martian simulant soil as well as biofilms, cyanobacteria were exposed to the conditions of low earth orbit and a simulated Martian environment for 1.5 years. Upon return to Earth they were able to be re-cultured. Biofilms in the BIOMEX experiment that were exposed in the presence of Mars soil simulant P-Mars showed the highest viability out of all conditions and the clearest biomarker Raman signal. Although biofilms without additional P-Mars were also able to be cultured after space and simulated-Mars exposure, fluorescence microscopy revealed the higher extent of damage sustained by biofilms without P-Mars. While fluorescence microscopy of the planktonic cells in the BOSS experiment showed less damage than the biofilms, the culturing and Raman analysis showed extensive damage to the cells. These results demonstrate the increased protection from extreme radiation conditions conferred by biofilms as well as the supplementary protection that a small amount of regolith can provide. In addition to factors enabling the survival of single species exposed to multiple extreme conditions, the bacterial cultures themselves were shown to provide physical protection for smaller co-cultivated species. Together, these results illustrate the need for future planetary protection studies and protocols to take the threat of multi-species contaminants into consideration.
Chapter 7

Non-linear dose-risk relationship of bacterial growth in ultra-low radiation environments

7.1 Introduction

The effects of high radiation exposure on life have been extensively researched. However, the effects of the lack of radiation have received limited attention from the scientific community, and are still largely unclear. The widely accepted and implemented model of radiation-dose relationship is the linear no-threshold (LNT) model, which is currently challenged by various new models such as the hormesis model. The LNT model predicts a positive linear correlation between radiation dose and damage caused to an organism, whilst the hormesis model predicts high-dose inhibition but low-dose stimulation, suggesting that doses of low radiation have beneficial effects for an organism. Experiments to date have not yet been able to clearly dismiss competing models. The aim of our collaborative Subsurface Experiment of Life in Low Radiation (SELLR) project was to put the LNT model to the test, using bacterial growth assays performed under ultra-low ionising radiation in the Boulby International Subsurface Astrobiology Laboratory (BISAL) facilities of the Boulby mine (Northeast England). This chapter presents the initial findings of the SELLR experiments and how they contribute to the understanding of prokaryotic radiation-responses.
7.2 Background

Radiation research is often conducted in the context of effects of, and protection from, high radiation exposure such as radioactive fallout, medical appliances and space travel. The connection between irradiation from an ionising or an energetic radiation source and biological damage resulting in mutations or cell death has been well established \[5\–7\]. However, the effects of ultra-low level radiation on prokaryotic and eukaryotic organisms has yet to be thoroughly researched.

All life on Earth is constantly being subjected to a low dose of naturally occurring radiation in various forms. UVA (315 - 400 nm) and UVB (280 - 315 nm) light from the sun can pass through the Earth’s atmosphere and reach us, sometimes causing skin melanomas \[8\]. Rock radionuclides from various long-lived radioisotopes, such as uranium, thorium and potassium, and cosmic rays are further sources of natural radiation. Moreover, airborne radon and its decay radionuclides contribute to more than half the total average worldwide dose rate of naturally occurring radiation of \(\sim 2.8 \text{ mGy/y}\). With additional man-made radiation the average global surface radiation dose rate is estimated to be \(\sim 3.01 \text{ mGy/y} \) \[53\]. Usually, doses/dose rates below the global or local background dose average are referred to as low radiation doses. An exception of this can be found in medically relevant research, which often utilises the terminology “low” even when dose/dose rates above that of the average surface radiation are being discussed.

Since the mid-20\textsuperscript{th} century the preferred model describing an organism’s radiation dose-response relationship is the “linear no-threshold” (LNT) model. This model predicts a positive linear correlation between total radiation dose and risk of damage to cells, so even a single electron transversing a cell is considered to elevate the risk of cell damage. LNT is currently the most widely accepted model regarding the radiation dose-damage relationship, and is supported by the National Research Council’s recommendation of its use in their “Biological Effects of Ionising Radiation VII Report” \[236\], which also addresses the prediction of low-dose effects. The LNT model is largely based on studies on above-background, i.e. high radiation exposure. It was originally developed to describe radiation-induced mutations in rats in the early 20\textsuperscript{th} century \[237\] in the context of evolution, and subsequent research on fruit fly exposure to high radiation doses (\(10^3 \times \) background dose) served as the basis for the LNT model \[112\]. However, more recent studies have indicated that when dealing with lower doses, the LNT
model may not be applicable [116, 238, 239].

Despite the long-standing application of the LNT model, it is strongly contested in the literature by proponents of the hormesis (hormetic) model. The principle of hormesis is based on the low-dose stimulatory effect of a substance that, at high doses, has an inhibitory effect [23, 240, 241]. Various researchers present that the LNT model is not representative of results of cells’ behaviour at lower radiation doses and dose rates [116, 239]. Despite the research into alternative models to LNT to fit observed effects, there are multiple reasons why hormesis hasn’t successfully replaced LNT to date, as described by Mossman [242]:

- Pro-hormesis data regarding humans are limited and are often re-evaluations of previous epidemiological studies that had been designed for a different purpose.

- Documented hormetic results are often weak, inconsistent and have large statistical variation.

- There is still no consensus amongst scientists as how to define and quantify hormesis.

- It remains unclear as to how hormesis would be integrated into the health system’s regulatory framework, especially regarding exposure to doses that exceed safety limits.

The apparent lack of consensus and confusion in the literature with regard to the definition and quantification of the hormetic model also extends to the lack of disentanglement of eukaryotic and prokaryotic effects. It is reasonable to presume the LNT model is also applicable for prokaryotes at higher doses. However, there is still no agreement within the scientific community on the effects of low and ultra-low radiation exposure to microbes (fig. 7.1).

The experiments described in this chapter will solely focus on the effects on prokaryotic cells, which may not be representative of eukaryotic responses, in an attempt to fill in the unknown responses with data from a controlled environment. This study reports phenomenological observations, not mechanistic proposals or any attempt to fit data to a particular model.
Figure 7.1 Prokaryotic dose-risk model, based on the current standpoint of knowledge, with a linear relationship for higher doses & unknown effect of background and sub-background radiation doses.

See section 2.5 in the background chapter for an illustration of suggested radiation dose-response models.

7.3 Methods

There have been previous sub-background radiation experiments carried out in various facilities by research groups looking at a variety of cells and with various experimental set-ups. However, there have consistently been aspects of the set-up that were overlooked or not taken into account. These factors may have had an impact on the resulting data and should be re-evaluated before being used to construct the dose-risk model.

Factors such as:

- Lack of underground controls. Differences such as air pressure and humidity between surface and sub-surface environments can skew bacterial growth results.
- Model organisms used were known to be radio-resistant and would not show representative effects of low radiation exposure of the majority of microbes.
• Use of end-point analysis of growth curves, which are not representative of real time microbial reactions to the environment, and can include effects of complex biochemistry.

• Lack of replicates & time points for growth data.

• Only two doses tested (background radiation & low radiation); insufficient to plot or rule out models.

• Reliant on simulation of low radiation environment dose, with no actual in situ confirmation of actual dose environment.

In response to the previous sub-background radiation research, I designed simple, comprehensive bacterial growth experiments, that take the above-mentioned factors into account, and approached the Boulby Underground Laboratory for their collaboration in experimental set up and execution.

The following experimental design adjustments were adopted to avoid some of the potential variables unaccounted for in previous, similar studies.

• Underground controls were used with a simulated surface radiation dose exposure, all other parameters were identical.

• Well-characterised environmental model organisms were used to represent Gram positive & negative microbes.

• Analysis of radiation impact on the linear exponential growth phase, as microbes are most sensitive to environmental input during this phase.

• Continuous measurement of growth curves to match bacterial doubling time, large number of replicates, and experiment repetition.

• low, simulated background, 10× and 100× simulated background radiation doses were used in identical experimental set-ups for multiple dose data points.

• Simulation and in situ measurement of low and simulated background radiation environment.

Details of these new experimental measures are described in the following sections.
Unless specifically stated, all experiments were carried out in the Boulby Underground Lab at the Boulby Mine, Cleveland UK. At 1.1 km depth, the rock accounts for a cosmic ray attenuation by a factor of $10^6$. Boulby is particularly well-suited to providing radiation protection as the local salt is itself very radio-quiet, with low internal $\gamma$, neutron, and radon levels resulting in doses of $>3$ Bq/m$^3$. However, in order to establish an ultra-low radiation environment, the experiments were additionally encased in a lead-lined container. This resulted in a 0.01 mGy/y low dose rate; a 100-fold reduction of the average ionising surface radiation dose rate. A $^{137}$Cs source was placed above one of the plate readers to mimic a surface radiation dose rate of 1 mGy/y and served as a control to the low radiation environment.

In order to minimise any unknown variables, both the simulated background and low radiation experiment’s set up was as similar as possible. Two identical SPECTROstar Nano plate readers (BMG Labtech) were positioned in either half of the lead-lined box (or “castle”), separated by a 10 cm-thick lead internal wall. The temperature of both plate readers was internally controlled, the outside temperature was constant at $22^\circ$C ($\pm1^\circ$C) and 82 % humidity ($\pm2\%$). $B.\ subtilis$ or $E.\ coli$ were grown in the simulated background and low radiation environments for 24 and 48 h. The dose rate was also increased to 10× and 100× simulated background by adjusting the distance of the source to the samples (see section 7.3.4). Growth rate and gradient of the bacterial linear exponential growth phase were assessed, to establish whether either radiation environment had an effect on the bacterial growth. Additional UVC exposure after growth in the various radiation environments was performed to assess any difference in the cells’ ability to cope with sudden stress.
Figure 7.2 SELLR lead-lined “castle” with its lid being closed (a) and with an open lid. The white $^{137}$Cs source is visible on an elevated platform above plate reader. The platform height was altered to achieve higher radiation doses ($10 \times$ and $100 \times$ simulated background radiation (b)).
7.3.1 Model organism selection

*Bacillus subtilis* (strain 168, DM 402) and *Escherichia coli* (MG 1655) were chosen as model organisms to test the effect of low radiation doses on microbial growth. Both microbes are well-characterised environmental bacteria that can be cultured in the lab and were chosen to represent Gram positive and negative bacteria. Both have approximately the same, short doubling time of \(~20\) min, which allows for an efficient production of multiple growth curves. Neither is classed as radio-resistant, unlike other model organisms used in previous studies (e.g. extremophile *Deinococcus radiodurans*) [243], to maximise the likelihood of detecting a response to the low radiation doses.

7.3.2 Internal radiation

Due to the need to ensure an ultra-low radiation environment, the internal radiation of both the equipment and the samples had to be established to rule out any contribution to the final radiation dose. Germanium detectors were used (in Boulby Underground Laboratory) to measure the various radioactive nuclide contributions of the plate reader, plates, bacteria sample and media. The nuclide activity was determined from a \(\gamma\)-spectrum analysis. The detailed nuclide contribution of the various components is listed in appendix [B]. Table 7.1 shows the bacterial radiation contribution. The highest internal nuclide activity from the experimental components was potassium (\(^{40}K\)), which undergoes \(\beta\)-decay, and in most cases also \(\gamma\)-decay. Values of other nuclides are upper limits as they are below the sensitivity of the detector.

<table>
<thead>
<tr>
<th>Nuclide series</th>
<th>Activity in Bq/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{238})U(e)</td>
<td>-</td>
</tr>
<tr>
<td>(^{238})U(l)</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>(^{232})Th(e)</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>(^{232})Th(l)</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>(^{40})K</td>
<td>11.5 ± 1.5</td>
</tr>
<tr>
<td>(^{60})Co</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>(^{235})U</td>
<td>&lt;0.8</td>
</tr>
</tbody>
</table>

Table 7.1 Activities of nuclide series in the bacterial sample
The expected dose rate due to potassium is

\[ D_{K-40} = 0.0273 \text{ mGy/y} \pm 0.00352 \text{ mGy/y} \]  \hspace{1cm} (7.1)

and the expected maximum absorbed dose rate due to other nuclides of

\[ D_{\text{other}} < 0.17104 \text{ mGy/y} \]  \hspace{1cm} (7.2)

This value would be too high for the low-radiation experiments. However, the calculated dose rate of nuclides excluding potassium is the expected maximum. However, to be certain of the actual experimental radiation environment, additional in situ measurements were also carried out (section 7.3.4) to place more realistic constraints on the upper dose rate boundary.

All internal dose measurements were carried out by Emma Meehan in the Boulby Underground Lab germanium detector facility, Cleveland UK. All internal dose calculations were carried out by Leander Cascorbi (Trinity College Oxford University).

### 7.3.3 Radiation simulation

To begin to understand the probable radiation environment in the lead-lined “castle”, the entire experimental set up was constructed in GEANT4, and known radiation sources were added to simulate the resulting radiation. GEANT4 is a toolkit using Monte Carlo methods for simulating the passage of particles through matter. This simulation was performed to provide insight into possible daughter particle radiation that must be taken into consideration when establishing the simulated background and low radiation environments.

Radiation from a variety of sources was simulated, including the \(^{137}\text{Cs}\) source, radon from the air, the surrounding bedrock, and lead in the “castle”. It was concluded that gamma rays from the decay chains of uranium-thorium-potassium contamination in lead and the decay products of airborne radon would result in a \(\sim 0.1 \text{ mGy/y}\) contribution to the radiation background in both halves of the “castle”. However, as the samples would be shielded inside the plate readers from the airborne radon its contribution can be discounted. Additionally, the simulation showed the \(^{137}\text{Cs}\) source contributing the extra dose rate of 314 nGy/year to the shielded, low-radiation part of the “castle”. The summary
of simulated $\gamma$-radiation can be found in appendix B.

All GEANT4 simulations and calculations were carried out by Athoy Nilima (University of Edinburgh).

Figure 7.3 Geometry of lead-lined chamber in GEANT4, view from above. Lead wall is marked in red, separating the container into a low and simulated background radiation environment.

7.3.4 Simulated background and low radiation environments

Theoretical dose rates

In order to establish control samples that were exposed to the same environmental factors as the low-dose samples with the exception of radiation, samples were placed in the same lead “castle” as the low-dose experiments, 1.1 km underground. However, controls were exposed to a 15.5 MBq ($\pm$ 0.1 MBq) $^{137}$Cs $\gamma$-source to simulate a natural surface background radiation dose rate. The unattenuated source provided a dose rate of $\bar{D}=27.8$ mGy/y ($\pm$ 0.506 mGy/y) at a 59 cm height from the top of the 96-well plate, which was lowered to 1 mGy/y by placing lead sheets between the source and samples. It was calculated that 11 lead sheets (3.3 cm thickness) would be required to obtain the required dose rate. To generate higher dose rates the inverse square rule is used.

$$D(r) = \left(\frac{59 \text{ cm}}{r}\right)^2 \times D(59 \text{ cm}) \quad (7.3)$$

130
However, as the distance between source and sample is decreased, the difference in dose rate across the plate becomes more significant. It was calculated that for our maximum dose rate used (100 mGy/y) the maximum difference in dose rate across the plate would be 3.3 mGy/y.

**Measured dose rates**

A series of measurements were taken to establish the *in situ* dose rates of the simulated background and low radiation environments. The measurements were taken with a NaI detector (InSpector 1000 with an IRPOS-2: Stabilised 2” × 2” probe), which gives real time values with a minimum dose rate equivalent of 10 nSv/h (0.088 mSv/y). Measurements of the unattenuated source $\dot{D}(3.3 \text{ cm})$ yielded an absorbed dose rate of $\dot{D}(0) = 29.9 \text{ mGy/y} \pm 0.5 \text{ mGy/y}$ and an 11 lead sheet-shielded dose rate of $\dot{D}(3.3 \text{ cm})=1.067 \text{ mGy/y} \pm 0.2 \text{ mGy/y}$, which lies within our expected boundaries. The higher measured value for the unattenuated source dose rate can be explained by measurement errors and backscattering of photons from the walls of the lead “castle”. The measured dose rate within the “castle” with the lid closed and without the source was 0.0044 mGy/y, a reduction to $\sim 1\%$ natural background radiation (measured local surface dose rates ranged from 0.18 to 0.6 mGy/y).

All dose calculations were carried out by Leander Cascorbi (Trinity College Oxford University). *In situ* dose measurements were carried out by Leander Cascorbi and Sean Paling (Boulby Underground Lab, Cleveland UK).

### 7.3.5 Bacterial growth

Because of the limited access to the mine, I performed the growth experiments in Boulby in blocks of 5-6 days at a time. The terminology of “week 1, 2..” refers to the week in which the set of experiments was carried out, for ease of comparison (tab. 7.2). Vegetative cells of *Bacillus subtilis* strain 168 (DM 402) and *Escherichia coli* (MG 1655) were used in the experiments. These are well-characterised organisms that serve as general environmental model organisms that are not radio-resistant. To obtain aliquots for experiments, a monoculture of each was grown at 37°C before being frozen into 25% glycerol 1 mL aliquots and stored at $-80^\circ$C. To obtain overnight cultures for experiments, 40 mL nutrient broth was inoculated with 10 $\mu$L thawed aliquots, respectively, and cultured overnight.
at 37°C before use in the experiments. The concentration of starting cultures was always > 0.9 optical density (OD$_{600}$), approximately $10^7$ CFU/mL. At the beginning of each growth experiment, 5 µl of culture was added to 84 wells of a 96-well plate containing 200 µl fresh nutrient broth. The last row of the plate contained only nutrient broth to serve as a blank for the plate reader. _B. subtilis_ was grown at 30°C and _E.coli_ at 37°C respectively for 24 h, with OD$_{600}$ measurements being taken every 20 minutes to coincide with both bacterial doubling times. For the 48 h experiments, 5 µl of the 24 h culture was added to 84 wells of a 96-well plate containing 200 µl of fresh nutrient broth and grown for 24 h under the same conditions as it was in the previous 24 h. The reason for having both 24 and 48 h growth data was to determine whether an extended period of exposure to low radiation environments accentuated any observed responses in bacterial growth. This also gave us information on an increased number of generations, with 48 h resulting in $\sim 144$ generations for both _B. subtilis_ and _E. coli_.

### 7.3.6 UV exposure

In order to assess whether the microbes’ ability to cope with stress was influenced by growth in the various radiation environments, cells were exposed to a short pulse of UVC and their viability assessed by plating. After being grown for the specified amount of time in the respective radiation environments, the plates were irradiated with a monochromatic UVC lamp ($\lambda = 254$ nm; $I = 11.2$ W/m$^2$) at distance of 5 cm for 30 s. Triplicates were selected from random wells of each condition and serially diluted by a factor of $10^6$. One hundred microlitres of each triplicate was plated in triplicate on Oxoid (LP0011) nutrient agar and cultured overnight at 37°C.

### 7.3.7 Experiment nomenclature

Three replicate low and simulated background experiments were run with _B. subtilis_. For clarity when presenting these results (fig. 7.4), each set of experiments will be referred to with the nomenclature in table 7.2 to differentiate between experimental runs. Whether the data is from the low or simulated background environment, and whether it is from the 24 or 48 h growth curves, will be stated in the text. Henceforth, the one half of the experimental set up with 1%
background radiation environment will be referred to as the “low” condition. The other half of the experimental set up, containing the $^{137}$Cs source will henceforth be referred to as the “simulated background” condition.

Table 7.2  SELLR experiment nomenclature for B. subtilis replicate experiments (shown in fig. [7.4]) indicating growth time, radiation exposure and the week of experimental execution; sim = simulated.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Growth time</th>
<th>Radiation exposure</th>
<th>Week</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>24 and 48 h</td>
<td>Low and sim. background</td>
<td>Week 1</td>
<td>B. subtilis</td>
</tr>
<tr>
<td>B2</td>
<td>24 and 48 h</td>
<td>Low and sim. background</td>
<td>Week 2</td>
<td>B. subtilis</td>
</tr>
<tr>
<td>B3</td>
<td>24 and 48 h</td>
<td>Low and sim. background</td>
<td>Week 3</td>
<td>B. subtilis</td>
</tr>
</tbody>
</table>

7.3.8 Analysis

All plate reader experiments were performed using 84 replicates; UV experiments were performed in triplicate with each triplicate being plated on three agar plates. Numbers in figures show averages of replicates, error bars show standard error (s.e.) among replicates. Growth gradients of linear exponential growth phases were established by an automated, linearly-fit trendline in excel. Error of gradients was calculated using the LINEST function in excel. Growth rates were calculated with the equation:

$$
\mu = \frac{lnOD_2 - lnOD_1}{t_2 - t_1}
$$

(7.4)

which when converted to the decadic logarithm becomes

$$
\mu = 2.303 \times \frac{(logOD_2 - logOD_1)}{t_2 - t_1}
$$

(7.5)

OD$_1$ and OD$_2$ as well as respective $t_1$ and $t_2$ were determined in each growth curve as the beginning of the linear exponential phase. Statistical analysis was performed using one-way ANOVA and two-tailed unpaired equal variance Student’s t-tests, where $p < 0.05$ was considered significant. Analysis of the bacterial growth curves focused on the linear exponential growth phases, which were most comparable. When a “shoulder” in the exponential growth phase was observed, only data from the primary linear phase was used to calculate gradients.
7.4 Results

7.4.1 Bacterial growth at low and simulated background radiation

Initially, both plate readers were tested on the surface in the Cockell lab at Edinburgh University and/or on the surface facility at Boulby, to ensure there were no stochastic results between plate readers when identical experiments were simultaneously run, and to avoid any false positives of deviance in experimental data. Both cultures of isogenic *B. subtilis* and *E. coli* were grown up in both plate readers to obtain growth curves of at least the linear exponential growth phase. The surface growth curve data can be found in appendix B.

The goal of the initial growth experiments was to establish whether there was any discernible difference in growth rates and gradients of the linear exponential growth phase of bacteria grown in our low or simulated background radiation environments. This was carried out using *B. subtilis* and *E. coli* grown in the respective radiation environments for 24 and 48 h (fig. 7.4, fig. 7.5). Sample nomenclature for fig. 7.4 can be found in tab. 7.2.

An initial analysis of the growth phases indicated variation between each run. Firstly, a separation into groups occurred at the end of the lag phase, which was maintained throughout the exponential phase. However, it was grouped by experiment set and not by radiation condition. The upper cluster of endpoint growth data in fig. 7.4 contained all experiments from B1, B2 and 24 h B3, whilst experiments and the lower cluster contained all B3 48 h experiments. Additionally, the *B. subtilis* growth curves showed the presence of a “shoulder” in the exponential growth phase approximately between 480 and 600 minutes into growth after which all batches, except 48 h simulated background (B3), progressed to a secondary exponential phase. The stationary phase was reached with varying ODs from 1.4 to 1.8 with the exceptions of both B3 low and simulated background samples reaching between 1.4 and 1.5. Their OD also dropped off most during the death phase to just below 1 and 1.2 respectively. All other samples maintained a death phase OD between 1.5 and 1.8. In all cases the low radiation samples resulted in higher ODs than the corresponding simulated background samples. These variations between samples is addressed in the discussion.
To verify whether this lack of effect of the different radiation environments on exponential phase growth could be extended to other bacteria, experiments were replicated using *E. coli* (fig. 7.5). Once again, there was grouping according to batch (24 h and 48 h), not according to radiation environment, with the the 24 h batch having a shorter lag phase than that of the 48 h batch. Likewise, “shoulders” appeared the growth curves at \( \sim 200 \) minutes and at 300 - 400 minutes. However, unlike the wide variation of OD in the stationary and death phase of *B. subtilis*, the *E. coli* OD values remained between 1 and 1.2. Additionally, there was no obvious grouping in the maximum OD between the low and simulated background samples, in contrast to the *B. subtilis* samples. The gradients of *E. coli* revealed a steeper gradient incline than those of *B. subtilis* and also showed more variation between each batch. Importantly, there was no significant \( (p > 0.05) \) difference between the low and simulated background radiation growth within both time conditions. However, whilst the gradient of *B. subtilis* samples also didn’t significantly \( (p > 0.05) \) vary amongst and between conditions, the *E. coli* gradient values showed a significant \( (p < 0.05) \) difference between the time conditions. The growth gradients for 24 h in low and simulated background radiation were \( 0.0836 \pm 4.18 \times 10^{-3} \) and \( 0.0866 \pm 4.33 \times 10^{-3} \) respectively, in contrast to the values of \( 0.0693 \pm 3.92 \times 10^{-3} \) at 48 h in simulated background radiation. There was also a significant \( (p < 0.05) \) difference between the 24 h simulated background sample at \( 0.0866 \pm 4.33 \times 10^{-3} \) and the 48 h simulated background sample at \( 0.0693 \pm 3.92 \times 10^{-3} \). In spite of this, there was no trend towards a steeper gradient for either low or simulated background radiation.

Figure 7.8 illustrates the gradients and growth rates of the linear exponential growth phase of each experimental conditions. There was no significant \( (p > 0.05) \) difference in the growth gradient between any of the low radiation conditions in fig. 7.4 and their corresponding simulated background radiation conditions. There was slight trend of higher growth in the low radiation samples. However, this was not the case for all samples in this graph, and only appeared under the 48 h samples of *E. coli* gradients.
Figure 7.4. Average (n = 84) of B. subtilis growth curves at the determined low radiation (low) and simulated background radiation (sim. background) dose rate: 24 and 48 h denote time in hours that bacteria batch has spent in respective conditions; B1-3 = experiment run number. Error bars show ± standard error (n = 84).
Figure 7.5 Average (n = 84) of *E. coli* growth curves at the determined low radiation (“low”) and simulated background radiation (“sim. background”) dose rate: 24 and 48 h denote time in hours that bacteria batch has spent in respective conditions. Error bars show ± standard error (n = 84).

7.4.2 Exposure to higher radiation

The aim of the following experiment was to establish whether an increase in simulated background radiation would result in an impact on bacterial growth. As mentioned in the background section of this chapter, many previous experiments solely focused on low vs. control/simulated background radiation environments, despite the necessity of multiple data points to verify any of the models. By raising our simulated background dose to 10-fold and 100-fold the original simulated background dose, we wanted to provide more data points higher up the “dose-axis” of the models to establish a more robust analysis for the “risk-axis” trend.

*B. subtilis* were grown for 24 and 48 h under increased simulated background radiation conditions. Simultaneously, a low-radiation growth assay in the identical plate reader was run with the same bacterial batch for comparison (fig. 7.6). The growth curves displayed the same characteristics as the previous experiments, exhibiting a grouping in the exponential phase according to time condition, not radiation environment, in addition to a “shoulder” in the growth
curve, and variation in OD during the stationary and death phases. In accordance with the previous experiments, neither the 10× or 100× simulated background radiation’s growth gradients were significantly (p > 0.05) different from the corresponding low-radiation gradients that were run simultaneously. However, when comparing the 10× and 100× gradients, there is a visible difference in gradients with the 100× gradient being lower than the 10× gradient (p = 0.13), which may be construed as being caused by the higher radiation negatively influencing cell growth (fig. 7.8). Although this is also mirrored in the lower growth rate for the 100× simulated background (0.27 μ/h), the simultaneously run low radiation sample also showed lower growth rate (0.29 μ/h).

![Graph of growth curves](image)

**Figure 7.6** Average (n = 84) of B. subtilis growth curves at increased radiation dose rates: 10× = 10× the simulated background dose rate; 100× = 100× the simulated background dose rate; Low paired = samples run parallel at the low dose rate. Error bars show ± standard error (n = 84).

### 7.4.3 Response to stress

The final experiment was carried out to determine whether cells grown in a low radiation environment are more susceptible to sudden stress than cells exposed to simulated background radiation dose. B. subtilis and E. coli cultures that had been growing in low and simulated background radiation environments for 24 and 48 h respectively from week 4 were exposed to a short burst of UVC radiation (λ = 254 nm) for 30 s and plated to assess their post-irradiation viability (fig. 7.7).
Generally, there was no significant \((p > 0.05)\) difference between any of the cells’ viability. Thereby, there was no significant \((p > 0.05)\) difference in viability between the cells grown in low or simulated background radiation doses, or indeed between the two organisms. Despite this, fig. 7.7 illustrates certain trends in viability that are of interest. There was a consistent trend that \(E. coli\) had higher viability than \(B. subtilis\) under all conditions. Additionally, \(E. coli\) cells grown in a simulated background radiation environment showed slightly higher viability than those grown under low radiation conditions (1.03-fold for 24 h and 1.06-fold for 48 h). This behaviour was replicated by \(B. subtilis\) at 24 h (1.07-fold higher viability) but was not the case for cells at 48 h. There was also a trend of higher viability in cells that had been growing for 48 h under low and simulated background conditions, with the exception of \(B. subtilis\) in simulated background.

**Figure 7.7** Averages \((n = 9\) per condition\) of post-UV irradiation cell counts of \(B. subtilis\) and \(E. coli\) grown under the determined low radiation \(\text{\textquotedblleft low\textquotedblright}\) and simulated background radiation \(\text{\textquotedblleft sim. background\textquotedblright}\) dose rate: 24 and 48 h denote time in hours that bacteria batch has spent in respective conditions. Error bars show \(\pm\) standard error \((n = 9\) per condition\).
(a) Growth gradients of *B. subtilis* and *E. coli* from the linear exponential growth phase.

(b) Growth rates of *B. subtilis* and *E. coli* from the linear exponential growth phase.

**Figure 7.8** Growth gradients of *B. subtilis* and *E. coli* linear exponential growth phases at the determined low radiation (“low”) and simulated background radiation (“sim. background”) dose rate: 24 and 48 h denote time in hours that bacteria batch has spent in respective conditions; B1-3 denote week number when the multiple *B. subtilis* samples were run; 10× = 10× the simulated background dose rate; 100× = 100× the simulated background dose rate; Low paired = low dose rate samples run parallel to 10× and 100× dose rates. Error bars show ± error of gradient. “B.s” and “E.c.” stand for *B. subtilis* and *E. coli*, respectively.
7.5 Discussion

When the effects of high doses and dose rates of radiation are investigated, there is a general consensus on the lethal consequences for both prokaryotic and eukaryotic organisms. From early experiments with radioactivity [244] to sterilisation techniques utilising the energetic shortwave UV [245], exposure to high radiation environments has been proven to be deleterious to life. However, there is a grey area once the dose/dose rate is lowered, prompting much discussion as to the potential harm and benefits of low-dose irradiation [246]. Previously conducted studies show mixed results of effects on a variety of organisms in varying low radiation environments [24, 25, 247]. However, gaining access to or establishing well-characterised environments of low radiation, as well as detangling radiation-induced effects from others, is not a straightforward task. The experiments described in this chapter were carried out in response to previous work, with the aim of conducting simple growth experiments in a well-characterised and controlled environment with as few variables as possible.

7.5.1 Boulby results

Growth of *B. subtilis* and *E. coli* cultures were monitored in well-characterised sub-background and simulated background levels of radiation. Bacteria in the low radiation environment were confirmed by *in situ* measurements to experience a dose rate of 0.0044 mGy/y, whereas the control bacteria in the simulated background radiation environment were exposed to a 1 mGy/y dose rate using an attenuated $^{137}$Cs source. The primary finding of the low and simulated background radiation environment experiments is that, at the low dose rates utilised in the set up, there is no significant, observable effect on either kinds of bacteria with regard to their exponential growth phase in low radiation when compared to the simulated background controls. This directly challenges both the LNT and hormesis model.

Although there is variation present between the samples, as described in the results section, these discrepancies are unlikely to be a result of the radiation environment. The variation of lag phase length is observed in all experiments performed. However, the variation in samples is grouped by experiment set and not by radiation environment, making the difference in dose rate unlikely to be the
cause. The variation in lag phase length is most likely due to a varying starting concentration amongst the batches, possibly caused by pipetting error or different starting culture concentrations. Even a small error can have an observable effect on the bacterial growth curve during the exponential phase, with lower starting concentrations exhibiting an extended lag phase. This is an example of the benefits of choosing the linear exponential growth phase for analysis over the curve as a whole as an indicator of environmental effects. It gives the ability to normalise multiple bacterial growth data in spite of minor discrepancies. Aside from the methodology, the benefit of focusing analysis on the exponential phase is that it genuinely represents the measurement of growth rate.

An observable characteristic of \textit{B. subtilis} growth curves is the wide distribution of OD values in all samples in the stationary and death phase. This may be due to a number of causes. Complex biochemistry in these phases caused, amongst other things, by cell lysis resulting in the release of various organics is likely to interfere with the optical density measurement and influence cell growth. A further characteristic of the \textit{B. subtilis} growth curves is the higher end-point OD of low radiation samples compared to their respective simulated background controls. Identical surface experiments (data shown in appendix B), performed to show any differences in plate reader data, show no trends that can explain this behaviour. However, the aforementioned complex biochemistry taking place during stationary and death phase may heavily influence end-point OD and may not be representative of responses to radiation environment. These are prime reasons why solely end-point focused analysis is inadequate to inform on cells’ response to a single environmental factor. Despite this, previous research efforts have chosen to adopt end-point focused analysis.

An additional artefact in the \textit{B. subtilis} and \textit{E. coli} growth curves is the presence of a “shoulder” in the exponential growth phase. This may be a result of the bacteria exhausting the primary nutrient source and switching to a secondary one, which is probable in the nutrient-rich media used. Previous research has also tested the hypothesis of growth in low radiation causing heightened sensitivity to consequent stress factors. Satta \textit{et al.} [25] demonstrate \textit{Saccharomyces cerevisiae} yeast’s increased sensitivity to carcinogen methyl methanesulfonate after growing in a low radiation environment (0.6 $\mu$Gy/d, compared to Boulby’s 0.012 $\mu$Gy/d). This prompted our own experiment using UVC exposure as a stress factor to determine whether there was any difference in viability of cells after exposure. The results show no significant difference in either \textit{B. subtilis} or \textit{E. coli} viability.
grown at low or simulated background radiation dose rates. This is unsurprising, as the likelihood of low dose rates over 24 and 48 h having an impact on cells’ essential repair pathways is small, especially since additional environmental and internal factors can exert stress on an organism thus utilising these pathways. This is further discussed in section following section (7.5.2).

7.5.2 Context of previous research

Although the Boulby data challenges the LNT and hormesis models for low radiation doses, it is somewhat unsurprising that at such low dose rates the bacteria fail to show any sign of their growth being influenced. The empirical lethal dose necessary to sterilise 90% (“lethal dose” LD$_{90}$) of *E. coli* MG 1655 is 700 Gy [248] ($2.59 \times 10^8$ higher than the 24 h total dose received by Boulby control cells). Although no LD$_{90}$ data could be found for vegetative *B. subtilis* cells, the majority of life is not radio-resistant and can be killed by $< 500$ Gy [249, 250].

The cells subjected to 24 h of simulated background radiation in the Boulby set up are exposed to a total dose of 2.7 µGy (or 5.4 µGy for 48 h experiments), whereas the cells’ total dose after 24 h in the low radiation environment amounts to 0.012 µGy (0.024 µGy for 48 h experiments). For radiation to affect the cells in any significant manner, the expected dose would have to be $6-8$ orders of magnitude higher than simulated background and $10^9$ times higher to reach LD$_{90}$ (fig. 7.9). This also explains why, when the simulated background dose rate is increased by $10 \times$ and $100 \times$, there is no effect on bacterial growth. Although this may not rule out sublethal biochemistry, it illustrates the scale of background and sub-background dose rates in comparison to higher dose rates and their documented impact.

At ultra low dose rates, it becomes increasingly illogical to prescribe effects on bacteria to the radiation exposure and not to additional environmental factors. Growth and growth rate measurements have been key to investigating the initial effects that a specific dose rate may have on cells’ health. There have been various growth studies at low radiation. Planel *et al.* [24] report lower growth at low dose rates and “growth-stimulation” at dose rates up to 50 mGy/y in protozoa *Paramecium tetraurelia* and cyanobacteria *Synechococcus lividus*. However, both organisms have a high LD$_{90}$ dose, making their sensitivity to low doses questionable. In addition, the low radiation environment was still exposed to surface cosmic ray doses and ionising radiation two orders of magnitude higher.
than the Boulby low radiation environment. The trend of low radiation being actively detrimental to cells is continuously discussed in the low radiation papers, citing work such as Satta et al. [25] and Smith et al. [26] that underpin this. However, their work is with eukaryotic cells (hamster cells and lung fibroblasts, respectively), which may not be representative of prokaryotic responses.

Castillo et al. [243] acknowledge the limitations of small data point growth assays, and additionally look at regulation of genes associated with stress response in *Shewanella oneidensis*. Their low dose rate is defined as 0.16 nGy/h (as opposed to Boulby’s measured 0.5 nGy/h dose rate), yet was only simulated and not verified *in situ*. They report up-regulation of marker genes for oxidative stress and SOS-response of cells grown in low radiation. However, this up-regulation is mostly observed during late exponential phase and stationary/death phase. There are many stresses on cells during this growth phase, such as nutrient shortage, that may be reflected in stress gene up-regulation. Concurrently, they suggest that cultures grown in low radiation for 24 h are less able to respond to reactive oxygen species (ROS) within the cell. ROS can be produced by radiation interacting with water [5], with higher dose rates causing higher ROS production. Castillo *et al.* insinuate that the lack of radiation, and by extension ROS production, fails to “prime” cells’ response pathways for internal ROS removal, resulting in ROS accumulation and consequent stress. Exposure to sub-lethal radiation doses has been shown to reduce subsequent sensitivity in hamster cells [252]. Notwithstanding that eukaryotic cells from higher organisms

![Figure 7.9](image.png)

**Figure 7.9** Survival-dose relationship of various cell types exposed to γ-radiation illustrating the lethal dose required to kill 90% of cells; DSB = DNA double strand break. Graph from Daly (2012) [251].
are not representative of prokaryotic responses, this hormetic effect is often projected onto lower organisms. Although hormesis is not entirely ruled out, a loss of the essential ability to react to ROS is unlikely to be demonstrated in an experiment spanning fewer than 40 generations. In addition, the ROS-removal pathways would not be redundant at low dose rates, as ROS are continuously produced by aerobic bacterial metabolism [253]. The Boulby UVC exposure experiments verified the absence of significant difference between low and simulated background cells’ viability, inferring no difference in their repair pathway responses after 24 and 48 h of a low dose rate exposure.

In addition to secondary effects of radiation such as ROS production in aqueous environments, primary effects such as mutations and DNA strand breaks can be measured to demonstrate the radiation dose-damage relationship. This has been done for higher radiation exposure [254], however it lends less clarity at the scale of below-background radiation doses. The likelihood of radiation-cell interaction has been calculated by Lampe et al. [255] for evolutionary research in the Modane Underground Laboratory (LSM) at 1.7 km below the Fréjus Peak (4.8 km water equivalent [256], Boulby is 3 km water equivalent [257]). Using $7.4 \times 10^{-4}$ mutations per generation as an upper point for spontaneous point mutation occurrence in *E. coli* [258], they calculate that such natural mutation occurrences would happen at a $1.04 \times 10^2$ higher frequency than what they calculate for radiation-cell interactions. They compute that a dose rate of $> 20 \mu\text{Gy/}h (= 175 \text{mGy/y})$ is required for the radiation-cell interactions to have a detectable impact above the natural mutation “noise”. These calculations are in accordance with the lack of observable difference between the Boulby low, simulated background, and $100\times$ simulated background (100 mGy/y) radiation dose rates on bacterial exponential growth.

### 7.5.3 New model

Taking all previous research along with the Boulby findings into consideration, it becomes apparent that a singular radiation dose-response relationship model may be impossible to obtain. Parts of the model addressing ultra-high and ultra-low radiation responses can be argued to be universal with few exceptions. However, accurate predictions of an organism’s response to the intermediate doses may depend on multiple factors such as:
- Total dose versus dose-rate
- Prokaryotic or eukaryotic cells
- Type of radiation (high or low linear energy transfer)
- Growth phase of cells

Fig. 7.10 represents an updated version of fig. 7.1 for radiation dose-response in prokaryotes.

![Graph showing dose-response in prokaryotes](image)

**Figure 7.10** Updated prokaryotic dose-response model with a linear relationship for higher doses & added Boulby observations from sub-background to 100x background. Black dotted lines indicate possible, hypothetical risk of damage at intermediate radiation exposure.

Fig. 7.10 illustrates the dose threshold discussed in accordance with the low dose rate Boulby growth observations and Lampe *et al.* calculations. Like the LNT model, the new model displays risk of damage to an organism in relationship to *total dose*, not dose rate. Total doses of the Boulby samples were calculated, with 24 h exposure at the simulated background dose rate of 1 mGy/y resulting in a total dose of $2.74 \times 10^{-6}$ Gy. Hypersensitivity and/or hormetic responses are not entirely ruled out at intermediate above-background radiation exposure,
and this requires further research to confirm. There may be many additional saddle points in the graph depending on factors previously listed such as dose-rate and organism. Although it is unlikely that something as simplistic as the LNT model is applicable to such a complex set of variables, it has been argued that LNT can still serve a purpose as a radiation protection standard rather than a mechanistic, true representation of cells’ responses to a certain dose \[259\]. However, even this may be proven an invalid argument when concerning eukaryotic responses in light of the mounting body of research dedicated to cancer radiation therapy and empirical evidence of hormesis \[260–262\].

With regard to the significance of this research for astrobiology, the results presented in this chapter show that a wide range of low radiation dose rates, spanning at least four orders of magnitude, have no real-time effect on the growth of microbes. This points to a hypothesis that the presence of low radiation dose rates are not necessary for life to thrive. Not only does this inform on the factors required to sustain life and the habitability of low radiation environments on Earth, but it also allows for predictions of habitability of extraterrestrial environments for Earth-like lifeforms with regard to extreme, low radiation.

### 7.5.4 Limitations

Work at low radiation often involves both technical and physical limitations. Despite the Boulby Underground Lab’s excellent collaboration and assistance on the SELLR project, all parties involved were still subject to Boulby mine regulations, as it is still an operational mine. This limited the kinds of ionising radiation sources that could be used when establishing the simulated background and increased simulated background radiation environments, limiting the increase to 100× simulated background. Additionally, as the mine is located in Cleveland, England, experiments were conducted at 1-2 weeks at a time, with each trip involving extensive planning of materials. Limitations regarding establishing an ultra-low radiation environment were mostly overcome, however the pre-experimental radiation analysis highlighted some potential aspects that could be improved upon. One stand-out factor was the high potassium contribution to the overall internal radiation dose. Future experiments may wish to adjust the potassium content of growth media, if possible, to ensure even lower radiation environments. The main focus for analysis of the SELLR experiments was the linear exponential growth phase of bacteria (for reasons discussed in section 7.5.1).
Although effects on growth phases can be used as indicators of fitness, further research such as DNA damage analysis and gene regulation during this growth phase would give a more in-depth picture of mechanistic aspects to bacterial responses, or lack thereof.

7.5.5 Future work

As highlighted in the previous section, there are still many aspects of low radiation effects on life to explore. A direct follow-up experiment could consist of using a more powerful radiation source to mimic the $10^{-4}$ to $10^{2}$ Gy dose range and establish the “dose death curve” of *B. subtilis* and *E. coli*. The SELLR experiments utilised these organisms to represent Gram negative and positive bacteria, however experimental repeats with a larger range of bacteria, including particularly radio-sensitive bacteria, as well as respective DNA/gene analysis, would contribute to a more generally-applicable model. With this in mind, experimental repeats using simple eukaryotic model organisms would allow for a comparable study, and may highlight response differences between the two domains.

Radiation-induced mutations and mutation rates would also be of interest in the framework of evolution. The focus of SELLR was real-time bacterial responses to low radiation, however this does not rule out the possible requirement of low-dose radiation to drive evolution. It is outside the scope of this research to elucidate low radiation evolutionary effects. Future long-term experiments of mutation rates at low dose rates would add an extra dimension to low radiation research and be of astrobiological interest.
7.6 Conclusions

Research on the effects of low doses and dose rates of radiation on organisms is mostly overshadowed by research efforts investigating the consequences of high-radiation exposure. There is a body of scientific literature concerning low radiation and life, but it consists of convoluted and often incomparable data lacking in rigorous model definitions and experimental set-ups. The SELLR project was initiated in response to this, and was dedicated to establishing a well-characterised experimental set-up, that focused on one indicator of bacterial health, namely the linear exponential growth phase. The resulting data of *B. subtilis* and *E. coli* growth showed no significant difference in the exponential growth phases at either low, simulated background, or increased simulated background dose rates, and displayed no difference in sensitivity to stress in the form of shortwave UV exposure. This challenges previously established models such as LNT and hormesis, and suggests a dose threshold for radiation with regard to real-time effects on bacterial fitness.
Chapter 8

Conclusions

This chapter will summarise the general conclusions of the thesis. Detailed conclusions of experimental results and specific remarks on future work can be found at the end of each results chapter, respectively.

8.1 Bacterial responses to photochemical products

The deleterious effects of direct radiation exposure on life are well-documented, yet still not fully understood. Radiation can indirectly impact cellular viability by a range of methods, one of these being radiation-induced photochemistry and subsequent photoproducts. Chapters 4 & 5 focused on the observed effects of UV-irradiated iron oxide and perchlorate on bacterial survival. Iron oxide is characterised to have UV-absorbing abilities but is also known for its role as a catalyst in the free radical-producing Photo-Fenton reaction. While perchlorate is characterised as being particularly stable at ambient temperatures, despite also having strongly oxidising properties when activated at elevated temperatures. Both compounds are components of the Martian surface regolith and consequently exposed to high doses of ionising and UV radiation. Experiments in chapter 4 provided further evidence for the ability of various iron oxides to confer substantial protection to vegetative cells of bacterial species *Bacillus subtilis* when exposed to high-energy UVC for multiple hours. However, these experiments also supported evidence for irradiated iron oxide’s catalytic function when in the presence of additional Fenton reagent, hydrogen peroxide. Within a particular concentration
range, the protective property of iron oxide was usurped by its catalytic role, resulting in a significantly greater loss of cell viability than under UV-only exposure.

A similarly bacteriocidal effect was observed in chapter 5, when perchlorate was irradiated with the same high-energy UVC radiation, causing a significant decrease in bacterial viability on the order of seconds in comparison to UV-only controls. Furthermore, a synergistic bacteriocidal effect was observed when organisms were exposed to Martian concentrations of perchlorate in the presence of Fenton compounds used in chapter 4. These findings provide insight into the probable photochemical reactions taking place on the Martian surface and near-surface regolith layers and reveal the radiation-induced bacteriocidal nature of the geochemical environment. Specifically, the effects of combined, irradiated surface components result in an enhanced toxicity of the Martian surface that, in the absence of specific adaptations, is lethal to life.

8.2 Bacterial responses to low Earth orbit exposure

Following on from ground-based radiation exposure, chapter 6 presented post-flight analysis results of cyanobacterial sample cultures exposed to the multiple extremes of low Earth orbit on the outside of the International Space Station for 1.5 years. Cyanobacterial *Gloeocapsa* samples were exposed to the environment in various configurations depending on the investigation focus. Bacterial biofilms within the Biology Mars Experiment (BIOMEX) set up were exposed to space and a simulated Martian environment with and without the presence of Martian analogue soil to investigate its effect on bacterial survival. A similar set up was used in the Biofilm Organisms Surfing Space (BOSS) experiment to test the protective properties of biofilms in comparison to singular, planktonic cells when exposed to space and simulated Martian conditions. In addition to testing the potential for protection of a single species, these experiments served as a platform to test the ability of one species to protect another. An environmental, bacterial co-culture was sent to low Earth orbit within the cyanobacteria samples to establish the protective abilities of the primary bacterial culture in the framework of BIOMEX and BOSS. Post-flight analysis of the samples showed the best preservation of viability and structural integrity were found in biofilm
samples with the additional protection conferred by the Martian simulant soil in comparison to samples lacking the soil, thus complementing the protective iron oxide characteristics presented in chapter 4. Planktonic bacterial samples incurred the most severe damage compared to all other experimental set ups, showing that, although not optimal, biofilms provided enhanced protection and allowed for a subsection of cells to be re-cultured upon return to favourable conditions. Moreover, the co-cultured bacteria species was shown to have been successfully protected by the cyanobacteria.

The results of this work show the capacity of biogenic and abiogenic substances to protect a species from extreme radiation exposure. Even primitive cellular clumping, resulting in an amorphous cellular mass, confers sufficient radiation protection to maintain culture viability. Furthermore, this work demonstrates the ability of larger cells, even if dead, to protect at least one additional species that may otherwise not have survived radiation exposure. These findings additionally support planetary protection concerns regarding the contamination risk of non-sterile planetary exploration equipment. Moreover, they highlight the need to reassess risk with regard to multiple, non-resistant organisms surviving harsh conditions due to protection of robust, co-cultured species. Conversely, this provides reinforcement for the intriguing hypothesis of panspermia.

8.3 Bacterial responses to sub-background radiation environments

The majority of radiation research is focused on the effects of high radiation exposure. However, the absence of radiation must also be considered when endeavouring to gain a complete understanding of its effects on life. Despite previous and continuing scientific studies of life in low-radiation conditions, the field is pervaded by confusing and inconsistent terminology, as well as the lack of standardised methodologies for comparable results. With this in mind, a well-characterised, low-radiation experimental set up was constructed within the Boulby mine facility (Cleveland, UK) with the objective of measuring the effects of prokaryotic growth at low radiation doses in a controlled environment with minimal variables. At 1.1 km underground, the Earth’s regolith provides a $10^6$ attenuation of cosmic rays. This exposure was further reduced by utilising lead casing around the experiment within the underground lab, achieving a radiation
dose of 1% of the surface dose rate. *Bacillus subtilis* and *Escherichia coli* cells were grown under these low radiation conditions for multiple weeks at a time with *in situ* controls exposed to a simulated surface radiation dose rate provided by a $^{137}$Cs source. As presented in chapter 7, growth results from the multiple experimental runs show no significant differences in growth during the exponential growth phase of the bacteria in any of the conditions. These results stand in contrast to current, but disputed dose-response models. Additionally, the bacterial cultures were subjected to a short exposure of UVC, to test for a heightened sensitivity in low-radiation cells described in the literature. However, no difference was shown in either species when compared to the controls. In spite of observations and claims made in previous research, the Boulby findings are unsurprising. While surface dose rates induce mutation rates indistinguishable from those occurring by replication error, significantly lower dose rates and thereby lower probability of cell-photon interaction would be unlikely to induce an observable response in bacterial cells. This work not only provides a well-characterised basis for comparison with future prokaryotic low dose exposure, but also illustrates the necessity for a new model to accurately represent low dose responses.

### 8.4 Final remarks

The experiments presented in this thesis show the extensive, direct influence that radiation has on prokaryotic organisms, as well as its varied and equally extensive indirect effects. Radiation can be effectively blocked or absorbed by iron oxides and other Martian compounds, as well as by biogenic material such as biofilms or other species, thus enabling the retention of cell viability. However, heightened irradiation of chemical and biological structures can also induce an array of secondary bacteriocidal effects that extend the deleterious impact of radiation exposure. Although high-radiation research is essential for advancement in multiple fields, it is also important to establish appropriate experimental protocols for intermediate and low radiation research in lieu of exclusively relying on high-radiation data extrapolation. Understanding how radiation contributes to establishing the limits of life enables us to incorporate these parameters into current and future exploration missions and thus take an evidence-based approach to the search for life elsewhere in the universe.
Appendix A

Numerical perchlorate data

A.1 Numerical results of B. subtilis viability

Table A.1  Numerical results from figures in the main thesis body. Results are in the unit $N/N_0 \%$, with $N$ as the number of surviving cells after a given treatment and $N_0$ is the number of cells in the starting concentration, calculated using the average of triplicates. The results for each condition are paired with the results from the respective UV-irradiated control ($\lambda = 254$ nm, unless stated). Mg(ClO$_4$)$_2$ = magnesium perchlorate at given wt%; Ca(ClO$_4$)$_2$ = calcium perchlorate at given wt%; NaClO$_4$ = sodium perchlorate at given wt%; MgSO$_4$ = magnesium sulphate at given wt %; H = 1 g/L hematite (5 µm grain diameter); HP = hydrogen peroxide at final concentration of 10 mM; polychromatic UV = 200 - 400 nm; anaerob. = anaerobic; rock = rock analogue; liq. = liquid system; n.a. = not applicable.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>1 min irradiation</th>
<th>2 min irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV control</td>
<td>1.43</td>
<td>0.09</td>
</tr>
<tr>
<td>UV + 0.6 wt% Mg(ClO$_4$)$_2$ at 4°C</td>
<td>1.31</td>
<td>0.008</td>
</tr>
<tr>
<td>UV control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UV + 0.6 wt% Mg(ClO$_4$)$_2$ at 4°C</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

3 min irradiation  | 4 min irradiation
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>UV control</td>
<td>0</td>
</tr>
<tr>
<td>UV + 0.6 wt% Mg(ClO$_4$)$_2$ at 4°C</td>
<td>0</td>
</tr>
</tbody>
</table>

10 s irradiation

<table>
<thead>
<tr>
<th>Conditions</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Polychromatic UV control</td>
<td>3.13</td>
</tr>
<tr>
<td>Polychromatic UV + 0.6 wt% Mg(ClO$_4$)$_2$</td>
<td>0.29</td>
</tr>
</tbody>
</table>
Table A.2  Continuation of numerical results of *B. subtilis* viability.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>30 s irradiation</th>
<th>60 s irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV control</td>
<td>1.53</td>
<td>0</td>
</tr>
<tr>
<td>UV + 0.6 wt% Mg(ClO$_4$)$_2$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UV control</td>
<td>45.86</td>
<td>22.22</td>
</tr>
<tr>
<td>UV + 0.6 wt% Mg(ClO$_4$)$_2$ rock</td>
<td>26.67</td>
<td>2.9</td>
</tr>
<tr>
<td>UV control</td>
<td>n.a.</td>
<td>0.12</td>
</tr>
<tr>
<td>UV + 0.6 wt% Mg(ClO$_4$)$_2$, anaerob. liq.</td>
<td>n.a.</td>
<td>0</td>
</tr>
<tr>
<td>UV control</td>
<td>n.a.</td>
<td>8.23</td>
</tr>
<tr>
<td>UV + 0.6 wt% Mg(ClO$_4$)$_2$, anaerob. rock</td>
<td>n.a.</td>
<td>0.85</td>
</tr>
<tr>
<td>UV control</td>
<td>0.36</td>
<td>0.15</td>
</tr>
<tr>
<td>UV + 1 wt% Mg(ClO$_4$)$_2$</td>
<td>0.003</td>
<td>0</td>
</tr>
<tr>
<td>UV + 2.5 wt% Mg(ClO$_4$)$_2$</td>
<td>0.003</td>
<td>0</td>
</tr>
<tr>
<td>UV + 5 wt% Mg(ClO$_4$)$_2$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UV control</td>
<td>3.93</td>
<td>0</td>
</tr>
<tr>
<td>UV + 0.6 wt% Ca(ClO$_4$)$_2$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UV + 0.6 wt% NaClO$_4$</td>
<td>0.09</td>
<td>0</td>
</tr>
<tr>
<td>UV control (16× less irradiance)</td>
<td>5.91</td>
<td>1.45</td>
</tr>
<tr>
<td>UV + 0.6 wt% Ca(ClO$_4$)$_2$ (16× less irradiance)</td>
<td>7.36</td>
<td>0.54</td>
</tr>
<tr>
<td>UV + 0.6 wt% NaClO$_4$ (16× less irradiance)</td>
<td>6.07</td>
<td>0.69</td>
</tr>
<tr>
<td>UV control</td>
<td>n.a.</td>
<td>2.7</td>
</tr>
<tr>
<td>UV + 30 wt% MgSO$_4$</td>
<td>n.a.</td>
<td>2.41</td>
</tr>
<tr>
<td>UV + 0.6 wt% Mg(ClO$_4$)$_2$ + 30 wt% MgSO$_4$</td>
<td>n.a.</td>
<td>2.58</td>
</tr>
<tr>
<td>UV control</td>
<td>3.34</td>
<td>2.26</td>
</tr>
<tr>
<td>UV + H</td>
<td>4.61</td>
<td>3.1</td>
</tr>
<tr>
<td>UV + HP</td>
<td>2.33</td>
<td>1.2</td>
</tr>
<tr>
<td>UV + 0.6 wt% Mg(ClO$_4$)$_2$</td>
<td>2.37</td>
<td>1.39</td>
</tr>
<tr>
<td>UV + H + HP</td>
<td>3.36</td>
<td>0.84</td>
</tr>
<tr>
<td>UV + H + 0.6 wt% Mg(ClO$_4$)$_2$</td>
<td>4.74</td>
<td>1.44</td>
</tr>
<tr>
<td>UV + HP + 0.6 wt% Mg(ClO$_4$)$_2$</td>
<td>4.08</td>
<td>0.79</td>
</tr>
<tr>
<td>UV + H + HP + 0.6 wt% Mg(ClO$_4$)$_2$</td>
<td>4.16</td>
<td>0.21</td>
</tr>
<tr>
<td>UV control at 4°C</td>
<td>1.7</td>
<td>1.27</td>
</tr>
<tr>
<td>UV + 0.6 wt% Mg(ClO$_4$)$_2$ at 4°C</td>
<td>3.32</td>
<td>1.69</td>
</tr>
</tbody>
</table>
Appendix B

SELLR additional data

B.1 Radiation environment analysis

B.1.1 Radiation simulation

<table>
<thead>
<tr>
<th>Plate *</th>
<th>Mass of Plate (Kg)</th>
<th>Time (Sec)</th>
<th>Total Energy Deposition (MeV)</th>
<th>Energy Deposited Per Unit Mass (J/ Kg - Gy)</th>
<th>Gamma Dose Eq. Rate (mSv/year)</th>
<th>Gamma Dose Eq. Rate (mSv/year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate 1</td>
<td>0.1567</td>
<td>664000</td>
<td>81.80</td>
<td>8.36E-11</td>
<td>3.05</td>
<td>0.00</td>
</tr>
<tr>
<td>Plate 2</td>
<td>0.1567</td>
<td>119.86</td>
<td>1.23E-10</td>
<td>4.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plate 1</td>
<td>0.1567</td>
<td>664000</td>
<td>1700.23</td>
<td>1.74E-09</td>
<td>91371.50</td>
<td>0.09</td>
</tr>
<tr>
<td>Plate 2</td>
<td>0.1567</td>
<td>2110.35</td>
<td>2.16E-09</td>
<td>113411.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plate 1</td>
<td>0.1567</td>
<td>38</td>
<td>174.49</td>
<td>1.78E-10</td>
<td>1.56E+05</td>
<td>0.16</td>
</tr>
<tr>
<td>Plate 2</td>
<td>0.1567</td>
<td>0.35</td>
<td>3.58E-13</td>
<td>313.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plate 1</td>
<td>0.1567</td>
<td>664000</td>
<td>293.37</td>
<td>3.00E-10</td>
<td>10.95</td>
<td>0.00</td>
</tr>
<tr>
<td>Plate 2</td>
<td>0.1567</td>
<td>339.49</td>
<td>3.47E-10</td>
<td>12.67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure B.1** Summary of simulated gamma doses. “Plate 1” refers to 96-well plate containing microbes exposed to the $^{137}$Cs source, in the “background” radiation environment; “Plate 2” refers to 96-well plate containing microbes in the “low” radiation environment. All GEANT4 simulations and calculations were carried out by Athoy Nilima (University of Edinburgh).
B.1.2 Internal dose measurements

See table B.1.

B.2 SELLR experiment layout

B.2.1 Radiation source alter set up

Figure B.2 Alter set up for adjusting height of $^{137}$Cs source from samples, including lead sheet shielding. Measurements and drawing done by Leander Cascorbi (Trinity College Oxford University).
Table B.1

| Sample | Plate Reader | Bacteria Re-run | Bacteria | Media | Media = Nutrient Broth | Plate = 96-well plate | Bacteria = B. subtilis
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate</td>
<td>6.73 ± 0.35</td>
<td>4.16 ± 0.03</td>
<td>5.74 ± 0.02</td>
<td>5.62 ± 0.04</td>
<td>10.9 ± 0.1</td>
<td>0.29 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Media</td>
<td>&lt; 0.2</td>
<td>&lt; 0.05</td>
<td>&lt; 0.001</td>
<td>0.40 ± 0.02</td>
<td>4.05 ± 0.06</td>
<td>2.37 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td>&lt; 0.03</td>
<td>&lt; 0.005</td>
<td>&lt; 0.0005</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
<td>6.0 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

All internal dose measurements were carried out by Emma Meehan in the Boulby Underground Lab germanium detector facility, Cleveland, UK.

Sample U238(e) U238(l) Th232(e) Th232(l) K40 Co60 U235

| Sample | Plate Reader | Bacteria Re-run | Bacteria | Media | Media = Nutrient Broth | Plate = 96-well plate | Bacteria = B. subtilis
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Plate</td>
<td>6.73 ± 0.35</td>
<td>4.16 ± 0.03</td>
<td>5.74 ± 0.02</td>
<td>5.62 ± 0.04</td>
<td>10.9 ± 0.1</td>
<td>0.29 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Media</td>
<td>&lt; 0.2</td>
<td>&lt; 0.05</td>
<td>&lt; 0.001</td>
<td>0.40 ± 0.02</td>
<td>4.05 ± 0.06</td>
<td>2.37 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td>&lt; 0.03</td>
<td>&lt; 0.005</td>
<td>&lt; 0.0005</td>
<td>&lt; 0.005</td>
<td>&lt; 0.005</td>
<td>6.0 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

All internal dose measurements were carried out by Emma Meehan in the Boulby Underground Lab germanium detector facility, Cleveland, UK.
B.3 Biological analysis

B.3.1 Surface growth controls

Figure B.3 Growth curves of isogenic *B. subtilis* cultures in plate readers 1 and 2 (PR1 & 2) on the surface at Edinburgh and Boulby, as references for plate reader deviations and underground values.

Figure B.4 Growth curves of isogenic *E. coli* cultures in plate readers 1 and 2 (PR1 & 2) on the surface at Edinburgh and Boulby, as references for plate reader deviations and underground values.
### B.3.2 Growth curve numerical values

**Table B.2** Bacterial growth experiment numerical values of *B. subtilis* and *E. coli* of linear exponential growth phases at the determined low radiation ("l") and background radiation ("b") dose rate: B.s. and E.c. = *B. subtilis* and *E. coli*, respectively; 24 and 48 h denote time in hours that bacteria batch has spent in respective conditions; B1-3 denote week number when the sample was run, as in tab. 7.2; 10× = 10× at the determined background dose rate; 100× = 100× at the determined background dose rate; l ctr = controls exposed to the determined low dose rate and ran in parallel with 10× and 100× dose rates; E = Edinburgh surface control; B = Boulby surface control; PR 1,2 = plate reader 1,2.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Growth Curve</th>
<th>$R^2$</th>
<th>Gradient error</th>
<th>Growth Rate [μ/h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. s. 48h l (B1)</td>
<td>0.0602</td>
<td>0.99</td>
<td>3.01 × 10$^{-3}$</td>
<td>0.44</td>
</tr>
<tr>
<td>B. s. 48h b (B1)</td>
<td>0.0618</td>
<td>0.99</td>
<td>3.09 × 10$^{-3}$</td>
<td>0.45</td>
</tr>
<tr>
<td>B. s. 24h l (B2)</td>
<td>0.0636</td>
<td>0.99</td>
<td>3.18 × 10$^{-3}$</td>
<td>0.38</td>
</tr>
<tr>
<td>B. s. 24h b (B2)</td>
<td>0.0632</td>
<td>0.99</td>
<td>3.16 × 10$^{-3}$</td>
<td>0.39</td>
</tr>
<tr>
<td>B. s. 48h l (B2)</td>
<td>0.0666</td>
<td>0.99</td>
<td>3.33 × 10$^{-3}$</td>
<td>0.47</td>
</tr>
<tr>
<td>B. s. 48h b (B2)</td>
<td>0.0637</td>
<td>0.99</td>
<td>3.15 × 10$^{-3}$</td>
<td>0.42</td>
</tr>
<tr>
<td>B. s. 24h l (B3)</td>
<td>0.0667</td>
<td>0.99</td>
<td>3.28 × 10$^{-3}$</td>
<td>0.46</td>
</tr>
<tr>
<td>B. s. 24h b (B3)</td>
<td>0.0624</td>
<td>0.99</td>
<td>3.12 × 10$^{-3}$</td>
<td>0.46</td>
</tr>
<tr>
<td>B. s. 48h l (B3)</td>
<td>0.0626</td>
<td>0.98</td>
<td>3.13 × 10$^{-3}$</td>
<td>0.48</td>
</tr>
<tr>
<td>B. s. 48h b (B3)</td>
<td>0.0612</td>
<td>0.99</td>
<td>3.06 × 10$^{-3}$</td>
<td>0.47</td>
</tr>
<tr>
<td>B. s. (10×) l ctr</td>
<td>0.0630</td>
<td>0.99</td>
<td>3.15 × 10$^{-3}$</td>
<td>0.42</td>
</tr>
<tr>
<td>B. s. 10× b</td>
<td>0.0677</td>
<td>0.99</td>
<td>3.35 × 10$^{-3}$</td>
<td>0.42</td>
</tr>
<tr>
<td>B. s. (100×) l ctr</td>
<td>0.0638</td>
<td>0.99</td>
<td>3.19 × 10$^{-3}$</td>
<td>0.29</td>
</tr>
<tr>
<td>B. s. 100× b</td>
<td>0.0593</td>
<td>0.99</td>
<td>2.96 × 10$^{-3}$</td>
<td>0.27</td>
</tr>
<tr>
<td>E. c. 24 h l</td>
<td>0.0836</td>
<td>0.99</td>
<td>4.18 × 10$^{-3}$</td>
<td>0.27</td>
</tr>
<tr>
<td>E. c. 48 h l</td>
<td>0.0769</td>
<td>0.99</td>
<td>3.22 × 10$^{-3}$</td>
<td>0.28</td>
</tr>
<tr>
<td>E. c. 24 h b</td>
<td>0.0866</td>
<td>0.99</td>
<td>4.33 × 10$^{-3}$</td>
<td>0.29</td>
</tr>
<tr>
<td>E. c. 48 h b</td>
<td>0.0693</td>
<td>0.99</td>
<td>3.92 × 10$^{-3}$</td>
<td>0.26</td>
</tr>
<tr>
<td>B. s. E (PR 1)</td>
<td>0.0566</td>
<td>0.98</td>
<td>2.62 × 10$^{-3}$</td>
<td>0.34</td>
</tr>
<tr>
<td>B. s. E (PR 2)</td>
<td>0.0533</td>
<td>0.99</td>
<td>2.58 × 10$^{-3}$</td>
<td>0.39</td>
</tr>
<tr>
<td>B. s. B (PR 1)</td>
<td>0.0524</td>
<td>0.99</td>
<td>2.54 × 10$^{-3}$</td>
<td>0.27</td>
</tr>
<tr>
<td>B. s. B (PR 2)</td>
<td>0.0516</td>
<td>0.98</td>
<td>2.66 × 10$^{-3}$</td>
<td>0.28</td>
</tr>
<tr>
<td>E. c. B (PR 1)</td>
<td>0.0762</td>
<td>0.99</td>
<td>3.90 × 10$^{-3}$</td>
<td>0.24</td>
</tr>
<tr>
<td>E. c. B (PR 2)</td>
<td>0.0746</td>
<td>0.99</td>
<td>3.73 × 10$^{-3}$</td>
<td>0.24</td>
</tr>
</tbody>
</table>


