The impact of ionizing radiation on microbial cells pertinent to the storage, disposal and remediation of radioactive waste

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Engineering and Physical Sciences

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# Table of Contents

Table of Contents ........................................................................................................... 2  
Table of Figures ............................................................................................................... 8  
List of Tables ................................................................................................................ 16  
Abstract .......................................................................................................................... 18  
Declaration ..................................................................................................................... 19  
Copyright statement ..................................................................................................... 20  
Acknowledgements ........................................................................................................ 22  
The Author ..................................................................................................................... 23

1. Thesis content ............................................................................................................. 25  
   1.1. Thesis layout ......................................................................................................... 25  
   1.2. Thesis content, status of manuscripts and author contributions .................... 25

2. Ionizing radiation processes in biogeochemistry relevant to the storage, 
   geodisposal and bioremediation of radioactive waste ............................................. 29
   2.1. Environmental radioactivity ............................................................................... 29  
   2.2. Storage of spent nuclear fuel and geological disposal of radioactive waste 31  
   2.3. Microbial ecology of nuclear infrastructure and repository environment ... 32  
   2.4. Waste legacy and contaminated land ................................................................. 36  
       2.4.1. Bioremediation .............................................................................................. 36  
   2.5. Radiation dose .................................................................................................... 37  
       2.5.1. Radiation doses in geodisposal scenarios .................................................... 38  
       2.5.2. Ionizing radiation doses in remediation scenarios and 
            contaminated land .............................................................................................. 40  
   2.6. Ionizing radiation ............................................................................................... 42  
       2.6.1. Charged particles .......................................................................................... 44  
       2.6.2. Electromagnetic radiation .......................................................................... 45  
       2.6.3. Ionizing radiation interaction with biomolecules ....................................... 47  
       2.6.4. The direct effect ........................................................................................... 48  
       2.6.5. The indirect effect ........................................................................................ 48
2.7. The impact of ionizing radiation on microbial physiology – the intracellular environment ..............................50
  2.7.1. Radiation resistance and sensitivity ........................................50
  2.7.2. The impact of ionizing radiation on DNA ..........................50
  2.7.3. Conventional DNA repair mechanisms ..............................51
  2.7.4. *Deinococcus*: extreme radioresistance ..........................52
2.8. The metabolic impact of radiation ........................................53
  2.8.1. Alternative targets of ionizing radiation: Protein ...............53
  2.8.2. Protection of proteins by manganese ................................54
  2.8.3. Radiation damage in lipids ..............................................55
2.9. The response of model organisms to ionizing radiation............58
  2.9.1. The metabolic response of *S. oneidensis* .........................58
  2.9.2. Ionizing radiation and eukaryotic microorganisms ...............59
  2.9.3. Radiation induced growth of microorganisms ....................60
2.10. Ionizing radiation interactions with the extracellular environment ......61
  2.10.1. The direct effect in the extracellular environment ..............61
  2.10.2. The indirect effect in the extracellular environment ..........61
2.11. Conclusions .............................................................................65
2.12. Aims and objectives .................................................................66
2.13. References ................................................................................68

3. Methodology ..................................................................................83

3.1. Irradiation ..................................................................................83
  3.1.1. X-irradiation ........................................................................83
  3.1.2. Gamma irradiation ...............................................................84
  3.1.3. Fricke dosimeter ..................................................................84
3.2. Microbiological techniques .........................................................85
  3.2.1. Fourier transform infrared spectroscopy ...............................85
  3.2.2. Multivariate statistical analysis .............................................88
      3.2.2.1. Principal component analysis ..................................88
      3.2.2.2. Discriminant function analysis ..................................89
      3.2.2.3. Hierarchical cluster analysis ..................................89
      3.2.2.4. Partial least squares regression ..............................90

3
3.2.3. Matrix-assisted laser desorption/ionization mass spectrometry 90
3.2.4. Microbial community analysis .............................................. 92
   3.2.4.1. DNA extraction .......................................................... 92
   3.2.4.2. Amplification and Polymerase Chain Reaction (PCR) ........... 92
   3.2.4.3. Sequencing ............................................................... 93
3.3. Mineralogical characterisation .................................................. 94
   3.3.1. X-ray Diffraction ............................................................ 94
   3.3.2. Transmission electron microscopy ....................................... 94
   3.3.3. Selected area electron diffraction ...................................... 95
   3.3.4. Mössbauer Spectroscopy .................................................. 95
      3.3.4.1. Isomer shift ............................................................. 96
      3.3.4.2. Quadrupole splitting ................................................ 96
      3.3.4.3. Magnetic splitting .................................................... 97
3.4. Ion Chromatography .................................................................. 98
3.5. Chemical Fe Assay ..................................................................... 99
3.6. References ................................................................................ 100

4. Phenotypic characterisation of *Shewanella oneidensis* MR-1 exposed to X-radiation .......................................................... 104

4.1. Abstract ....................................................................................... 104
4.2. Introduction .................................................................................. 105
4.3. Materials and Methods ............................................................... 107
   4.3.1. Growth of cells ..................................................................... 107
   4.3.2. Irradiation ............................................................................ 108
   4.3.3. Quantification of growth ...................................................... 108
   4.3.4. Viability ............................................................................... 108
   4.3.5. Analysis of metabolism by FT-IR Spectroscopy .................... 108
   4.3.6. Data processing .................................................................... 109
   4.3.7. Partial least-squares regression ........................................... 109
   4.3.8. Discriminant analysis ......................................................... 110
   4.3.9. Quantification of spectral peak areas .................................... 110
   4.3.10. MALDI-TOF-MS ................................................................. 111
4.3.11. Fe(III) reduction by irradiated *S. oneidensis* .............................................. 112
4.4. Results and Discussion ......................................................................................... 112
  4.4.1. Growth and survival of *S. oneidensis* after irradiation ......................... 112
  4.4.2. Post irradiation metabolism ................................................................. 113
  4.4.3. Dose related phenotypes estimated from FT-IR spectra ................. 117
  4.4.4. Radiation induced changes to proteins .............................................. 120
4.5. Conclusions ........................................................................................................... 125
4.6. Acknowledgements ............................................................................................... 125
4.7. Supplementary information ................................................................................ 126
4.8. References ............................................................................................................ 126

5. The impact of ionizing radiation on the bioavailability of Fe(III) minerals for microbial respiration ......................................................................................... 138

  5.1. Abstract .............................................................................................................. 138
  5.2. Introduction ........................................................................................................ 139
  5.3. Experimental Section ......................................................................................... 141
    5.3.1. Mineral synthesis and irradiation ......................................................... 141
    5.3.2. Mineralogical characterisation .......................................................... 141
    5.3.3. Microbial Fe(III) reduction ................................................................. 142
  5.4. Results and discussion ......................................................................................... 143
    5.4.1. Mineralogical characterisation of irradiated Fe(III) oxides ........ 143
    5.4.2. Microbial reduction of irradiated oxides ........................................... 150
    5.4.3. Fate of microbially reduced iron ......................................................... 152
  5.5. Conclusions ........................................................................................................... 153
  5.6. Acknowledgements ............................................................................................... 154
  5.7. Supplementary information ................................................................................ 154
  5.8. References ............................................................................................................ 155

6. Stimulation of microbial Fe(III)-reducing communities by gamma radiation ................................................................................................................................. 160

  6.1. Abstract .............................................................................................................. 160
  6.2. Introduction ........................................................................................................ 161
  6.3. Methods .............................................................................................................. 165
### 6.3.1. Sediment collection .......................................................... 165
### 6.3.2. Sediment microcosms ...................................................... 165
### 6.3.3. Irradiations ................................................................. 166
### 6.3.4. Geochemical analyses .................................................... 167
### 6.3.5. Ion chromatography ...................................................... 167
### 6.3.6. 16S amplicon pyrosequencing and data analysis ............... 167

### 6.4. Results and Discussion .................................................... 169
### 6.4.1. Biogeochemistry of irradiated microcosms containing added electron donor .......................................................... 169
### 6.4.2. Microbial ecology of Sellafield sediments ....................... 174
### 6.4.3. Microbial ecology of Sellafield sediments with added electron donor .......................................................... 174
### 6.4.4. Biogeochemistry in microcosms containing no added electron donor .......................................................... 177
### 6.4.5. Microbial ecology of Sellafield sediments with no added electron donor .......................................................... 180
### 6.4.6. Fe(III)-reduction in irradiated microcosms inoculated with *G. sulfurreducens* ...................................................... 183
### 6.4.7. Implications to the geodisposal of nuclear waste ............. 186

### 6.5. Acknowledgements .......................................................... 187

### 6.6. References .................................................................... 187

### 7. The morphological and physiological effects of ionizing radiation on the freshwater alga *Haematococcus pluvialis* ......................................................... 195
### 7.1. Abstract ........................................................................ 195
### 7.2. Introduction ................................................................. 196
### 7.3. Materials and methods .................................................... 199
### 7.3.1. Organism and culture conditions ................................. 199
### 7.3.2. Irradiation of cultures .................................................. 199
### 7.3.3. Algal cell number and viability .................................... 199
### 7.3.4. Analysis of metabolism by FT-IR spectroscopy .............. 200
### 7.3.5. Multivariate statistical analyses ................................... 200
### 7.4. Results and discussion .................................................... 201
### Table of Figures

1. **Thesis content** .................................................................................................................. 25

2. **Ionizing radiation processes in biogeochemistry relevant to the storage, geodisposal and bioremediation of radioactive waste** ................................................................. 29

**Figure 1.** The nuclear fuel cycle.......................................................................................... 29

**Figure 2.** The multi-barrier concept for the geological disposal of intermediate level radioactive waste. ........................................................................................................... 32

**Figure 3.** Total absorbed doses and dose rates relevant to the geodisposal of radioactive waste and bioremediation of radionuclide contaminated land. .................. 39

**Figure 4.** The photoelectric effect (a) and subsequent monochromatic photon production: X-ray fluorescence (b). ................................................................................. 43

**Figure 5.** Bremsstrahlung; broad spectrum X-rays generated from fast electron deceleration near a nucleus. ......................................................................................... 43

**Figure 6.** The interactions of charged particles with matter results in ionization and excitation of molecules along the particle track. Secondary electrons may cause additional excitations and ionizations. Modified from Spinks and Woods (1964). .................................................................................................................. 45

**Figure 7.** Electromagnetic radiation interactions with matter: the photoelectric effect (a); the Compton effect (b) and pair production (c). Modified from Spinks and Woods (1964). .................................................................................................................. 47

**Figure 8.** Initial processes in the decomposition of water by ionizing radiation. Modified from Garrett et al., (2005) and Lefticariu et al., (2010). ................................. 48

**Figure 9.** The direct interaction of ionizing radiation with biologically important molecules (Ward, 1981; Ghosal et al., 2005; Bank et al., 2008; Chivian et al., 2008; Schaller et al., 2011). Question marks represent unknown or uncharacterised mechanisms and/or the extent to which they contribute to the resultant effect. .................................................................................................................. 56
Figure 10. The indirect interaction of ionizing radiation with biologically important molecules (Ward, 1981; Gálès et al., 2004; Ghosal et al., 2005; Lin et al., 2005; Lin et al., 2005a; Lin et al., 2005b; Lefticariu et al., 2006; Dadachova et al., 2007; Daly et al., 2007; Chivian et al., 2008; Dadachova and Casadevall, 2008; Lefticariu et al., 2010; Daub et al., 2011; Libert et al., 2011; Yakabuskie et al., 2011). Question marks represent unknown or uncharacterised mechanisms and/or the extent to which they contribute to the resultant effect.

Figure 11. (A) A schematic of the impact of ionizing radiation on microbial interactions with key minerals (c). Experimental systems allow observation of a few select targets, including: cellular physiology (a) and growth substrates, such as Fe(III) bearing minerals (b). (B) The complex interaction of ionizing radiation with environmental systems involves multiple targets.

3. Methodology

Figure 1. Schematic of an interferometer configured for FT-IR. Adapted from Naumann (2000).

Figure 2. A typical FT-IR spectrum of S. oneidensis MR-1. See Table 1 for characteristic band assignments.

Figure 3. (a) MALDI and (b) Time-of-flight (TOF) mass analyzer. Adapted from Vaidyanathan and Goodacre (2003).

Figure 4. Nuclear energy levels and hypothetical Mössbauer spectra. (Image from Cornell and Schwertmann (2003)). The isomer shift (δ) in the absorber (A) relative to the source (S) is observed as a one line spectrum with a positive deviation of the absorption spectrum away from 0 mm s⁻¹. The quadrupole splitting (ΔE_Q) is observed as a two line spectrum. The six line spectrum is the result of magnetic splitting in samples which exhibit magnetic ordering.

4. Phenotypic characterisation of Shewanella oneidensis MR-1 exposed to X-radiation

Figure 1. Mean time difference in lag phase duration between irradiated cultures and respective controls (measured at mid exponential phase). Error bars depict 95% confidence intervals.
**Figure 2.** Euclidean distances between PC-DFA clusters of control and irradiated cultures at the three growth phases sampled using (A) FT-IR spectra and (B) MALDI mass spectra. PCs 1 to 5 (FT-IR) and 1 to 30 (MALDI-MS) were used by the DFA algorithm with *a priori* knowledge of machine replicates, i.e. 1 class per sample point and treatment, giving 6 classes in total for each dose.

**Figure 3.** Principal component scores from partial least squares regression performed on FT-IR data of control and irradiated cultures at lag phase. Solid black circles represent control samples and crosses represent irradiated samples.

**Figure 4.** (A) FT-IR spectra of cells of lag phase *S. oneidensis* MR-1 cultures exposed to X-radiation. These spectra are offset so that the spectral features are clearly visible. The bars at the top of the panel relate to the following biochemical regions: CH, aliphatic CH vibrations; CH$_{2/3}$, CH$_2$/CH$_3$ asymmetric stretch; Ad, amide I and II; Am, amine. Quantitative analysis of highest peak intensities in absorbance bands of lag phase FT-IR spectra: (B) Amide I + II / amine ratio, ratio of amide I and II peak intensity to amine peak intensity; (C) Protein / lipid ratio, ratio of summed amide and amine region peak intensities to summed peak intensities of CH region and CH$_2$/CH$_3$ asymmetric stretches. Data points show the mean of 9 measurements and error bars depict the standard error of the mean.

**Figure 5.** Mean MALDI-MS spectra of *S. oneidensis* MR-1 exposed to (A) 12 Gy X-radiation and (B) 95 Gy X-radiation. Spectra are offset to so that spectral features are clearly visible. Asterisks (*) show mass peaks in irradiated spectra that are discriminant with respect to batch controls, as observed in subtraction spectra (mean irradiated spectrum minus mean control spectrum) (Figure S6.). The mass range has been limited in the figure to only include peaks which are discriminant and to which tentative annotations can be assigned, displayed in Table 1.

**Figure 6.** Fe(III) reduction by *S. oneidensis* MR-1 with and without exposure to 50 Gy X-radiation. Error bars depict the standard error of the mean of triplicate experiments.
**Figure S1.** Growth profiles of *S. oneidensis* MR-1 after exposure to 12, 24, 48, 72 and 95 Gy X-radiation. Data points show mean of triplicate batch cultures and error bars depict 95% confidence intervals.

**Figure S2.** Survival of *S. oneidensis* MR-1 exposed to acute doses of X-radiation. Error bars depict standard error of the mean CFU mL\(^{-1}\).

**Figure S3.** Principal component scores from partial least squares regression performed on FT-IR data of control and irradiated cultures at lag phase (left panel) and validation plots for each model (right panel). Solid black circles represent control samples and crosses represent irradiated samples.

**Figure S4.** Principal component scores from partial least squares regression performed on FT-IR data of control and irradiated cultures at exponential phase (left panel) and validation plots for each model (right panel). Solid black circles represent control samples and crosses represent irradiated samples.

**Figure S5.** Principal component scores from partial least squares regression performed on FT-IR data of control and irradiated cultures at stationary phase (left panel) and validation plots for each model (right panel). Solid black circles represent control samples and crosses represent irradiated samples.

**Figure S6.** MALDI-MS subtraction spectra of *S. oneidensis* MR-1 exposed to 12 Gy (top panel) and 95 Gy X-radiation (bottom panel). Spectra show the result of the mean irradiated spectrum minus the mean control spectrum with labels indicating the masses of peaks that show a deviation away from zero. The mass range has been limited in the figure to only include peaks which show a difference and to which tentative annotations can be assigned, displayed in Table 1.

5. The impact of ionizing radiation on the bioavailability of Fe(III) minerals for microbial respiration

**Figure 1.** (A) Schematic representation of the 2-line ferrihydrite structure determined by electron nanodiffraction. (B) The structure of akaganeite.

**Figure 2.** Transmission electron micrographs of (a) non-irradiated ferrihydrite and (c) irradiated ferrihydrite. The corresponding SAED pattern for non-irradiated ferrihydrite is shown in (b) with indexed lines for 2-line ferrihydrite.
The corresponding SAED pattern for irradiated ferrihydrite is shown in (d) with measured interplanar spacings for irradiated suspensions in the bottom left segment and remaining segments displaying previously reported indexed patterns for selected Fe(III)-(oxy)hydroxides. .................................................................145

**Figure 3.** Mössbauer spectra of non-irradiated (top) and irradiated (bottom) ferrihydrite showing calculated fits (black curve) and component fits (red and blue curves). Respective $\chi^2 = 1.27$ and 1.51. .................................................................146

**Figure 4.** Transmission electron micrographs of non-irradiated (a) and irradiated (c) hematite. Corresponding SAED patterns show indexed lines for hematite in (b) and (d) respectively. .................................................................148

**Figure 5.** Mössbauer spectra of non-irradiated (top) and irradiated (bottom) hematite showing calculated fits (black curve) and component fits (red and blue curves). Respective reduced $\chi^2 = 214.92$ and 2.45. .................................................................149

**Figure 6.** Microbial Fe(III)-reduction in media containing irradiated (red) and non-irradiated (blue) ferrihydrite. Error bars depict the standard error of the mean. .................................................................151

**Figure 7.** Microbial Fe(III)-reduction in media containing irradiated (red) and non-irradiated (blue) hematite. Error bars depict the standard error of the mean. .................................................................152

**SI Figure S1.** Sequential extractions of iron in ferrihydrite-containing systems. Error bars indicate the standard error of the mean. .................................................................154

**SI Figure S2.** Sequential extractions of iron in hematite-containing systems. Error bars indicate the standard error of the mean. .................................................................155

6. **Stimulation of microbial Fe(III)-reducing communities by gamma radiation**

...........................................................................................................................................160

**Figure 1.** Concentrations of nitrate, 0.5 N HCl extractable Fe(II) and sulphate in microcosms containing added lactate (7 mM) and acetate (7 mM). Dashed line indicates removal of microcosms from the irradiation cell. Error bars represent the standard error of the mean of triplicate measurements and where not visible, error bars are within the symbol size. .................................................................171
Figure 2. Concentrations of lactate, acetate, propionate and formate in microcosms containing added lactate (7 mM) and acetate (7 mM). Malate equivalent represents the concentration of an organic acid which elutes with a very similar retention time to malate on the ion chromatography system used in these experiments. The exact identity of the molecule remains undetermined and the quantities are therefore calculated using the molar mass of malate. The dashed line indicates removal of microcosms from the irradiation cell. Error bars represent the standard error of the mean of triplicate measurements and where not visible, error bars are within the symbol size.

Figure 3. Microbial community analysis of microcosms containing added lactate (7 mM) and acetate (7 mM).

Figure 4. Concentrations of nitrate, 0.5 N HCl extractable Fe(II) and sulphate in microcosms containing no added electron donor. The dashed line indicates the removal of the microcosms from the irradiation cell. Error bars represent the standard error of the mean of triplicate measurements and where not visible, error bars are within the symbol size.

Figure 5. Concentrations of lactate, acetate and formate in microcosms containing no added electron donor. The dashed line indicates the removal of microcosms from the irradiation cell. Error bars represent the standard error of the mean of triplicate measurements and where not visible, error bars are within the symbol size.

Figure 6. Microbial community analysis of microcosms with no added electron donor. Microcosms were removed from the irradiation cell at T = 56.

Figure 7. 0.5 N HCl extractable Fe(II) concentrations in control and irradiated microcosms inoculated with *G. sulfurreducens*. Microcosms were removed from the irradiation cell and inoculated at T = 0. Error bars represent the standard error of the mean of triplicate measurements and where not visible, error bars are within the symbol size.

7. The morphological and physiological effects of ionizing radiation on the freshwater alga *Haematococcus pluvialis*
Figure 1. The effect of ionizing radiation on the growth, survival and morphology of *H. pluvialis* cultures determined immediately after irradiation on each day. (A) Cell numbers (cells mL\(^{-1}\)). (B) Percentage of viable cells in X-irradiated and control cultures. (C) Percentage of encysted cells in X-irradiated and control cultures.

Figure 2. (A) X-treated culture after three consecutive irradiations (over three days) showing palmelloid green cells. (B) X-treated culture after five consecutive irradiations over five days showing encysted cells and ghost/nonviable cells. (C) X-treated culture showing high concentrations of ghost cells and cell debris after 5 consecutive days of X-radiation. (D) Control culture after five consecutive days showing small palmelloid green cells. Image B and C are both from the same sample; however, large coagulations of cell debris and ghost cells appeared to accumulate in conjunction with encysted cells in selected areas. Scale bar represents 50µm.

Figure 3. Raw mean FT-IR spectra obtained from control (C) and irradiated (X) cultures throughout the five day experiment (day of treatment = 1, 2, 3, 4, 5). The spectra are offset in the y-axis for ease of visualisation.

Figure 4. PC-DFA score plots of FT-IR spectra from all treatments. Scores for discriminant function 1 (DF1) versus discriminant function 2 (DF2) are shown in (A), whilst the scores of DF1 versus DF3 are plotted in (B). 20 PCs were extracted from PCA and passed to the DFA algorithm.

Figure 5. HCA dendrogram constructed from Euclidean distances between DF clusters in PC-DFA score plots of FT-IR spectra. Each treatment category represents the mean from each treatment class.

8. Conclusions and future directions

Appendix

Figure 1. (A) Fe(II) concentrations in solutions containing irradiated and non-irradiated single crystals of hematite (Hm). (B) Fe(II) associated with the solid crystalline phase in experiments containing irradiated and non-irradiated hematite. Where present, error bars depict the standard error of the mean of triplicate experiments.
Figure 2. AFM images representative of (a) non-irradiated hematite (sterile); (b) non-irradiated hematite incubated with *G. sulfurreducens*; (c) irradiated hematite (sterile) and (d) irradiated hematite incubated with *G. sulfurreducens*. Colour intensities indicate relative height in the z-dimension as indicated by the scale bars to the right of each panel.

Figure 3. ESEM images of (a) non-irradiated hematite; (b) non-irradiated hematite incubated with *G. sulfurreducens*; (c) irradiated hematite and (d) irradiated hematite incubated with *G. sulfurreducens*.

Figure 4. EDX spectra of (A) ‘non-amorphous’ surface site on irradiated hematite and (B) ‘amorphous’ surface site on irradiated hematite.

Figure 5. XPS spectra of Fe 2p from the cleaved surface of (a) non-irradiated hematite; (b) non-irradiated hematite incubated with *G. sulfurreducens*; (c) irradiated hematite and (d) irradiated hematite incubated with *G. sulfurreducens*.
List of Tables

1. Thesis content ..........................................................................................................................25

2. Ionizing radiation processes in biogeochemistry relevant to the storage, geodisposal and bioremediation of radioactive waste .................................................29

   Table 1. Important actinides and fission products in the nuclear fuel cycle and radioactive wastes. .................................................................31
   Table 2. Primary radiolytic reactions. .....................................................................................49

3. Methodology ............................................................................................................................83

   Table 1. Assignments to biochemical bands identified by FT-IR spectroscopy. Adopted from Ellis et al. (2003). See Figure 2 for typical FT-IR spectrum of S. oneidensis. ............................................................................................................86

4. Phenotypic characterisation of Shewanella oneidensis MR-1 exposed to X-radiation .........................................................................................................................104

   Table 1. Annotations of protein peaks in irradiated S. oneidensis from UniprotKB/Swiss-Prot and UniProtKB/TrEMBL protein sequence databases...121

5. The impact of ionizing radiation on the bioavailability of Fe(III) minerals for microbial respiration ..........................................................................................................................138

   Table 1. Mössbauer parameters of all samples and parameters previously reported for akaganeite. .........................................................................................................................147

6. Stimulation of microbial Fe(III)-reducing communities by gamma radiation .................................................................160

   Table 1. Initial microcosm compositions and treatments. .............................................166
   Table 2. Concentrations of bioavailable Fe, inorganic anions and organic acid salts in Sellafield sediment microcosms immediately after irradiation and prior to
addition of *G. sulfurreducens*. Errors indicate the standard error of the mean of triplicate measurements. ..........................................................185

7. The morphological and physiological effects of ionizing radiation on the freshwater alga *Haematococcus pluvialis* .............................................195

8. Conclusions and future directions ................................................................214

Appendix ...........................................................................................................228
Abstract

The University of Manchester

Ashley Richards Brown

Doctor of Philosophy (PhD)

The impact of ionizing radiation on microbial cells pertinent to the storage, disposal and remediation of radioactive waste.

2013

Microorganisms control many processes pertinent to the stability of radwaste inventories in nuclear storage and disposal facilities. Furthermore, numerous subsurface bacteria, such as Shewanella spp. have the ability to couple the oxidation of organic matter to the reduction of a range of metals, anions and radionuclides, thus providing the potential for the use of such versatile species in the bioremediation of radionuclide contaminated land. However, the organisms promoting these processes will likely be subject to significant radiation doses. Hence, the impact of acute doses of ionizing radiation on the physiological status of a key Fe(III)-reducing organism, Shewanella oneidensis, was assessed. FT-IR spectroscopy and MALDI-TOF-MS suggested that the metabolic response to radiation is underpinned by alterations to proteins and lipids. Multivariate statistical analysis indicated that the phenotypic response was somewhat predictable although dependent upon radiation dose and stage of recovery. In addition to the cellular environment, the impact of radiation on the extracellular environment was also assessed. Gamma radiation activated ferrihydrite and the usually recalcitrant hematite for reduction by S. oneidensis. TEM, SAED and Mössbauer spectroscopy revealed that this was a result of radiation induced changes to crystallinity. Despite these observations, environments exposed to radiation fluxes will be much more complex, with a range of electron acceptors, electron donors and a diverse microbial community. In addition, environmental dose rates will be much lower than those used in previous experiments. Sediment microcosms irradiated over a two month period at chronic dose rates exhibited enhanced Fe(III)-reduction despite receiving potentially lethal doses. The microbial ecology was probed throughout irradiations using pyrosequencing to reveal significant shifts in the microbial communities, dependent on dose and availability of organic electron donors. The radiation tolerance of an algal contaminant of a spent nuclear fuel pond was also assessed. FT-IR spectroscopy revealed a resistant phenotype of Haematococcus pluvialis, whose metabolism may be protected by the radiation induced production of an astaxanthin carotenoid. The experiments of this thesis provide evidence for a range of impacts of ionizing radiation on microorganisms, including the potential for radiation to provide the basis for novel ecosystems. These results have important implications to the long-term storage of nuclear waste and the geomicrobiology of nuclear environments.
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For Mum and Dad
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The Author

The author graduated from the University of Manchester in 2009 with a BSc (Hons) in Environmental Science. The author then joined the Geomicrobiology research group in the School of Earth, Atmospheric and Environmental Sciences to pursue a postgraduate research degree and has been engaged in the research reported in this thesis since October 2009 under the supervision of Prof. Jon Lloyd, Prof. Roy Goodacre and Prof. Simon Pimblott.
Chapter 1

Introduction

Introduction and thesis content
1. Thesis content

1.1. Thesis layout
This thesis is presented in the alternative format style, with each research chapter presented as an independent body of work. Due to the nature of the alternative style, there is some degree of repetition in each of the chapters, in particular, the introduction and methodology sections, however, this is to enable the submission of each chapter for publication in a scientific journal.

1.2. Thesis content, status of manuscripts and author contributions

Chapter 1.
Introduction and Thesis Content.
A. R. Brown.

Chapter 2.
Ionizing radiation processes in biogeochemistry relevant to the storage, geodisposal and bioremediation of radioactive waste
A. R. Brown.

Chapter 3.
Methodology
A. R. Brown.

Chapter 4.
Phenotypic characterisation of Shewanella oneidensis MR-1 exposed to X-radiation.
Status:
Manuscript in final preparation for submission to Applied and Environmental Microbiology, American Society for Microbiology.
Author contributions:
A. R. Brown - Principal author, irradiation of cultures, data collection, FT-IR spectroscopy, MALDI-MS data collection and statistical analyses; E. Correa – Statistical analysis of FT-IR spectra; Y. Xu – drift correction of MALDI mass spectra; S. M. Pimblott – Manuscript review and co-supervisor; R. Goodacre – Manuscript review and co-supervisor; J. R. Lloyd – Manuscript review and principal supervisor.

Chapter 5.
The impact of ionizing radiation on the bioavailability of Fe(III) minerals for microbial respiration.
Status:
Manuscript in final preparation for submission to Environmental Science and Technology, ACS.
Author contributions:
A. R. Brown - Principal author, microbiological and geochemistry sample preparation and data collection, TEM and SAED analysis, assisted with Mössbauer analysis; P. L. Wincott – Mössbauer data collection and fitting; D. J. Vaughan – Manuscript review and Mössbauer analysis; S. M. Pimblott – Manuscript review and co-supervisor; J. R. Lloyd – Manuscript review and principal supervisor.

Chapter 6.
Stimulation of microbial Fe(III)-reducing communities by gamma radiation.
A. R. Brown, C. Boothman and J. R. Lloyd.
Status:
Manuscript in final preparation for submission to Applied and Environmental Microbiology, American Society for Microbiology.
Author contributions:
A. R. Brown - Principal author, preparation of soil microcosms, microbiological and geochemistry sample preparation and data collection; C. Boothman – Microbial community analysis and manuscript review; J. R. Lloyd – Manuscript review and principal supervisor.
Chapter 7.
The morphological and physiological effects of ionizing radiation on the freshwater alga *Haematococcus pluvialis.*
*A. R. Brown and V. E. Evans contributed equally to the authorship of this work.
Status:
Manuscript in preparation for submission to *Applied and Environmental Microbiology, American Society for Microbiology.*
Author contributions:
**A. R. Brown** - Co-author, irradiation of algal cultures, FT-IR spectroscopy and multivariate statistical analyses; **V. E. Evans** – Co-author, microbial culturing, fluorescence microscopy, UV-Vis spectroscopy; **E. Correa** – IR data analysis; **D. Sigee** – Manuscript review; **R. Goodacre** – Manuscript review and co-supervisor; **J. R. Lloyd** – Manuscript review and principal supervisor.

Chapter 8.
Conclusions and future directions.
**A. R. Brown**

Appendix
i. The bioavailability of Fe(III) for microbial respiration in irradiated hematite single crystals.

Author contributions:
**A. R. Brown** - Principal author, microbiological and geochemistry sample preparation and data collection, ESEM, EDX and AFM; **P. L. Wincott** – AFM and XPS data collection and fitting; **D. J. Vaughan** – manuscript review; **J. R. Lloyd** – Principal supervisor.
Chapter 2

Literature Review

Ionizing radiation processes in biogeochemistry relevant to the storage, geodisposal and bioremediation of radioactive waste
2. Ionizing radiation processes in biogeochemistry relevant to the storage, geodisposal and bioremediation of radioactive waste

2.1. Environmental radioactivity

Sources of radiation in the environment are both diverse and pervasive. Naturally occurring radionuclides; cosmic rays; nuclear weapons testing and accidental release, for example, the 1986 explosion at the Chernobyl atomic energy power station, account for significant quantities of radionuclides present in the environment (Aarkrog, 1988; Lloyd and Renshaw, 2005; Merroun and Selenska-Pobell, 2008). However, perhaps of most concern is radiation from fission products; spent fuel waste of high activity from nuclear plant operation and fuel reprocessing (Lloyd et al., 2005) and associated contaminated infrastructure and materials of lower and intermediate activity (Nirex, 2005). Indeed, more than 50 years of civil nuclear power generation in the UK has generated a significant waste legacy which will increase as a result of the UK commitment to nuclear new build.

Figure 1. The nuclear fuel cycle. SNF, spent nuclear fuel; MOX, mixed oxide.

Much radioactive waste is derived from the preparation and processing of uranium fuel for nuclear reactors: the so-called nuclear fuel cycle (Figure 1). Initially, this involves the abstraction of uranium ore from open-cast mining facilities, often requiring acid leaching. As such, significant quantities of low activity wastes are generated during the mining and milling processes (Sharrad et al., 2011).
Fission reactors rely on the thermal neutron induced fission of $^{235}\text{U}$. However, the natural abundance of this isotope is only $\sim 0.72 \text{ atom}\%$, hence enrichment is required to increase the concentration to such a level at which a chain reaction can be sustained: for a pressurized water reactor this is typically $\sim 3$ to 5 atom\%. Once this is achieved, oxide fuel pellets are formed and then clad in metal/alloy tubes for insertion into the reactor.

In addition to the fissile $^{235}\text{U}$, the dominant uranium isotope, $^{238}\text{U}$, is “fertile” since its neutron irradiation inside a reactor leads to its conversion to other fissile isotopes, including $^{239}\text{Pu}$. As such, typically 40\% of the total energy from a uranium-fuelled reactor is generated from the fission of plutonium isotopes produced in situ (Sharrad et al., 2011). In addition to plutonium, significant activity concentrations of fission products are generated. These are often highly efficient neutron absorbers and thus act to ‘poison’ the fuel. Hence, the decreasing proportion of fissile material and the increasing concentrations of fission products result in the fuel becoming ‘spent’. This highly radioactive waste product can then either be stored for eventual disposal, or reprocessed in order to remove fission products and re-enrich the concentration of fissile uranium to produce more fuel. For example, the PUREX process (‘plutonium uranium extraction’) involves the nitric acid extraction of these elements into a solvent phase. The extracted plutonium can then also be recycled to produce a mixed uranium-plutonium oxide pellet: MOX fuel. Again, reprocessing of this kind also generates significant quantities of radioactive wastes (Sharrad et al., 2011).

The waste materials generated throughout the nuclear fuel cycle are highly varied and thus produce complex mixed wastes with various concentrations of radionuclides, their daughter products, heavy metals and organics. The concentration, activity, half-life and chemical behaviour of radionuclides in such mixed wastes poses a great challenge for the containment, storage and disposal of radioactive materials.

Further to the challenges of management of the scale and complexity of radioactive wastes is the obvious risk posed to the biosphere by emissions associated with the decay of radioactive nuclei (Nirex, 2005). The activities and half-lives of
radionuclides in higher activity wastes are such that there is a requirement to isolate waste from the biosphere (Table 1).

**Table 1.** Important actinides and fission products in the nuclear fuel cycle and radioactive wastes (Wilson, 1996; Ewing, 1999; Choppin et al., 2001).

<table>
<thead>
<tr>
<th>Radioisotope</th>
<th>Decay mode</th>
<th>Energy (MeV)</th>
<th>Half-life (years)</th>
<th>Activity (TBq t⁻¹ U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{235}$U</td>
<td>Alpha, gamma</td>
<td>4.40</td>
<td>$7.04 \times 10^8$</td>
<td></td>
</tr>
<tr>
<td>$^{239}$Pu</td>
<td>Alpha</td>
<td>5.15</td>
<td>$2.41 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>$^{237}$Np</td>
<td>Alpha</td>
<td>4.78</td>
<td>$2.14 \times 10^6$</td>
<td></td>
</tr>
<tr>
<td>$^{137}$Cs</td>
<td>Beta, gamma</td>
<td>0.51 β; 0.66 γ</td>
<td>30</td>
<td>3060</td>
</tr>
<tr>
<td>$^{90}$Sr</td>
<td>Beta</td>
<td>0.54</td>
<td>28.5</td>
<td>2180</td>
</tr>
<tr>
<td>$^{99}$Tc</td>
<td>Beta</td>
<td>0.29</td>
<td>$2.13 \times 10^3$</td>
<td>0.484</td>
</tr>
<tr>
<td>$^{129}$I</td>
<td>Beta, gamma</td>
<td>0.15 β; 0.04 γ</td>
<td>$1.57 \times 10^7$</td>
<td>0.0012</td>
</tr>
</tbody>
</table>

2.2. **Storage of spent nuclear fuel and geological disposal of radioactive waste**

In order to satisfy the above requirement, the implementation of deep geological disposal of wastes with various activities has taken place or is being planned in a number of countries (IAEA, 1997; Nirex, 2005). In the UK, this policy has been adopted for the disposal of intermediate level waste (ILW; contaminated components, cladding and plutonium contaminated material with activities >4 GBq α t⁻¹ and >12 GBq β/γ t⁻¹) and high level waste (HLW; fission products from irradiated fuel with the same activity distributions as for ILW, but also with the requirement to take heating into account) (Richardson et al., 1996; DEFRA, 2008). The geological disposal facility (GDF) design is based on a multiple barrier concept, whereby several engineered barriers are employed to restrict radionuclide mobility (Figure 2) (West et al., 2002; Morris et al., 2011). These generally consist of (i) the conditioned waste form; (ii) the waste container; (iii) buffer materials around the waste package, to control the evolution of chemical or hydrogeological conditions; (iv) ‘backfill’ to pack repository caverns: a material with mechanical strength and the ability to further condition repository conditions to retard radionuclide mobility; (v) sealing systems to moderate groundwater flow after emplacement and (vi) the host rock geology (Morris et al., 2011). In the case of HLW, this will likely involve
vitrification in glass, ceramics or other known stable mineral phases prior to encapsulation in stainless steel containers and backfilling with bentonite (NDA, 2007). ILW, on the other hand, will be grouted in stainless steel containers prior to backfilling with cement (Nirex, 2003b; Morris et al., 2011). It is likely that both these waste forms would then be placed in excavated vaults in a highly crystalline granitic host rock, approximately 200-1000 m deep in the subsurface (Figure 2) (Nirex, 2003b). Such a repository would have a range of access shafts and drift tunnels to aid waste emplacement during the operational period of the facility before the vaults would be permanently sealed.

Upon re-saturation of the GDF with groundwater and reaction with the cementitious backfill, a hyperalkaline region (pH 12-14) will develop in the repository vicinity (Pedersen et al., 2004) providing an alkaline and reducing environment in which most radionuclides are predicted to be insoluble (Nirex, 2003a). It is intended that these engineered structures will safeguard the integrity of the waste forms over the geological timeframes necessary for the storage of long half-life wastes. Hence, performance assessments of proposed repositories focus on mitigation of scenarios which may compromise the containment of wastes over these timeframes.

Figure 2. The multi-barrier concept for the geological disposal of intermediate level radioactive waste (Nirex, 2003b; Morris et al., 2011).

2.3. Microbial ecology of nuclear infrastructure and repository environment
As mentioned previously, the primary objective of waste disposal is the isolation of waste from the biosphere and as such, radioactive wastes have been stored
underground for numerous years with this intention (Dayal et al., 1984; Brady and Kozak, 1995; Sears, 1998; Bergström et al., 2011). However, microorganisms are known to be both viable and active at great depth in many subsurface geological formations and associated groundwaters (Fredrickson and Onstott, 1996; Pedersen, 2000; Fredrickson and Fletcher, 2001). In addition, the sterility of waste components and encapsulants will not be guaranteed and thus, the potential exists for inoculation of the host media during repository construction (Fredrickson and Fletcher, 2001; Merroun and Selenska-Pobell, 2008).

Microbial colonisation of surface environments relevant to nuclear power generation has also been documented. For example, a variety of bacteria have been isolated from nuclear reactor coolant water and from spent nuclear fuel storage ponds (Sinilova et al., 1969; Bruhn et al., 2009). The autotrophic microalga *Coccomyxa actinabiotis* has also been identified and isolated from a highly radioactive spent nuclear fuel storage pond, which is highly unusual for a eukaryotic organism (Rivasseau et al., 2013).

The quantification of microbial activity in nuclear and repository environments is an important consideration in risk and performance assessments as the development of microbial communities in such environments could influence the evolution of biogeochemical conditions, waste stability and radionuclide mobility (Lloyd, 2003; Lloyd and Renshaw, 2005; Lloyd et al., 2005; Rizoulis et al., 2012). Numerous studies have focused on the behaviour and survival of microorganisms in disposal environments, with reviews of the Canadian concept of waste disposal providing particularly good examples (Stroes-Gascoyne and West, 1996; Stroes-Gascoyne and Sargent, 1998). This concept involves the permanent geological disposal of nuclear waste in an engineered vault 500-1000 m deep in the granitic rock of the Canadian Shield. This is similar to the proposed multibarrier system of the U.K., however, fuel waste will be packaged in titanium or copper containers surrounded by a clay-based buffer material and then backfilled with a mix of crushed host rock and glacial lake clay. Thus, such studies also provide an appropriate analogue for the U.K. concept of disposal.
Microbial interactions with the whole array of materials associated with nuclear power generation and waste processing and storage have also been the subject of several reviews and studies (Pedersen, 1999; West et al., 2002; Lloyd, 2003). For instance, microbially influenced corrosion (MIC) of repository infrastructure and waste containers, and also microbial transformation of metals and radionuclides in the waste itself may be of detriment to the stability of the waste inventory (West et al., 2002). For example, some sulphate-reducing bacteria (SRB) display the ability to corrode copper; the material of choice for many waste canisters (King and Stroes-Gascoyne, 1997). On the other hand, the interactions of such microbes with radionuclides may promote the stability of wastes. For instance, the SRB Desulfovibrio desulfuricans is also capable of the reduction of U(VI) to insoluble U(IV) (Lovley and Phillips, 1992). In addition, biofilm and ligand production by microbes may lead to biosorption and enhanced chemisorption of radionuclides (West et al., 2002), whilst the incorporation of radionuclides into biominerals may also restrict their solubility (Lloyd and Macaskie, 2002).

The availability of electron donors for microbial metabolism in these environments will likely not be limited. For example, hydrogen generation from the anaerobic corrosion of steel may be a significant electron donor for microbial metabolism (Libert et al., 2011). Similarly, organic products from the degradation of cellulose, which may be present in the form of paper, filters and cotton (Keith-Roach, 2008), may act as both electron donors and carbon sources (Glaus and Van Loon, 2008). Of particular importance is the formation of complexing agents, such as NTA and isosaccharinic acid (ISA), the latter produced by alkaline hydrolysis of cellulose (Keith-Roach, 2008). These organic acids have the potential to enhance the solubility and migration of radionuclides with which they complex (Knill and Kennedy, 2003). However, microbial degradation of these complexing agents may release the inorganic form of the radionuclide and restrict its mobility (Reed et al., 1999; Keith-Roach, 2008).

Gas generation in a repository due to microbial degradation of organic materials in intermediate level waste is also of concern to repository performance (Nirex, 1994). For instance, cellulose may be a significant gas-generating component in organic containing wastes (Askarieh et al., 2000). The production of gases, such as
hydrogen, carbon dioxide, methane and hydrogen sulphide, via microbial metabolism is an important consideration due to the explosive potential of H\textsubscript{2} and (radio)toxicity of \textsuperscript{14}CO\textsubscript{2} and \textsuperscript{14}CH\textsubscript{4}. Indeed, over-pressurisation may lead to enhanced radionuclide transport in both the gas phase and in groundwater.

The biogeochemical cycling of iron in repository environments is also of particular interest as this may impart a control on the redox potential of such environments. Iron will be present in large quantities in a repository, as a canister material; in infrastructure, such as rock bolts, and in the indigenous mineralogy of the host rock. Thus, the availability of Fe(II), coupled with an increase in organic matter from cellulose degradation, may stimulate the development of an Fe(II)-reducing microbial community. In addition to the reduction of Fe(II), iron-reducing bacteria, such as Geobacter and Shewanella spp. also have the ability to couple the oxidation of organic matter to the reduction of redox-active radionuclides. This may occur both directly, via enzymatic reduction; or indirectly, via the reduction of bioavailable Fe(II) and subsequent abiotic electron transfer from biogenic Fe(II)-bearing phases, such as magnetite (Lloyd and Macaskie, 1996; Lloyd et al., 2000; Anderson et al., 2003; Hansel et al., 2003; Wilkins et al., 2006). As the reduction of radionuclides, such as mobile Tc(VII), U(VI) and Np(V), results in their conversion to insoluble Tc(IV), U(IV) and Np(IV) species, subsequent precipitation will restrict the mobility of these radionuclides (Wildung et al., 2000; Law et al., 2010; Williams et al., 2011).

Furthermore, as such bacteria are able to couple the reduction of Fe(III) to the oxidation of hydrogen, significant hydrogen generation from the anaerobic corrosion of steel (Libert et al., 2011) may be moderated via microbial metabolism. Indeed, this and other forms of anaerobic metabolism (e.g. sulphate reduction) may also limit hydrogen availability for methanogenesis and alleviate the potential for over-pressurisation.

Despite the high pH associated with the disposal of cementitious ILW, a few recent studies have demonstrated that a range of anaerobic redox pathways, including microbial Fe(III)-reduction, may still occur in these environments (Rizoulis et al., 2012; Williamson et al., 2013).
2.4. Waste legacy and contaminated land

In addition to the challenges posed by the long-term storage of legacy wastes, civil nuclear energy generation and nuclear weapon production since 1945 has generated significant volumes of legacy wastes and contaminated land (Brim et al., 2003). For example, a US Department of Energy (DOE) survey of ninety one contaminated sites, out of around three thousand in total, contained millions of cubic meters of mixed wastes including toxic organics, heavy metals and radionuclides (Riley et al., 1992). The requirement for remediation has led to US government investment in the order of billions of dollars (Macilwain, 1996). However, physico-chemical methods of remediation may be costly and hence, natural attenuation and bioremediation technologies may present a more versatile and cost-effective alternative (Tiedje, 2002; Lovley, 2003).

2.4.1. Bioremediation

In addition to the reduction of radionuclides by some Fe(III)-reducing bacteria, some species are also able to couple the oxidation of organic matter to the reduction of a variety of metal cations and anions (Nealson and Saffarini, 1994; Wade and DiChristina, 2000; Liu et al., 2002; Daulton et al., 2007). This metabolic versatility provides the potential for the use of such species in the bioremediation of radionuclide contaminated land (Heidelberg et al., 2002; Liu et al., 2002; Tiedje, 2002; Lloyd, 2003; Fredrickson et al., 2008). Similarly, redox-active co-contaminants often associated with radioactive wastes may also be reduced by such species (Fredrickson et al., 2004). For example, the toxic and mobile Cr(VI) can also be removed from solution by reduction to the less toxic and less mobile Cr(III) (Hansel et al., 2003). Thus, dissimilatory Fe(III)-reducing species such as *Shewanella* sp. and *Geobacter* sp. have been considered for the remediation of complex wastes comprising contaminant metals, radionuclides and toxic organic compounds that can be degraded by these organisms (Lloyd, 2003).

Phytoremediation of radionuclide contaminated storage ponds using green eukaryotic species has also been considered. For instance, the desmid *Closterium moniliferum* shows a high level of Sr incorporation in to BaSO$_4$ crystals which it precipitates in to vacuoles (Wilcock et al., 1989; Krejci et al., 2011). *Coccomyxa actinabiotis* also provides an attractive method of decontamination as it strongly
accumulates $^{238}\text{U}$, $^{137}\text{Cs}$, $^{60}\text{Co}$ and $^{14}\text{C}$ (Rivasseau et al., 2013). Cultures of this microalga also proved effective as ion exchangers in purifying nuclear effluents contaminated with the above radionuclides over a 1 hour period. Such biodecontamination techniques may therefore prove to be more feasible than expensive physico-chemical processes.

2.5. Radiation dose

Despite the importance of microbial processes in a geological repository and radwaste contaminated environments, the characterisation of such processes has largely been conducted with little consideration of the presence of ionizing radiation fluxes, one of the key environmental stresses associated with such environments (Bruhn et al., 2009). Radiation fluxes will be present in both environments, although the doses in these environments will be different due to the nature of the radioactive sources involved. For instance, dose rates and total absorbed doses will be governed by the concentration of radionuclides, the distance from the source and the half-life of present radionuclides, including unstable daughter products. The decay mode ($\alpha$, $\beta$, $\gamma$, n) and energy is also of importance as these affect penetration depth (also dependent on the absorber) and energy deposition in absorbing molecules (Section 2.6). The relationship of decay dynamics to geodisposal timescales is particularly important, as this will govern dose rates at the time of potential failure of the encapsulant, potentially affecting the dose to a microbial community. Indeed, in both geodisposal and remediation scenarios, the dynamics of radioactive decay coupled to the heterogeneity of radioactive material in these environments means dose rates may vary over a range of 17 orders of magnitude (Allard and Calas, 2009; Allard et al., 2012). Thus, when examining the interaction of ionizing radiation with biogeochemical processes in such environments, there is a requirement to reconcile experimental doses with measured or predicted dose rates and their coincidence with microbial populations.

The following sections discuss both predicted and measured dose rates and total absorbed doses in the environments of interest. The S.I. unit of ionizing radiation dose is the Gray (Gy). This unit defines the absorption of energy (in Joules) per unit mass of the absorbing matter (in kg); whereby $1 \text{ Gy} = 1 \text{ J kg}^{-1}$.  

37
2.5.1. Radiation doses in geodisposal scenarios

In HLW forms, the major sources of dose are the beta decay of fission products, for example $^{90}$Sr and $^{137}$Cs, and the alpha decay of actinides: e.g. $^{235}$U, $^{237}$Np, $^{239}$Pu, $^{241}$Am and $^{244}$Cm (Ewing et al., 1995). The decay of fission products dominates the radiation flux in the first 500 years of the repository lifetime due to their shorter lifetimes; subsequently the alpha decay of actinides will be the primary source due to the much longer half-lives of the actinides (Table 1). This is illustrated by predictions of total absorbed doses for the wastes of the Savannah River Plant, South Carolina (Figure 3) (after Weber, 1991; Ewing et al., 1995). After $10^3$ years, the total absorbed dose of beta/gamma radiation is predicted to be 600 MGy. This gives an average dose rate of 68.5 Gy h$^{-1}$. Indeed, the initial dose rate would be significantly higher and will tail off quickly, such that, after $10^6$ years the total absorbed dose would be 1 GGy, giving an average dose rate of 0.11 Gy h$^{-1}$. For alpha radiation, after $10^3$ years, the total absorbed dose is predicted to be 90 MGy, giving an average dose rate of 10 Gy h$^{-1}$. After $10^6$ years the total absorbed dose would be 800 MGy, resulting in an average dose rate of 0.09 Gy h$^{-1}$. Indeed, these figures only provide an indication of doses in the vitrified waste forms themselves, and in the case of alpha radiation, doses at the container surface and further afield will be significantly lower.
Figure 3. Total absorbed doses and dose rates relevant to the geodisposal of radioactive waste and bioremediation of radionuclide contaminated land. 1, Bruhn et al. (2009); 2, Niedree et al. (2013); 3, Pitonzo et al. (1999); 4, Bank et al. (2008); 5, Daub et al. (2011); 6, Plotze et al. (2003) after Reed et al. (1987); 7, Ewing et al. (1995) after Weber (1991); 8, Allard and Calas (2009); 9, Allard et al. (2012); 10, Stroes-Gascoyne et al. (1994); 11, Noynaert et al. (1998); 12, Rivasseau et al. (2013); 13, Daly et al. (2004).
Predictions of doses at disposal canister surfaces vary wildly. For instance, a study of dose from a simulated HLW container in Boom Clay suggested that dose rates may be as high as 400 Gy h\(^{-1}\) at the clay-canister interface, decreasing to 25 Gy h\(^{-1}\) at 20 cm away (Figure 3) (Noynaert et al., 1998). The study was carried out over 5 years and therefore, this figure may represent the upper limits of dose rates near a canister. On the other hand, gamma and neutron dose rates of 2 Gy h\(^{-1}\) dropping off after 200 years toward 0.2 Gy h\(^{-1}\) have been reported (Reed et al., 1987; Plotze et al., 2003). Despite this lower estimation of dose rates, the total absorbed doses are still significant, with 0.7 MGy gamma and 140 MGy alpha radiation predicted after 10\(^4\) years near the container surface (Figure 3). In clays, total absorbed doses of MGy to GGy have been suggested (Allard et al., 2012) and other estimations of surface dose rates generally fall in between previous predictions: 71 Gy h\(^{-1}\) (Allard and Calas, 2009) and 52 Gy h\(^{-1}\) (Stroes-Gascoyne et al., 1994; Stroes-Gascoyne and West, 1996) have been suggested for canister surfaces.

It is difficult to predict the coincidence of these doses with microbial populations, however, as previously mentioned, the waste packages will likely not be sterile at emplacement. Furthermore, the predicted failure of the engineered barrier after several thousand years (DEFRA, 2008; Morris et al., 2011) will lead to the ingress of groundwaters and inoculation with associated microbes will occur.

### 2.5.2. Ionizing radiation doses in remediation scenarios and contaminated land

The radioactivity of contaminated land and surface storage facilities varies significantly between sites, with the Hanford site, Washington State, representing one of the most contaminated sites in the U.S. Activities of up to 0.37 GBq kg\(^{-1}\) of \(^{137}\)Cs have been recorded at one location (Riley et al., 1992; Fredrickson et al., 2004).

The radioactivity at such sites tends to be quantified by activity, i.e. decays s\(^{-1}\) (Bq), though in order to assess the importance of radiation fluxes on potential remediation schemes, there is a requirement to relate activity (e.g. Bq kg\(^{-1}\)) to dose (Gy; J kg\(^{-1}\)). This is a complex relationship as these two units cannot be converted mathematically. However, the relationship can be modelled relatively easily in simple systems, i.e. with known activities of single radionuclides and the known
attenuation coefficients of the medium, e.g. air. In reality, the relationship is compounded by the heterogeneity of contaminated environments, both in the spatial distribution of (multiple) emitters and the parameters of the contaminated medium, e.g. soils of various water contents.

A study by Niedree et al. (2013) provides a useful case study of the dose-activity relationship in a ploughed soil from the Chernobyl exclusion zone (Figure 3). Doses were calculated from activities of $^{90}$Sr and $^{137}$Cs using particle track calculations and Monte Carlo simulation. For instance, activities representative of the most contaminated soils, i.e. 0.5 MBq kg$^{-1}$ $^{90}$Sr and 0.9 MBq kg$^{-1}$ $^{137}$Cs, gave simulated doses of 3.2 Gy y$^{-1}$ and 1.5 Gy y$^{-1}$ respectively. In addition, higher experimental activities of 8.2 MBq kg$^{-1}$ $^{90}$Sr and 9.6 MBq kg$^{-1}$ $^{137}$Cs gave calculated doses of 51.7 Gy y$^{-1}$ and 14.8 Gy y$^{-1}$ respectively. Indeed, these simulated dose rates are only characteristic of the soil properties and contamination of the Chernobyl locality and thus, it would be inappropriate to extend these calculations to other contaminated sites, such as Hanford. However, these data provide useful context for experimentation and offer insight in to doses after radionuclide release in a GDF when the radiation is no longer a point source.

In general, dose rates and total absorbed doses by microbes in near surface contaminated environments will likely be lower than in geodisposal scenarios (Figure 3). Nevertheless, in relative terms, dose rates in both environments may be considered chronic. However, there are scenarios in non-geodisposal environments where acute doses may be encountered by microbes, such as reactor waters (Sinilova et al., 1969) and fuel cladding (Bruhn et al., 2009). Furthermore, microbial colonisation of spent nuclear fuel ponds has also been observed (Santo Domingo et al., 1998; Sarro et al., 2003; Galès et al., 2004). One such study reported dose rates of 70 mGy h$^{-1}$ near the pool extremities and surfaces, rising to several hundred Gy h$^{-1}$ near fuel elements (Figure 3) (Rivasseau et al., 2013). However, the practice of deionisation and filtering of pool waters (Galès et al., 2004) may result in water circulation, such that cycles of acute doses are delivered to organisms at fuel elements.
It is evident that the occurrence of microorganisms in nuclear environments may potentially coincide with significant radiation fluxes. Furthermore, the utility of specialist metal-reducing bacteria for remediation may be determined by the ability of a species to survive radiation stress (Brim et al., 2000). Alternatively, engineering of already radioresistant microorganisms for the remediation radionuclides has also been considered (Lange et al., 1998; Brim et al., 2000). In both these cases, there is a requirement to deliver fundamental physiological information on the biochemical processes which underpin the cellular response to radiation (Lovley, 2003; Gao et al., 2004). Furthermore, an evaluation of the wider effects of radiation on microbially catalysed processes in a GDF is critical to the geological disposal risk assessment. However, such an investigation first requires an assessment of ionizing radiation effects.

2.6. Ionizing radiation

Ionizing radiation refers to radiation, in the form of alpha, beta and gamma rays from the decay of radioactive nuclei; high-energy charged particles and also photons of short-wavelength electromagnetic radiation (with energies greater than around 50 eV) (Spinks and Woods, 1964). Such particles or photons, by definition, have the capacity to ionize molecules during their collision, whereby the energy of the incident particle or photon exceeds the ionization potential of the molecule incurring the collision (Sonntag, 1987). Energy deposition by ionizing radiation may produce a range of excited or ionized states in a large number of molecules in the vicinity of the radiation track. This is in contrast to the low energy of UV photons for instance, which interact with one molecule only and do not generate ionized species (Spinks and Woods, 1964).
Figure 4. The photoelectric effect (a) and subsequent monochromatic photon production: X-ray fluorescence (b).

X-rays of shorter wavelength also have the capacity to ionize. Unlike gamma rays, which are emitted by a decaying nucleus, X-rays are emitted during electron collisions outside the nucleus (the photoelectric effect; Figure 4 and section 2.6.2) and during the deceleration of high energy charged particles in matter, or ‘bremsstrahlung’ (braking radiation) (Figure 5) (Dertinger and Jung, 1970).

Figure 5. Bremsstrahlung; broad spectrum X-rays generated from fast electron deceleration near a nucleus.

Although the sources of the different forms of radiation are different, the character of X and gamma radiation is the same, i.e. ionization of target molecules is through the action of secondary electrons (Spinks and Woods, 1964). However, the effect of the radiation, i.e. the number and types of species produced, depends on the energy of
the incident photons, which is related to wavelength by the modified Planck equation:

\[ E = \frac{hc}{\lambda} \]

where \( E \) equates to the photon energy; \( h \) is the Planck constant; \( c \) is equal to the speed of light and \( \lambda \) is the photon wavelength (Spinks and Woods, 1964). Whilst the energy of photons can be quantified by such direct means, this does not give a full indication of the production, distribution or concentration of products by the incident photons, nor is there any distinction between the effects of various radiation types. Rather, the subsequent chemistry caused by the radiation is ultimately dependent upon the rate of energy deposition in matter. Hence, different types of radiations are commonly classified in terms of their linear energy transfer (LET); that is, the linear-rate of energy loss by an ionizing particle traversing matter (Spinks and Woods, 1964). Due to the variation in the rate of energy deposition and the various mechanisms by which different radiation types interact with other molecules, it is appropriate to discuss the radiation chemistry of each type separately.

2.6.1. Charged particles

Alpha (helium atom nuclei) and beta particles generated by a decaying nucleus lose energy during their interaction with matter via three main mechanisms. Bremsstrahlung is only important at very high energies and elastic scattering is only important for particles of low mass and energy. Rather, inelastic collisions with the electrons of the stopping material lead to raised energy levels and excited states. Ionization results if the electron energy is increased such that an electron is ejected (Spinks and Woods, 1964).
Figure 6. The interactions of charged particles with matter results in ionization and excitation of molecules along the particle track. Secondary electrons may cause additional excitations and ionizations. Modified from Spinks and Woods (1964).

Furthermore, ejected electrons may themselves be sufficiently energetic to produce ‘secondary’ ionizations and excitations (Figure 6). The distribution of these secondary ionizations is again dependent on the energy and scatter angle and thus, small clusters of ionizations, or ‘spurs’, occur as energy is continually lost. Due to the large difference in mass between an alpha particle and the electrons with which it collides, there will be many collisions of small energy losses which produce little deflection of the particle track. Hence, high LET radiation such as alpha particles generate dense clusters of ionizations, whilst fast electrons and beta particles lead to tortuous tracks comprising well separated clusters (Spinks and Woods, 1964).

2.6.2. Electromagnetic radiation
Unlike alpha particles, electromagnetic radiation photons (gamma and X-radiation) tend to incur relatively large energy losses during their interaction with matter. The energy of those which interact may be attenuated according to the Beer-Lambert law:

\[ I = I_0 e^{-\mu x} \]

where \( I \) equates to the residual photon energy; \( I_0 \) is the initial photon energy; \( \mu \) is the attenuation co-efficient of the absorber and \( x \) is the photon path length. Depending on the energy loss of the initial interaction, there may be residual photon energy; however, this may not be sufficient energy to cause further ionizations. Those
photons which do not interact will continue their transmittance with no loss of energy or change of direction (Spinks and Woods, 1964).

The interactions of photons with atomic electrons are mainly via the photoelectric effect, the Compton Effect and pair production (Figure 7) (Spinks and Woods, 1964). In the photoelectric effect (Figure 7a), the entire energy of the photon is transferred to an atomic electron with which it collides, causing it to be ejected with energy \((E_e)\) equal to the difference between the initial photon energy \((E_0)\) and the binding energy \((E_s)\) of the atomic electron:

\[
E_e = E_0 - E_s
\]

In addition, energy is usually released from the atom in the form of characteristic X-radiation due to the filling of the vacancy (from the ejected electron) in the inner electron shell by an electron from an outer orbital. Alternatively, a second valence electron, an Auger electron, may be ejected, resulting in a 2+ cation. The predominant outcome is dependent on the identity of the atom.

The Compton effect, on the other hand, occurs when a photon interacts with a free or loosely bound electron, causing the photon to be deflected with a lower energy (Figure 7b). The energy difference between the photon’s original energy and its deflected energy is divided between the scattered electron (the initial electron to which energy was transferred) and the recoil electron (which allows for the conservation of energy and momentum) (Spinks and Woods, 1964).

Finally, pair production, which only occurs at energies greater than 1.02 MeV, is caused by the complete absorption of the incident photon in the region of the nucleus resulting in the ejection of an electron and a positron (Figure 7c). The positron will eventually recombine with an electron, accompanied by the release of two 511 keV gamma rays (annihilation radiation) (Spinks and Woods, 1964).
2.6.3. Ionizing radiation interaction with biomolecules

The inelastic collisions of charged particles and the coulomb interactions of electromagnetic radiation all result in ionization and excitations of the atoms or molecules with which the photons interact. Although, the chemical effects of each radiation type will, therefore, be qualitatively similar, the differences in energy transfer rates will result in variations in the quantity and distribution of chemical species. It is this which influences the numerous different chemical effects induced in the absorbing material (Spinks and Woods, 1964).

Figure 7. Electromagnetic radiation interactions with matter: the photoelectric effect (a); the Compton effect (b) and pair production (c). Modified from Spinks and Woods (1964).
In order to understand the potential chemical effects of these interactions in biological systems and the subsequent effect on microbial physiology, the result of ionizations must first be considered. The nature of the chemical consequences can be examined in terms of the ‘direct’ effects produced by energy deposition in biomolecules and the ‘indirect’ effects resulting from radicals and reactive oxygen species (ROS).

2.6.4. The direct effect

The direct effects of ionizing radiation on microbial cells arise from the deposition of energy directly into biomolecules (Dertinger and Jung, 1970). This may result in the radiolysis of bonds and radical production within molecules, due to the ejection of electrons (Spinks and Woods, 1964). The biological implications of direct damage are generally considered to be relatively minor compared to indirect effects. For instance, only ~20% of damage in DNA is incurred from direct energy transfer (Ghosal et al., 2005).

2.6.5. The indirect effect

The indirect effect considers the impact of secondary reactive species produced by ionizing radiation. Hence, in biological systems, as the cytosol has a high water content, the indirect effect primarily concerns the aqueous solution chemistry of water radiolysis products (Dertinger and Jung, 1970) (Figure 8). Such products contribute to ~80% of the radiation damage observed in DNA (Ghosal et al., 2005).

![Figure 8. Initial processes in the decomposition of water by ionizing radiation. Modified from Garrett et al., (2005) and Lefticariu et al., (2010).]
As the table below indicates, a large diversity of ROS, molecular species and oxidizing and reducing radicals are produced by the radiolysis of water (Riley, 1994). The rate constants of these reactions are such that many of these species rapidly lead to the proliferation of reactive species, the most biologically important being the hydroxyl radical, hydrogen peroxide and superoxide (Daly, 2009).

**Table 2.** Primary radiolytic reactions (Pimblott and LaVerne, 1992; Hill and Smith, 1994; Pastina and LaVerne, 2001; Daly, 2009)

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rate constant (mol(^{-1}) s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 H(<em>2)O (\rightarrow) HO(^{\cdot}) + H(^{+}) + e(^{-})(</em>{aq})</td>
<td>5.9 \times 10(^{-8})</td>
</tr>
<tr>
<td>2 2 HO(^{\cdot}) (\rightarrow) H(_2)O(_2)</td>
<td>3.6 \times 10(^9)</td>
</tr>
<tr>
<td>3 O(<em>2) + e(^{-})(</em>{aq}) (\rightarrow) O(<em>2^{\cdot})(</em>{aq})</td>
<td>2.0 \times 10(^{10})</td>
</tr>
<tr>
<td>4 H(^{+}) + O(<em>2^{\cdot})(</em>{aq}) (\leftrightarrow) HO(_2^{\cdot})</td>
<td>2.0 \times 10(^{10})</td>
</tr>
<tr>
<td>5 HO(_2^{\cdot}) + H(^{+}) (\rightarrow) H(_2)O(_2)</td>
<td>2.0 \times 10(^{10})</td>
</tr>
<tr>
<td>6 e(^{-})(_{aq}) + H(_2)O(_2) (\rightarrow) HO(^{\cdot}) + OH(^{-})</td>
<td>1.1 \times 10(^{10})</td>
</tr>
<tr>
<td>7 HO(^{\cdot}) + HO(_2^{\cdot}) (\rightarrow) H(_2)O + O(_2)</td>
<td>6.0 \times 10(^9)</td>
</tr>
<tr>
<td>8 H(_2)O(_2) + HO(^{\cdot}) (\rightarrow) H(_2)O + O(_2^{\cdot}) + H(^{+})</td>
<td>2.7 \times 10(^7)</td>
</tr>
<tr>
<td>9 H(^{+}) + OH(^{-}) (\leftrightarrow) H(_2)O</td>
<td>1.4 \times 10(^{11})</td>
</tr>
<tr>
<td>10 HO(_2^{\cdot}) + HO(_2^{\cdot}) (\rightarrow) H(_2)O(_2) + O(_2)</td>
<td>7.1 \times 10(^5)</td>
</tr>
<tr>
<td>11 H + H(_2)O(_2) (\rightarrow) OH + H(_2)O</td>
<td>1.6 \times 10(^8)</td>
</tr>
<tr>
<td>12 H + O(_2) (\rightarrow) HO(_2)</td>
<td>1.9 \times 10(^{10})</td>
</tr>
</tbody>
</table>
2.7. The impact of ionizing radiation on microbial physiology – the intracellular environment

The hydroxyl radical is the most prolific of damage agents and reacts indiscriminately with biomolecules, causing the oxidation of DNA, RNA, proteins and lipids (Riley, 1994; Daly, 2009). However, the extreme reactivity and short lifetime of hydroxyl radicals prevents damage beyond the locality in which it is formed. Hydrogen peroxide and superoxide, on the other hand, are considerably less reactive, though they are still able to oxidize proteins (Daly, 2009).

When the generation of reactive species exceeds the capacity of endogenous scavengers (e.g. glutathione, superoxide dismutase (SOD), catalase and peroxidase) to neutralize them, oxidative stress is incurred (Ghosal et al., 2005). Hence, the mechanisms to reduce and reverse oxidative stress and the direct and indirect damage incurred are vital components of radiation resistance (Whitehead et al., 2006).

2.7.1. Radiation resistance and sensitivity

The early focus of research into radiation sensitivity was medical; namely radiotherapy (Sonntag, 1987). This developed into research of the sterilisation of pathogens in the food industry, through to present research into the antioxidant systems of the extremely radioresistant bacterium Deinococcus radiodurans, which has implications for drug discovery (Zhang et al., 2009). Hence, since the 1960s, the interactions of radiation with biological molecules and protective mechanisms have been evaluated (Bruce, 1964).

2.7.2. The impact of ionizing radiation on DNA

As all molecules within a cell perform a specific function, any modification to the functionality of a molecule is likely to impinge upon the normal physiology of the cells. Thus, the overall impact of ionizing radiation on physiology is likely to depend not only on the sensitivity of a specific class of molecules and the intracellular distribution of such molecules (Goodhead et al., 1993), but also on the effect such loss of biological functionality will have on the cell. As cellular physiology and proliferation is heavily dependent on the conservation of genetic material, it was considered that the biological effects of ionizing radiation originate
from damage to DNA (Ward, 1994). Indeed, early assays for the detection of DNA
damage in bacterial cells displayed a strong correlation between damage and
sensitivity to ionizing radiation (Dertinger and Jung, 1970). Consequently, classical
models of radiation toxicity regard DNA as the molecule most affected by ionizing
radiation and hence, damage to DNA has become a hallmark indicator of the effects
of ionizing radiation (Daly, 2009).

Early research indicated that the initial spectrum of direct DNA damage and
subsequent mutation induction depends on the LET of the radiation, with high-LET
radiations being much more severe (Thacker and Cox, 1975; Goodhead et al., 1993).
Moreover, DNA damage depends on the complexity of initial damage. This in turn,
is dependent on the amount of energy deposited (in the case of direct damage), the
number and frequency of events in the locality and the type of damage, of which
there are four main types (Ward, 1985; Goodhead et al., 1993). Single strand breaks
are lesions in the sugar-phosphate backbone in one strand of the DNA double helix,
whereas double strand breaks traverse both strands, causing an unravelling of the
double helix. Crosslinks and base damage are also detrimental, although it is the
relative contribution of each damage type and their spatial distribution that controls
complexity of damage. For instance, single strand breaks have a negligible effect
(Goodhead, 1994); however, clustering of single strand breaks may lead to double
strand breaks, which are more difficult to repair (Goodhead, 1989; Zhang et al.,
2009; Alloni et al., 2010). Furthermore, secondary modifications of the damage site
by, for instance, thiols, oxygen and hydroxyl radicals, also increase damage
complexity (Goodhead, 1989; Goodhead et al., 1993).

2.7.3. Conventional DNA repair mechanisms
The capacity and ability of repair mechanisms to repair the complex initial damage
ultimately dictates the longevity of damage and the potential for cell death. In
addition to ROS scavengers, most bacteria encode conventional enzymatic DNA
repair mechanisms to repair both direct damage and damage resulting from oxidative
stress (Teoule, 1987). Indeed, some mechanisms, such as excision repair, are
ubiquitous in organisms and are essential for repairing the damage incurred by
numerous stresses, including ionizing radiation (Sancar, 1994).
For single strand breaks, the universal enzyme DNA polymerase acts to recombine the sugar phosphate chain. This is carried out by DNA Pol I and not the replicating DNA Pol III as DNA Pol I possesses two exonuclease domains enabling it to undertake nucleotide excision repair (Patel et al., 2001). Homologous recombination mechanisms rejoin double strand breaks and where required, excinuclease and glycosylases, which comprise the base excision repair process, act to remove any damaged bases in the DNA molecule (Teoule, 1987; Sancar, 1994). In addition to conventional repair mechanisms, some organisms also exhibit the upregulation of a range of genes related to unique repair pathways when exposed to radiation. For instance, *Escherichia coli* displays a characteristic regulatory system known as the SOS response when irradiated. This phenotype is characterised by the induction of stable DNA replication, enhanced repair capacity and inhibition of cell division and respiration; processes which may inhibit recovery (Little and Mount, 1982).

### 2.7.4. *Deinococcus*: extreme radioresistance

*D. radiodurans* is capable of growing at 5 kGy and surviving 16 kGy and is thus considered extremely radioresistant (Moseley and Mattingly, 1971; Cox and Battista, 2005). Indeed, this extremophile has been found at nuclear waste contaminated sites, including the arid Hanford site (Fredrickson et al., 2004). However, it is unlikely that it is this environment that led to this organism’s radioresistance. Mattimore and Battista (1996) argue that there are no terrestrial environments that generate such high fluxes of ionizing radiation and as such, there would be no selective advantage in having such extreme radioresistance. Rather, such resistance is likely incidental; a result of this organism’s adaptation to desiccation, which also yields significant DNA damage. Indeed, many species isolated from arid deserts show a similar response to ionizing radiation (Rainey et al., 2005).

The mechanism of ionizing radiation resistance in *D. radiodurans* appears to lie in the efficient repair of DNA damage incurred during irradiation (Moseley, 1983; Smith et al., 1992; Minton, 1994). Furthermore, the genome of *D. radiodurans* is polydiploid (Dertinger and Jung, 1970), consisting of 4-10 copies of the genome (Hansen, 1978). These ‘spare’ copies may restrict the relative amount of DNA damage. However, *D. radiodurans* does not exhibit an SOS response or non-homologous end-joining (Daly and Minton, 1996; Makarova et al., 2001).
On the other hand, the Fe(III)-reducing bacterium *Shewanella oneidensis* is comparatively sensitive to radiation. For instance, *D. radiodurans* can tolerate doses 200 times higher than that of *S. oneidensis* (Figure 3; 16 kGy yields 10% survival, c.f. 70 Gy for *S. oneidensis*) (Daly et al., 2004; Daly et al., 2007). Despite the fact that *S. oneidensis* encodes a conventional set of DNA repair mechanisms, including the SOS response, that are strongly upregulated upon irradiation (Brown et al., 2006; Qiu et al., 2006), it is killed by doses of radiation which yield less than one double strand break per genome (Daly et al., 2004). Thus, it is evident that for the same dose of ionizing radiation, the range of bacterial sensitivity is very diverse (Qiu et al., 2006). Furthermore, it is apparent that sensitivity is not quantifiably related to DNA damage. This, therefore, suggests that radiation damage to molecules other than DNA may characterise the impact of ionizing radiation on cellular physiology.

### 2.8. The metabolic impact of radiation

#### 2.8.1. Alternative targets of ionizing radiation: Protein

Reactive oxygen species react with a range of biomolecules, with proteins likely to incur the most initial damage (Ward, 1994; Du and Gebicki, 2004). For instance, radiation may cause fragmentation of proteins as a result of peptide bond cleavage (Stadtman, 1993; Berlett and Stadtman, 1997; Stadtman and Levine, 2003). This reaction is thought to be mediated by HO’, which extracts the hydrogen atom from the amino acid residue to form water (Berlett and Stadtman, 1997). This, in turn, leads to the production of a carbon-centred radical which is converted in to various radical intermediaries, or reacts with another carbon-centred radical to form a protein-protein cross-link. If no cross-link is formed, the formation of an alkoxy radical will lead to cleavage of the peptide bond.

Amine groups may also incur reaction with oxygen free radicals generated during radiolysis, with oxidation leading to the formation of α-ketoacids, aldehydes or carboxylic acids (Stadtman, 1993). Furthermore, the carboxyl moiety of amino acids has also been shown to be susceptible to free radical mediated oxidation (Stadtman and Levine, 2003).
In addition to these oxidation reactions, carbonylation may also occur and collectively, these reactions result in modifications, which may cause conformational changes that may alter the activity and function of the protein (Maher and Schubert, 2000; Daly et al., 2007; An et al., 2010). As enzymes catalyze the majority of metabolic reactions, if there is significant generation of ROS by ionizing radiation these modifications may cause significant disruption to downstream metabolism as well as protein turnover (Stadtman, 1993; Stadtman and Levine, 2003).

Moreover, it has been hypothesized that cells which are susceptible to oxidation of enzymes responsible for DNA repair may be killed by a relatively minor degree of DNA damage (Daly, 2009). Hence, cells which are better protected against protein oxidation may be better equipped to repair DNA damage which would otherwise lead to a loss in viability (Daly et al., 2010). Indeed, protein oxidation has been quantifiably related to bacterial sensitivity to ionizing radiation (Daly et al., 2007; Krisko and Radman, 2010).

### 2.8.2. Protection of proteins by manganese

The importance of manganese complexes in scavenging ROS has been noted for some time (Archibald and Fridovich, 1982). Daly et al. (2007) have since presented a model of manganese redox cycling in bacterial cells, which protects proteins by scavenging superoxide and other peroxyl radicals and catalyzes the formation of hydrogen peroxide. This works antagonistically to the catalytic proliferation of ROS (particularly the very reactive hydroxyl radical) by cellular iron redox cycling (Fenton and Haber-Weiss reactions). As such, Mn accumulating bacteria commonly exhibit high ionizing radiation resistance, with the exception of *Neisseria gonorrhoea* which is radiation sensitive (Daly et al., 2004). However, this organism also accumulates very high concentrations of cellular iron, resulting in a very low Mn/Fe concentration ratio of 0.004. This emphasizes that it is the accumulation of manganese with respect to cellular iron, which underpins radiation resistance. The work of Chen et al. (2009) also supports this hypothesis, demonstrating that *RecQ* (responsible for genomic stability) mutants exhibit high ROS concentrations, which are correlated with intracellular iron and manganese concentrations.
In addition, the Mn/Fe ratio correlates with the extent of protein oxidation in a range of bacteria (Daly et al., 2004). It has therefore been proposed that manganese complexes may associate with protein structures to protect them from oxidative modification by ROS. Indeed, Daly et al. (2010) demonstrated that Mn\(^{2+}\)-P\(_i\) complexes protected essential DNA repair enzymes from damage by doses of up to 50 kGy. Hence, Mn concentrations are observed to be highest near the nucleoid of *D. radiodurans*, suggesting that this mechanism leads to the highly efficient repair of DNA synonymous with *D. radiodurans* (Daly et al., 2007).

With regard to the proliferation of ROS by cellular Fe redox cycling, it is not surprising that Mn-poor and Fe-rich bacteria, such as *Shewanella* sp. exhibit high levels of protein oxidation correlated with rapid loss of viability (Daly et al., 2007; Shuryak and Brenner, 2009). Furthermore, whilst Mn was distributed near the nucleoid of *D. radiodurans*, Fe was partitioned at the septum of dividing cells (Daly et al., 2007); potentially predisposing the membrane region to oxidative stress via HO’’. This could have important implications to the respiratory capabilities of *S. oneidensis* and other Fe(III)-reducing bacteria, particularly as the respiration of extracellular terminal electron acceptors, including metals, relies on respiratory chain components and iron-rich cytochromes situated across both membranes and in the periplasm (Ehrlich, 2002).

### 2.8.3. Radiation damage in lipids

In addition to proteins, ionizing radiation has also been shown to damage lipids and lipoproteins, for example, via radiolysis, fragmentation and peroxidation of unsaturated fatty acid residues (Shadyro et al., 2002; Shadyro et al., 2004). Previous studies have suggested that lipid peroxidation can be disregarded as a cause of lethality in irradiated bacterial cells (Wolters and Konings, 1982; Nauser et al., 2005). However, as these molecules are major components of membranes, this could alter the integrity and function of membranes, for instance, by changes in viscosity and permeability (Stark, 1991). Again, these changes could have implications to the respiration of alternative extracellular electron acceptors by species such as *S. oneidensis*. 


Figures 9 and 10 illustrate the range of possible interactions of ionizing radiation with the biological system with respect to the direct and indirect effect. Coupled with the discussion of radiation impacts on the extracellular environment (Section 2.10), the figures highlight the complexity of radiation driven processes and indicate where detailed information on the mechanisms involved is lacking.

Figure 9. The direct interaction of ionizing radiation with biologically important molecules (Ward, 1981; Ghosal et al., 2005; Bank et al., 2008; Chivian et al., 2008; Schaller et al., 2011). Question marks represent unknown or uncharacterised mechanisms and/or the extent to which they contribute to the resultant effect.
Figure 10. The indirect interaction of ionizing radiation with biologically important molecules (Ward, 1981; Galès et al., 2004; Ghosal et al., 2005; Lin et al., 2005a; Lin et al., 2005b; Lefticariu et al., 2006; Dadachova et al., 2007; Daly et al., 2007; Chivian et al., 2008; Dadachova and Casadevall, 2008; Lefticariu et al., 2010; Daub et al., 2011; Libert et al., 2011; Yakabuskie et al., 2011). Question marks represent unknown or uncharacterised mechanisms and/or the extent to which they contribute to the resultant effect.
2.9. The response of model organisms to ionizing radiation

2.9.1. The metabolic response of *S. oneidensis*

From an ecological and bioremediation perspective, systems biology, genomic and post-genomic techniques are playing a key role in determining the physiological status of microbial communities and more fundamentally, in elucidation of the cellular impacts of ionizing radiation (Lovley, 2003; Qiu et al., 2006; Hau and Gralnick, 2007). Indeed, the sequencing of the *S. oneidensis* MR-1 genome has led to it becoming one of the most intensively studied environmentally-relevant bacteria subject to such approaches (Heidelberg et al., 2002).

The transcriptional response of *S. oneidensis* MR-1 to acute doses of gamma radiation revealed the functional up-regulation of numerous genes encoding conventional antioxidant defences and DNA repair mechanisms (Qiu et al., 2006). Interestingly, genes associated with several prophages in the *S. oneidensis* MR-1 genome were also upregulated in a synchronised manner indicative of initiation of the prophage lytic cycle. This cell lysis may contribute to the sensitivity of this organism to ionizing radiation (Qiu et al., 2006). These findings indicate that the response of *S. oneidensis* to radiation is not necessarily coherent; indeed, many uncharacterized or poorly defined genes were also up and down regulated. Thus, the prediction of the impact of these changes in gene regulation on down-stream metabolism (the phenotype) was limited.

In summary, the characterization of the metabolic impact of ionizing radiation has been limited due to the large array of potential cellular targets alongside the complex response in gene regulation (Qiu et al., 2006). As the metabolic status of such species is fundamental to their role in the reduction of key electron acceptors in nuclear environments, further quantitative analysis of the effects of ionizing radiation fluxes on metabolism is required.
2.9.2. Ionizing radiation and eukaryotic microorganisms

In addition to the interactions of ionizing radiation with biomolecules described above, which are common to all organisms, the response of eukaryotic microorganisms to ionizing radiation is more complex. In growing cultures, the metabolism of algal species is initially inhibited by radiation. For instance, cultures of the green alga *Oedogonium cardiacum* irradiated to 12 Gy display a dose-dependent delay in cell division (Pujara et al., 1970). However, upon a second dose of radiation, many algal species appear to show an ‘adaptive response’, whereby radioresistance increases in later growth stages of the same culture (Joiner et al., 2001). In species such as *Chlorella*, radioresistance cannot be attributed to differences in sensitivity within the initial population or between cell cycles, rather, as with bacterial species, this is likely associated with upregulation of protective mechanisms (Santier et al., 1985).

A proteomic analysis of the response of the green alga *Haematococcus pluvialis* to oxidative stress revealed the up-regulation of ubiquitous enzymes involved in the anti-oxidant response of many microorganisms, including superoxide dismutase, catalase and peroxidise (Wang et al., 2004). This suggests that the metabolic response of many microorganisms is likely very similar. However, in addition to these conventional protective molecules, the response of eukaryotic algae to radiation is often accompanied by the production of protective carotenoids. Such pigments serve as antioxidants, capable of scavenging/quenching reactive oxygen species and radicals, including superoxide, hydrogen peroxide and peroxyl radical (Kobayashi, 2000). Indeed, *H. pluvialis* showed an increase in the expression of genes and proteins related to the production of the carotenoid astaxanthin, when exposed to conditions which generated oxidative stress (Hu et al., 2008; Wang et al., 2009). Furthermore, *Haematococcus lacustris* appears to accumulate these pigments in vacuoles around the nucleus, which may help to prevent the genome from accumulating irreparable DNA damage (Hagen et al., 1993). Interestingly, the enzymatic defence processes of *H. pluvialis* only appear up-regulated during the early stages of oxidative stress (Wang et al., 2004). After this period, astaxanthin was readily accumulated, suggesting that the response of such species is dynamic, involving the differential regulation of several anti-oxidative defence strategies. Such dynamic processes may have implications to the accumulation of damage...
throughout chronic irradiation of such species in spent nuclear fuel ponds or in bioremediation scenarios.

2.9.3. Radiation induced growth of microorganisms

There is recent evidence to suggest that ionizing radiation may stimulate microbial respiration without the need for conventional molecular electron donors, such as organic carbon substrates. Dadachova et al. (2007) demonstrated that ionizing radiation changed the electronic properties of fungal melanin which corresponded with a four-fold increase in its capacity to reduce NADH. In addition, irradiated melanised cells of Cryptococcus neoformans exhibited a three-fold increase in acetate incorporation, alongside a general increase in growth rate and total biomass levels. Thus, it has been suggested that radiation fluxes can fuel non-photosynthetic electron transfer chains and thus directly stimulate growth (Figure 10) (Dadachova and Casadevall, 2008). Indeed, the observation of radiation hormesis (directional growth towards an ionizing radiation source) is not new (Zhdanova et al., 2004; Tugay et al., 2006); however, the mechanisms which underpin these processes are poorly constrained.

In addition to the direct reduction of intracellular pigments, ionizing radiation may also interact with molecules involved in extracellular electron transfer processes. For instance, the solvated electron generated by ionizing radiation readily reduces oxidized flavins to semiquinones (Land and Swallow, 1969). This has been demonstrated for riboflavin, a molecule secreted by Shewanella spp. which is capable of shuttling electrons, via a semiquinone moiety, to extracellular electron acceptors, alleviating the need for direct enzymatic contact (von Canstein et al., 2008). Furthermore, there is also evidence of cytochrome c reduction by the solvated electron generated by radiolysis (Land and Swallow, 1971). However, it is stressed that no experimental evidence for energy conservation or Fe(III) reduction via these reactions currently exists; though the reaction rates have been calculated, suggesting that such reactions may be feasible.
2.10. Ionizing radiation interactions with the extracellular environment

Efforts to determine the impact of radiation on microorganisms have consistently focused on the intracellular milieu. However, more recent research has highlighted the significance of radiation processes in the extracellular environment. As non-photosynthetic microbes rely on their environment to acquire growth substrates and nutrients and also to exchange and remove metabolic products, any radiation driven process that impacts upon the extracellular environment may perturb these cellular functions.

2.10.1. The direct effect in the extracellular environment

In addition to the direct effect in the intracellular environment, such direct interactions may also occur in the extracellular environment (Figure 9). For instance, recent studies of the decay of naturally occurring radionuclides in the deep subsurface suggest that radiolysis of inorganic bicarbonate (HCO$_3^-$) results in formate (HCO$_2^-$) production (Lin et al., 2006; Chivian et al., 2008). As formate is a suitable electron donor for the reduction of metals and radionuclides, this process may have positive implications for the stimulation of Fe(III)-reducing communities. (Lovley, 1993; Lloyd et al., 1997; Lloyd et al., 2001).

In addition, the radiolysis of cellulose may also lead to a supply of respirable carbon sources, however, it is likely that the alkaline hydrolysis of cellulose in cementitious wasteforms (such as ILW) will be far more dominant (Askarieh et al., 2000; Humphreys et al., 2010). Further carbon substrates may also be supplied by the radiolysis of natural organic matter in soils (Bank et al., 2008), through which, increases in dissolved organic carbon have been observed in irradiated soils (Schaller et al., 2011). However, the mechanism of this release is not well constrained, nor have the organic end products been identified.

2.10.2. The indirect effect in the extracellular environment

As in biological systems, indirect interactions via water radiolysis products may also dominate radiation effects in the extracellular environment. The generation of molecular hydrogen by radiolysis is perhaps the most prominent of these processes as it may be used as an electron donor for a range of microbial electron accepting processes (Figure 10) (Pedersen, 1997; Lloyd, 2003; Galès et al., 2004; Lin et al.,
For example, dihydrogen concentrations in deep volcanic rock units in South Africa were consistent with its production via radiolysis from the decay of naturally occurring U, Th and K (Lin et al., 2005a). The rate of radiolytic hydrogen production suggested that a deep subsurface microbial community consisting of chemolithoautotrophs, including sulphate reducers and methanogens, could be sustained by this process (Lin et al., 2005b; Lin et al., 2006). Thus, in a geological repository, where electron donor availability may be limited, the generation of hydrogen by radiolysis could potentially fuel the reduction of Fe(III), or indeed other electron acceptors including radionuclides, as described in Section 2.3. Furthermore, the utilisation of such gas may mitigate overpressurisation as a result of radiolytic gas generation.

In addition, a study by Galès et al. (2004) proposed that hydrogen may be a dominant electron donor in a radioactive waste pool containing 10 kBq L\(^{-1}\) \(^{60}\)Co, with hydrogen concentrations estimated to be at saturation. Indeed, it was demonstrated that Ralstonia and Burkholderia isolates were capable of \(H_2\) oxidation coupled to \(O_2\) respiration. This form of metabolism suggests that the utility of hydrogen as an electron donor may also be possible in environments pertinent to the surface storage of nuclear waste or indeed, during the operational period of a geological repository during which oxic conditions will prevail.

With regard to potential electron acceptors, radiation may lead to the oxidation or reduction of Fe, dependent on the coordinating ligand in the molecular structure (Ladriere, 1998). For instance, Mössbauer spectroscopy of gamma sterilised sediments indicated that irradiation (20 kGy) led to the reduction of Fe(III) oxyhydroxides (Bank et al., 2008). Such a process may preclude the reduction of Fe(III) by iron-reducing microbes; though with respect to the mobility of radionuclides, the sorption of U(VI) was subsequently enhanced in these systems regardless.

Similarly, gamma irradiation of montmorillonite (84 kGy) led to partial reduction of structural Fe(III) to Fe(II) (Gournis et al., 2000). It was proposed that this reduction was mediated by the strongly reducing \(H^+\) generated by the radiolysis of interstitial water molecules. A similar effect is also observed in irradiated illite and smectites.
(1.1 MGy) which led to a decrease in the layer charges (Plotze et al., 2003). Whilst this has implications to hydration and cation exchange capacity of clays (particularly important for radionuclide sorption) (Allard and Calas, 2009; Allard et al., 2012), it is not clear what effect, if any, these processes would have on the bioavailability of Fe.

Equally, oxidation of Fe has also been documented. Raman spectroscopy has revealed the formation of unidentified higher oxidation states of iron during the enhanced corrosion of steel waste packages at 300 Gy h⁻¹ gamma irradiation (Smart et al., 2008). A similar study by Daub et al. (2011) suggests that these products may include magnetite (Fe(II),Fe(III)₂O₄), maghemite (γ-Fe(III)₂O₃) and lepidocrocite (γ-FeOOH), with the final product dependent on the electrochemical potential at the steel surface, as governed by the radiation flux. A similar array of mixed Fe(II)/(III) oxides is obtained from the introduction of H₂O₂ solutions to steel surfaces, suggesting that such reactions are mediated by oxidizing species from the radiolysis of water (Zhang et al., 2007). Lepidocrocite colloids were also observed upon gamma irradiation (up to 40 kGy) of Fe²⁺ sulphate solutions (Yakabuskie et al., 2011), indicating that production of these Fe(III) phases is not restricted to the irradiation of steel.

As Fe is likely to be a significant component of waste packaging and repository infrastructure, particularly in the form of steel, such radiation processes could lead to an increase in the availability of Fe(III) to subsurface microbial communities (Figure 10). Lepidocrocite, in particular, is readily reduced by Fe(III)-reducing microorganisms such as Geobacter spp. (Cutting et al., 2009). Whilst the reduction of Fe(III) within the magnetite structure is generally not considered thermodynamically favourable, there is limited evidence to suggest it may be susceptible to microbial reduction (Kostka and Nealson, 1995).

Further studies have demonstrated that the reaction of iron pyrite with hydrogen peroxide leads to the oxidation of Fe(II) and subsequent release of Fe³⁺ into solution (Lefticariu et al., 2006; Lefticariu et al., 2010). Subsequent precipitation of Fe(III) oxides is proposed, however, the mineralogy of the resultant phase is poorly defined
Thus, there is currently no information on bioavailability of Fe(III) generated via this process.

In addition to the production of Fe(III), the oxidation of pyrite by radiolysis products results in the concomitant dissolution of sulphate (Lefticariu et al., 2010). In this system, it appeared that this process was mediated by HO• and radiolytically generated Fe$^{3+}$. Hence, it was proposed that with a constant radiation source, this oxidation could be continuous due to radiation driven redox cycling of Fe. Furthermore, ratios of sulphur isotopes, which are fractionated by microbial metabolism, suggest that radiolytically produced sulphate, in combination with radiolytic H$_2$, may be sustaining the microbial community in the deep volcanic rock units mentioned earlier (Lin et al., 2005a; Lin et al., 2006). Indeed, biogenic sulphide, from sulphate reduction, may potentially be re-oxidized by radiolysis products, leading to the suggestion that this novel ecosystem could be self-sustaining (Chivian et al., 2008).

It is not clear how widespread these processes will be in the subsurface, as that depends on the distribution of pyrite. However, this process has been demonstrated in conditions more analogous with the disposal of nuclear waste. Increased thiosulphate concentrations in the interstitial water of an irradiated clay backfill material were observed after 5 years irradiation from a simulated high level nuclear waste canister (dose rates in the order of 10$^2$ Gy h$^{-1}$) (Noynaert et al., 1998). Again, this sulphate was attributed to pyrite oxidation, suggesting that alternative electron acceptors may be generated in the immediate vicinity of a nuclear waste canister. These findings have important implications to the development of microbial communities in these environments after waste emplacement, particularly as some sulphate-reducing species have the potential to enhance the corrosion of copper canisters (King and Stroes-Gascoyne, 1997).

In addition to electron acceptor generation, ionizing radiation may have the potential to influence the availability of trace elements required for growth. For instance, a review of gamma sterilization of soils revealed that in most studies, radiation led to an increase in the extractable concentrations of P, Mn and K (McNamara et al., 2003). Many studies also observed an increase in mineral N (NH$_4$ and NH$_3$), though
conversely, the loss of NO$_3^-$ was observed in almost all instances. As nitrate may also be an electron acceptor, this may alter the balance of terminal electron acceptor usage in a geological repository. In turn, this may have implications for microbial community structures and the evolution of biogeochemical conditions.

2.11. Conclusions
The interactions of ionizing radiation with microorganisms are complex and the resultant impacts are extremely diverse. With regard to the role microorganisms will play in influencing biogeochemical processes in nuclear storage and disposal environments and bioremediation scenarios, previous research has focused on the sensitivity of species to radiation fluxes. DNA and proteins appear particularly susceptible to irradiation, and this damage likely characterizes the responses of these organisms to radiation. However, there is a paucity of information on how these impacts affect the metabolism of these organisms and the consequences to microbe-metal-radionuclide interactions are not well defined.

Studies of the wider biological system collectively suggest that radiation may lead to the production of electron donors, either by the radiolysis of water (e.g. H$_2$) or via radiolysis of organic substrates. Similarly, alternative electron acceptors may also be generated or removed via radiation driven redox transformations. However, there is limited information on how these reactions may impact upon the respiration of key microorganisms, such as Fe(III)-reducing species for example. Thus, prediction of how such processes may influence the evolution of the biogeochemical conditions in a geological repository over the geological timescales in question has been restricted, though this would clearly form an important component of the facility’s performance assessment.
2.12. Aims and objectives

In light of the findings of this review, the aim of this research project was to make a quantitative assessment of the range of impacts of radiation on microorganisms pertinent to the storage, disposal and remediation of radioactive waste. In addition, such an assessment aims to constrain the mechanisms by which radiation may influence microbial processes in these settings. These aims were pursued via the following distinct objectives which are summarised in the figure below:

i) Characterise the metabolism and phenotypic response of organisms irradiated with acute doses of ionizing radiation, including an Fe(III)-reducing organism and an algal contaminant representative of a spent nuclear fuel storage pool.

ii) Assess the bioavailability of a range of irradiated Fe(III) oxides for microbial reduction and characterise the mechanisms by which alterations in bioavailability may occur.

iii) Reconcile the findings from acute dose experimental systems with environmentally relevant dose rates in complex sediments. Probe changes to the microbial community and relate these changes to the susceptibility of biogeochemical processes to radiation stress.
Figure 11. (A) A schematic of the impact of ionizing radiation on microbial interactions with key minerals (c). Experimental systems allow observation of a few select targets, including: cellular physiology (a) and growth substrates, such as Fe(III) bearing minerals (b). (B) The complex interaction of ionizing radiation with environmental systems involves multiple targets.
2.13. References


Chapter 3

Methodology
3. Methodology

This chapter comprises descriptions of the main analytical techniques and experimental facilities utilised throughout this research project. The aim is to provide a summary of the main principles involved with each technique and facility, rather than giving a detailed description of the methodology of each experiment. Specific details of protocols are given in the methods section of each experimental chapter.

3.1. Irradiation

The experiments documented in this thesis have made use of several radiation sources. The process by which a radiation flux is produced varies between methods and these are documented below. In all cases, the dose rate is adjusted according to the inverse square law:

\[ \text{Intensity} \propto \frac{1}{\text{distance}^2} \]

whereby, the intensity of the flux is proportional to the inverse square of the distance from the source to the sample.

3.1.1. X-irradiation

The X-radiation used in the experiments of chapters 4 and 7 was generated using a Faxitron CP-160 Cabinet X-radiator (Faxitron; Arizona, US). This machine relies on the principle of X-ray fluorescence to generate monochromatic X-rays at a few discrete frequencies, which are characteristic of the target material used. An electron source (cathode) generates electrons (6 mA) which are then accelerated via an applied voltage (160 kV). Collision of these electrons with a metal target (tungsten), the anode, typically results in ejection of core electrons (1s, 2s, 2p, etc.) from the target atoms. Subsequently, an electron from an outer orbital will drop down to fill the electron vacancy. This results in the release of an X photon with an energy equivalent to the energy lost by the valence electron as it drops down to a lower energy level.
3.1.2. Gamma irradiation

The gamma irradiators employed in the experiments of Chapter 5 made use of a $^{60}$Co source (812 Co-60 irradiator, Foss Therapy Services Inc; and JL-109, JL Shepherd and Associates). These self-contained irradiators comprise rods of $^{60}$Co, which are generated artificially by neutron activation of $^{59}$Co. The decay process is such that beta emission results in the decay of $^{60}$Co to the excited state of $^{60}$Ni. This excited state then returns to the ground state via the emission of two mono-energetic gamma rays at 1.17 MeV and 1.33 MeV.

Similarly, the gamma irradiator used in the experiments of Chapter 6, made use of a gamma cell (AMEC; Harwell, Oxfordshire). The principle is the same as the self-contained irradiators described above; however, the nature of a walk-in cell allows much lower dose rates to be achieved by application of the inverse square law to a much larger space.

3.1.3. Fricke dosimeter

Dose rates in all experiments were determined by Fricke dosimetry (Fricke and Hart, 1935). This chemical dosimeter involves the oxidation of an acidified solution of ferrous sulphate to its ferric salt by ionizing radiation in the presence of oxygen. As described by Spinks and Woods (1964) after Weiss et al. (1956), a solution of 1.4 mM FeSO$_4$.7H$_2$O; 1 mM NaCl; 0.4 M H$_2$SO$_4$ was irradiated for an appropriate length of time, in the same vessels as used for experimental samples. The optical density of this solution was then measured spectrophotometrically at 304 nm, immediately after irradiation, with respect to a blank solution containing no ferrous sulphate. Similarly, the optical density of a non-irradiated ferrous sulphate solution was also measured to allow the calculation of the dose delivered via the equation:

$$\text{Absorbed dose (Gy)} = 0.01 \times \frac{0.965 \times 10^9(OD_i - OD_{non})}{\epsilon \cdot d \cdot \rho \cdot G(Fe^{3+})}$$

Where, $OD_i$ = optical density of irradiated solution at 304 nm; $OD_{non}$ = optical density of non-irradiated solution at 304 nm; $\epsilon$ = molar extinction coefficient for ferric ions at the wavelength of maximum absorbance, typically 2174 L mole$^{-1}$ cm$^{-1}$.
3.2. Microbiological techniques

3.2.1. Fourier transform infrared spectroscopy
The metabolic profiles of irradiated microorganisms were determined using Fourier transform infrared (FT-IR) spectroscopy. This technique provides a global “fingerprint” of cellular metabolism, whilst also being able to quantify specific biochemical signatures. Indeed, other more targeted metabolic profiling techniques, such as GC-MS for example, may provide higher resolution for the quantification of individual metabolites and their fluxes. However, FT-IR spectroscopy offers the benefit of rapid, inexpensive, quantitative analysis of samples that require minimal sample preparation (Goodacre et al., 2004; Wang et al., 2010a).

In addition, the use of FT-IR spectroscopy in the characterization of microorganisms has been demonstrated in a number of eco-toxicology studies (Naumann et al., 1991; Saxena et al., 2005; Mecozzi et al., 2007; Wang et al., 2010b). Moreover, its use in the characterization of \textit{S. oneidensis} phenotypes cultured in a variety of environmental conditions has been well defined (Wang et al., 2010a; Wang et al., 2010b). Thus, the potential for its use in the characterization of ionizing radiation effects in microbial cells is attractive, particularly as an initial phenotypic screen.

The principle of FT-IR spectroscopy resides in the absorption and vibration of chemical bonds at discrete wavelengths of mid-IR light. The spectral features of these absorptions and vibrations are characteristic of the bonds or functional groups of key biomolecules. Assignments of these biochemical bands and the biomolecule groups to which they relate are shown in Table 1 (see Ellis et al. (2003), and Wang et al. (2010a) and the references therein for further assignments).
Table 1. Assignments to biochemical bands identified by FT-IR spectroscopy. Adopted from Ellis et al. (2003). See Figure 2 for typical FT-IR spectrum of *S. oneidensis*.

<table>
<thead>
<tr>
<th>Region</th>
<th>Wavenumber (cm(^{-1}))</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty acid region</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3400</td>
<td>OH</td>
</tr>
<tr>
<td></td>
<td>2956</td>
<td>CH(_3) asymmetric stretch</td>
</tr>
<tr>
<td></td>
<td>2920</td>
<td>CH(_2) asymmetric stretch</td>
</tr>
<tr>
<td></td>
<td>2870</td>
<td>CH(_3) symmetric stretch</td>
</tr>
<tr>
<td></td>
<td>2850</td>
<td>CH(_2) symmetric stretch</td>
</tr>
<tr>
<td></td>
<td>1745/1735</td>
<td>C=O stretch (fatty acid esters)</td>
</tr>
<tr>
<td><strong>Amide region</strong></td>
<td>1705</td>
<td>C=O stretch (esters, carboxylic groups)</td>
</tr>
<tr>
<td></td>
<td>1652-1648</td>
<td>Amide I, (C=O) different conformations</td>
</tr>
<tr>
<td></td>
<td>1550-1548</td>
<td>Amide II (NH, C-N)</td>
</tr>
<tr>
<td></td>
<td>1460-1454</td>
<td>CH(_2) bend</td>
</tr>
<tr>
<td><strong>Mixed region</strong></td>
<td>1400-1398</td>
<td>C-O bend (carboxylate ions)</td>
</tr>
<tr>
<td></td>
<td>1310-1240</td>
<td>Amide III (C-N)</td>
</tr>
<tr>
<td></td>
<td>1240</td>
<td>P=O (phosphate)</td>
</tr>
<tr>
<td></td>
<td>1222</td>
<td>P=O</td>
</tr>
<tr>
<td></td>
<td>1114</td>
<td>C-O-P, P-O-P</td>
</tr>
<tr>
<td><strong>Polysaccharide region</strong></td>
<td>1085</td>
<td>Sugar ring vibrations</td>
</tr>
<tr>
<td></td>
<td>1052</td>
<td>C-O, C-O-C (polysaccharide)</td>
</tr>
</tbody>
</table>

As stated above, the aim of IR spectroscopy is to measure the absorption of light across a range of wavelengths. The simplest way of achieving this is to generate a monochromatic beam and measure the absorbance at each wavelength (*cf.* UV-Vis spectroscopy). However, the incorporation of an interferometer, and application of a Fourier transform to the output signal, allows an infrared spectrum to be gathered over a range of wavelengths simultaneously.
The interferometer of an FT-IR spectrometer (Figure 1) comprises a beam splitter to split the beam in two, with one half of the beam reflected by a static mirror, and the other half reflected by a moving mirror (Naumann, 2000). The moving mirror generates waves of varying path lengths, such that at any one time, the IR beam contains light of different pathlengths, the distribution of which varies with mirror position. Upon recombination of the split beams, various degrees of constructive and destructive interference are caused with the implication that the light at the sample has various intensities of each wavelength. The Fourier transform is the mathematical function required to produce a coherent spectrum across all wavelengths, as shown in Figure 2.
3.2.2. **Multivariate statistical analysis**

The spectra generated from FT-IR spectroscopy are generally complex. Thus, it is often difficult to make qualitative statements about the differences between spectra based solely on observations with the naked eye. Therefore, quantitative measurement of the subtle changes to spectral features attributable to key biochemical components is required. Initially, multivariate statistical analyses provide a means by which to reduce the complexity of the data and highlight the occurrence of differences between spectra of various treatments. This then allows the relationship between the phenotype and the biochemical features of the spectra to be modelled (Wang et al., 2010a).

3.2.2.1. **Principal component analysis**

The first of such analyses used in this research was principal component analysis (PCA). PCA is an unsupervised technique used to explain the variance-covariance structure of a set of correlated variables through a few linear combinations of the variables. These linear combinations are the principal components (PCs) and are an uncorrelated set of new variables. The first PC is the new variable with the greatest
variance and the second PC is the variable with the second greatest variance, and so on. Usually, the first few PCs account for most of the variability in the original data and thus, the dimensionality of the data is significantly reduced. In the case of an FT-IR data set for example, whilst it may not be possible to state which of the original variables (i.e. wavenumbers) account for the most variability between spectra, it may be possible to show differences (and similarities) between samples based on how the samples ‘cluster’ according to the variability in the first two PCs. Hence, the differences between samples may be summarised in a two dimensional plot of the first two PC ‘scores’ for each sample, where samples with a similar covariance matrix cluster together (Chatfield and Collins, 1980). These scores are calculated by the equation:

\[ t_i = X \cdot p_i \]

where \( t \) = the PC score for each of the variables \( i_n \) (i.e. wavenumbers) of the data \( X \) (FT-IR spectra), and \( p \) = a loadings vector, by which the original data, \( X \), are multiplied. The loadings vectors are ‘weightings’ which preserves the distances between objects whilst maximizing the variance, such that the first PC has the first largest variance etc. (Chatfield and Collins, 1980; Correa et al., 2012).

3.2.2.2. Discriminant function analysis

The PCs extracted during PCA can further be used for discriminant function analysis (DFA and hence PC-DFA). DFA is a supervised method, whereby discrimination between groups is based on \textit{a priori} knowledge of the experimental class structure. The algorithm acts to find linear combinations of the variables (canonical variates of the PCs which are fed into the algorithm) which maximize the ratio of between-group variance to within-group variance (Chatfield and Collins, 1980; Correa et al., 2012). Again, similar to PCA, the first few canonical variates for each sample can be plotted against each other and the data may cluster according to sample class.

3.2.2.3. Hierarchical cluster analysis

In the experimental section of Chapter 7, discriminant function scores from PC-DFA were used for hierarchical cluster analysis (HCA). The HCA algorithm uses the mean of the DF scores of each group to assign each group to a cluster based on the Minkowski metric (Euclidean distance) for the variables of each group. Hence, a
measure of similarity is generated and this can be plotted as a dendrogram (Chatfield and Collins, 1980).

### 3.2.2.4. Partial least squares regression

In addition to PC-DFA, partial least squares regression (PLSR) is another supervised learning technique which utilises knowledge of sample treatment to create a model. The method relates a set of independent variables $X$ (the FT-IR data) to a set of dependent variables $Y$ (for instance, the radiation dose). The algorithm then projects these variables into a set of new (latent) variables so that the covariance matrix of these two variables is maximized. The method is a form of linear regression, such that the model takes the mathematical form:

$$Y = XB + E$$

where, $B$ is a matrix of regression coefficients (loadings) and $E$ is the difference between the observed and predicted values of $Y$. This “learning phase” is performed on a “training” data set, where the classes of treatments are known. The model then performs the regression such that maximum discrimination between classes is achieved. Validation is then performed with a “test” set of new samples. Thus, the objective of the PLS model is to predict the classification of the new samples and in doing so, the output (for instance, a plot of the component scores) may show any discrimination between treatments (Wold et al., 2001; Correa et al., 2012). It is important to validate the model to determine how successfully the model predicts the scores of new samples and this is classically achieved by cross validation. An $R^2$ (sum of the squares of the differences between predicted and actual output) is calculated for the model and test data by increasing the number of components and performing successive iterations of the model (Wold et al., 2001).

### 3.2.3. Matrix-assisted laser desorption/ionization mass spectrometry

Quantification of proteins in irradiated *Shewanella oneidensis* was achieved using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). As with all mass spectrometry, this involves the separation and quantification of gas-phase ions based on their mass/charge ratio ($m/z$). This principle requires an
ionization source, to generate the gas-phase ions; a mass analyzer, to separate the ions; and a detector.

In the case of MALDI-MS, desorption and ionization of a dried sample is achieved by a pulsed UV or IR laser with a wavelength at which the matrix absorbs. The use of a chemical matrix enhances the production of the gaseous ions from the sample-matrix co-precipitate (Figure 3a). A time-of-flight (TOF) mass analyzer was used to separate the ions prior to quantification at the detector (Figure 3b) (Vaidyanathan and Goodacre, 2003).

![Diagram](image)

**Figure 3.** (a) MALDI and (b) Time-of-flight (TOF) mass analyzer. Adapted from Vaidyanathan and Goodacre (2003).

The experiments contained in this thesis used whole cell preparations for MALDI-MS. This technique involves mixing washed cells with a matrix solution (such as 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) for high mass proteins, as used in these experiments) and spotting them on to a target plate prior to analysis (Vaidyanathan and Goodacre, 2003). In order to maximise information contained in spectra at various mass ranges, the procedure of matrix/sample mixing and spotting needs to be optimised (i.e. 1:1 mix; matrix/sample; sample/matrix; matrix/sample/matrix) (Vaidyanathan et al., 2002).
Once analysed, peak m/z values can be tentatively matched to molecular masses of predicted proteins in organisms with sequenced genomes, of which *S. oneidensis* is one such organism (Demirev and Fenselau, 2008). Search engines such as TagIdent (maintained by ExPASy Bioinformatics Resource Portal at http://web.expasy.org/tagident (Artimo et al., 2012)) can be used to facilitate the searching of proteome databases, such as the UniProt Knowledgebase (Swiss-Prot and TrEMBL) (UniProt, 2013).

### 3.2.4. Microbial community analysis

The experiments of Chapter 6 sought to determine the influence of radiation on the diversity and dominance of species of indigenous microbial communities in sediment microcosms. Changes to the ecology of these systems were probed under a range of gamma radiation dose rates using Pyrosequencing.

#### 3.2.4.1. DNA extraction

Initially, DNA was extracted from sediment slurries using a DNA isolation kit. Bacterial cells were then mechanically and chemically lysed by the addition of glass beads and sodium dodecyl sulphate in order to release DNA. The addition of a buffer solution prevents the decay of genetic material. Centrifugation and washing steps were then employed to remove cell debris, soil particulates and any contaminants from the DNA solution.

#### 3.2.4.2. Amplification and Polymerase Chain Reaction (PCR)

Microbial typing techniques commonly target the 16S rRNA gene. This gene is used as it is highly conserved across all prokaryotic taxonomic groups, whilst having the advantageous property that there are variable regions contained within it that are species specific. Thus, by analyzing a sequence of this gene, it is possible to taxonomically classify microbes and generate a phylogeny of an environmental sample based on the DNA of species present. Hence, primers, lengths of nucleotides of a known sequence, are added to the denatured (unravelled) DNA which are complementary to the semi-conserved region of the 16S rRNA gene. DNA polymerase then anneals fresh nucleotides to the primed DNA sequence to create complementary base pairs, prior to removal of the new DNA sequence. This process is cycled around 30 times, with each new complementary sequence forming the
template for the next cycle of polymerisation with fresh nucleotides: the so-called ‘chain reaction’. Gel-electrophoresis is then performed on the PCR products to obtain fragments of the correct size, approximately 410 bp. Once these gel bands have been excised and cleaned up, they can be taken for sequencing.

3.2.4.3. Sequencing

Pyrosequencing is a next generation sequencing technology that removes the need for laborious cloning steps and further gel-electrophoresis synonymous with Sanger-type sequencing techniques (Sanger et al., 1977). The principle of pyrosequencing relies on the detection of light that is released during a cascade of enzymatic reactions that occurs during nucleic acid polymerisation. First, upon the incorporation of a nucleotide base to the nucleic acid sequence by polymerase, inorganic pyrophosphate (PP$_i$) is released. ATP sulfurylase then rapidly converts the PP$_i$ to ATP, which in turn provides energy for the oxidation of luciferin by luciferase. It is this final reaction which generates photons with a wavelength of 560 nm. The intensity of light generated is proportional to the number of incorporated nucleotides during the reaction period of that particular nucleotide (A, T, C or G) with the nucleic acid sequence. Hence, the full sequence of the nucleic acid fragment can be derived from the iterative addition of known nucleotides to the fragment (Ronaghi, 2001)

Pyrosequencing was performed using a 454 Life Sciences Junior system (Roche) and sequence reads were analysed using the Qiime 1.6.0 software (Caporaso et al., 2010). Sequences were then matched to known 16S rRNA sequences in GenBank (Benson et al., 2013) to allow taxonomic classification and to create phylogenies for each sample.
3.3. Mineralogical characterisation
A range of techniques have been used in this research to characterise the chemistry and structure of mineral phases, before and after irradiation.

3.3.1. X-ray Diffraction
X-ray diffraction (XRD) is an analytical technique that provides valuable information on the crystalline structure of a mineral phase. This technique works on the principle that incident X-rays, produced by a Cu $K\alpha$ source, will be diffracted by successive atomic planes of a crystal lattice.

When the angle of incidence is such that scattered waves undergo constructive interference, the scattered waves of successive planes will remain in phase if their path difference is equal to an integral number of wavelengths, as governed by the Bragg equation:

$$2d\sin\theta = n\lambda$$

Where, $d$ is the interplanar spacing, $\theta$ is the scattering angle, $n$ is an integer and $\lambda$ is the wavelength. When the Bragg law is satisfied, an intense ‘reflection’ will be observed, known as a Bragg peak. The diffractogram of the mineral in question may consist of several reflections, dependent on its lattice structure, which are attributable to each set of lattice planes. The interplanar spacings are characteristic of each mineral phase and can be resolved using the Bragg equation. In turn, Miller indices $(h \ k \ l)$ can be assigned to the calculated “$d$-spacings” and the mineral phase identified by comparison to previously reported materials via a database, such as that maintained by the International Centre for Diffraction Data or the American Mineralogist Crystal Structure Database (Downs and Hall-Wallace, 2003).

3.3.2. Transmission electron microscopy
The morphologies of irradiated minerals were probed using transmission electron microscopy (TEM). This high resolution microscopy allows the lattice interference pattern to be observed and is therefore a useful tool to detect any localised structural changes or lattice defects.

A transmission electron microscope (TEM) consists of an electron gun, lenses, specimen and detector (fluorescent screen and camera), all situated in a vacuum
column, to prevent electron interaction with a gas phase. Suspensions of minerals are mounted on support grids and the electron beam focused on the specimen. Bright field TEM images are then acquired with the contrast arising from differences in electron transmission as a result of sample thickness or atomic number.

### 3.3.3. Selected area electron diffraction

By making use of the same electron source, selected area electron diffraction (SAED) can be performed in conjunction with TEM. The principle is the same as XRD; however, much smaller areas can be ‘selected’ by using an appropriate aperture to prevent reflections from excluded areas contributing to the output. As with TEM, this gives the benefit of allowing localised changes to the structure to be observed.

The pattern produced is a series of spots, with each spot corresponding to diffracted electrons consistent with the Bragg law. These lattice reflections are reciprocals of the original lattice and therefore, as the output is calibrated, the d-spacing can be obtained by recording the reciprocal of the distance between the reflection and the angle of incidence, measured in real space. As with XRD, the patterns can be indexed using Miller indices and hence, identification can be achieved.

### 3.3.4. Mössbauer Spectroscopy

Mössbauer spectroscopy is a technique used to characterise the nuclear environment of various elements that are ‘Mössbauer active’, that is, they have a naturally occurring isotope with a nuclear magnetic moment. $^{57}\text{Fe}$ is one such isotope and thus, Mössbauer spectroscopy lends itself well to the characterisation of a range of iron minerals. The principle relies on the recoil-free, resonant absorption of gamma rays by $^{57}\text{Fe}$ nuclei in a solid iron-bearing mineral.

A Mössbauer spectrometer consists of a gamma source ($^{57}\text{Co}$), an absorber (sample) and a detector. The technique involves the forward and reverse oscillation of the source, such that, gamma rays with a very narrow energy spectrum are generated as a result of the Döppler Effect. Absorbance by the sample occurs when the energy of the photons is resonant with the $^{57}\text{Fe}$ nucleus. It is the absorbance of the gamma rays as a function of the velocity of the source that is recorded.
The resonance of a $^{57}$Fe nucleus at different velocities depends upon the electronic and magnetic environment of the nucleus i.e. the interactions of the nuclear charge distribution with the extranuclear electric and magnetic fields (Bancroft, 1973). These interactions result in energy transitions which can be defined by three parameters: the isomer shift; quadrupole splitting and magnetic splitting.

3.3.4.1. **Isomer shift**

The isomer shift ($\delta$) arises from the interaction of the nuclear charge distribution with the electrons in the vicinity of the nucleus. Overlapping of the charge density of $s$ electrons with that of the nuclear charge density results in a shift in the Mössbauer energy levels of the absorber relative to the source (Figure 4) (Bancroft, 1973).

Thus, as the isomer shift depends on the $s$ electron density, which in turn is influenced by the $p$ and $d$ electron orbitals, the isomer shift can provide information about the oxidation state of the atom. For instance, ferrous iron has six electrons in its valence $d$ orbital, one more than ferric iron, therefore, the charge density in the $s$ orbital is larger in ferrous iron. This results in a larger isomer shift in a ferrous iron ion.

3.3.4.2. **Quadrupole splitting**

The interaction of an electric field gradient with a nucleus with non-spherical charge distribution (i.e. $I > 3/2$) leads to a splitting ($\Delta E_q$) of the $I = 3/2$ energy level. This gives a characteristic two line spectrum (Figure 4), with the separation of the peak maxima equating to the quadrupole splitting and the centroid of the peaks relative to the source (0 mm s$^{-1}$) equates to the isomer shift (Greenwood and Gibb, 1971). The quadrupole splitting provides information on the distortion of the nucleus affected by its chemical environment.
3.3.4.3. Magnetic splitting

In magnetically ordered materials, the interaction between the nucleus and a magnetic field causes the energy level of spin $I$ to split into $(2I + 1)$ sublevels (Greenwood and Gibb, 1971). In $^{57}$Fe, this results in six possible transitions between the $I = 1/2$ state and the $I = 3/2$ thus, giving rise to six absorbance peaks in a Mössbauer spectrum (Figure 4).

![Diagram of nuclear energy levels and Mössbauer spectra](Image)

**Figure 4.** Nuclear energy levels and hypothetical Mössbauer spectra. (Image from Cornell and Schwertmann (2003)). The isomer shift ($\delta$) in the absorber (A) relative to the source (S) is observed as a one line spectrum with a positive deviation of the absorption spectrum away from 0 mm s$^{-1}$. The quadrupole splitting ($\Delta E_Q$) is observed as a two line spectrum. The six line spectrum is the result of magnetic splitting in samples which exhibit magnetic ordering.
As iron oxides can be differentiated based upon their Mössbauer parameters, it is therefore possible to identify the iron phases present in a sample. The three Mössbauer parameters can be derived by fitting the Mössbauer spectrum with Lorentzian curves using the Recoil software (University of Ottawa). The isomer shift data is calibrated by reference to metallic Fe foil.

3.4. Ion Chromatography

The concentrations of various anions and organic acids in solution of selected experiments were determined using ion chromatography. This process separates anions and polar molecules based on their charge properties.

Typically, an autosampler introduces the analytes into a mobile phase: an aqueous solution with known ionic strength. The mobile phase is passed over an ion exchange column, the stationary phase, which contains covalently bonded, positively charged functional groups (in the case of measuring anions). The analytes are retained by the column but will be eluted once a certain concentration of similarly charged species in the mobile phase acts to displace the analytes. The time at which elution of different analytes occurs depends on the equilibrium between the analyte, mobile phase and stationary phase. To achieve optimum separation of analytes, the ionic strength of the mobile phase is adjusted (typically using a gradient) which shifts the equilibrium position and thus the retention time. Upon elution, identification and quantification of the analytes is achieved by comparing peak areas to concurrently run standards. Several means can be used to detect the analytes, with conductivity used in the experiments described here.

The experiments documented in Chapter 6 made use of a Dionex IC5000 system with a Dionex Capillary AS11-HC 4µ column. 0.4 µL samples were injected into a potassium hydroxide mobile phase with a flow rate of 0.015 mL min$^{-1}$ and a gradient of 1 mM – 36 mM KOH over 40 minutes.
3.5. Chemical Fe Assay

Microbial Fe(III)-reduction in mineral systems and sediment microcosms was probed using the ferrozine assay. Aqueous Fe(II) reacts readily with ferrozine, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4’,4”-disulfonic acid sodium salt, to produce a stable magenta complex. This complex is very soluble in water and absorbs light strongly at a wavelength of 562 nm and this can be used to determine Fe(II) concentrations effectively using a UV-Vis spectrophotometer (Stookey, 1970).

Typically, mineral/sediment slurries were digested for 1 hour in 0.5 N HCl, at 1:50 dilution, to allow dissolution of surface Fe(II) and biogenic Fe(II) minerals and prevent oxidation of the Fe(II) (Lovley and Phillips, 1986a; Lovley and Phillips, 1986b). 0.2 mL aliquots of the digest were then added to 2.3 mL ferrozine solution (1 g L\(^{-1}\) ferrozine salt; 11.96 g L\(^{-1}\) HEPES, N-(2-hydroxyethyl) piperazine-N-2-ethanesulfonic acid, organic buffer; pH 7). Solutions were prepared in cuvettes, mixed well and then left for 30 seconds to allow colour development before measuring the absorbance at 562 nm with a UV-Vis spectrophotometer. Absolute Fe(II) concentrations were then determined by comparing absorbance to calibration standards containing known concentrations of the Fe(II) salt, iron sulphate.

Total Fe in synthetic mineral systems was determined by digesting mineral suspensions in a solution containing 0.5 N or 3 N HCl for 24 hours. The addition of 0.25 N hydroxylamine-HCl, promoted the reduction of Fe(III) to Fe(II) in preparation for reaction with the ferrozine assay. Similarly, total bioavailable Fe concentrations in sediments were measured by digesting sediment-groundwater slurries in solutions containing 0.25 N HCl and 0.25 N hydroxylamine-HCl for 1 hour. This has been previously reported to extract Fe that is available for microbial reduction (Lovley and Phillips, 1987)
3.6. References


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Chapter 4

Research Paper

Phenotypic characterization of *Shewanella oneidensis* MR-1 exposed to X-radiation
4. Phenotypic characterisation of *Shewanella oneidensis* MR-1 exposed to X-radiation

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4.1. Abstract

Biogeochemical processes mediated by Fe(III)-reducing bacteria such as *Shewanella oneidensis* have the potential to impact on the stability of radwaste disposed of in a geological repository. Furthermore, their potential to reduce both Fe(III) and radionuclides can be harnessed for the bioremediation of radionuclide-contaminated land. As such sites are likely to have significant radiation fluxes, there is a requirement to characterise the impact of radiation stress on such microorganisms. There have, however, been few global cell analyses of the impact of ionizing radiation on subsurface bacteria, so here we address the metabolic response of *S. oneidensis* MR-1 to acute doses of X-radiation. UV/Vis spectroscopy and CFU counts showed that although X-radiation decreased initial viability and extended the lag phase of batch cultures, final biomass yields remained unchanged. FT-IR spectroscopy of whole cells indicated an increase in lipid content. MALDI-TOF-MS detected an increase in total protein expression in cultures exposed to 12 Gy. At 95 Gy, a decrease in total protein levels was generally observed, although an increase in a peak tentatively assigned to a cold shock protein was observed, which may be related to the radiation stress response of this organism. Multivariate statistical analyses applied to spectroscopy data suggested that an irradiated phenotype developed throughout subsequent generations. This study suggests that significant alteration to the metabolism of *S. oneidensis* MR-1 is incurred as a result of X-
irradiation and that dose dependent changes to specific biomolecules characterises this response.

4.2. Introduction

Civil nuclear energy generation and nuclear weapon production since 1945 has generated significant volumes of legacy radioactive wastes and contaminated land (Brim et al., 2003). As physico-chemical methods of remediation may incur great cost, the use of non-invasive in situ alternative technologies, such as bioremediation, may provide a more versatile and cost-effective substitute (Tiedje, 2002; Lovley, 2003).

Many subsurface bacteria, such as *Shewanella spp.* have the ability to couple the oxidation of organic matter to the reduction of a range of metal cations, anions and radionuclides (Nealson and Saffarini, 1994; Wade and DiChristina, 2000; Daulton et al., 2007), thus providing the potential for use in the bioremediation of radionuclide contaminated land (Tiedje, 2002; Lloyd, 2003). However, as many of the sites contaminated by radionuclides are likely to have significant radiation fluxes (Riley et al., 1992; Fredrickson et al., 2004; Niedree et al., 2013), the utility of microorganisms in the remediation of highly radioactive wastes will largely be determined by the ability to survive radiation stress (Brim et al., 2000).

Furthermore, microorganisms control many processes pertinent to the stability of the radwaste inventory of a geological disposal facility. For instance, microbial activity may play an important role in the generation and consumption of gases, such as methane and hydrogen, from the corrosion of waste. Microorganisms can also control radionuclide speciation and mobility and the biodegradation of potential radionuclide complexants (West et al., 2002; Lloyd, 2003; Keith-Roach, 2008). Microbial Fe(III) reduction is of particular interest in these environments as the mobility of redox-active radionuclides may be restricted via their reduction by biogenic Fe(II)-bearing phases (Lloyd, 2003; Lloyd and Renshaw, 2005). However, as noted before, the organisms promoting these processes may be subject to significant radiation doses. For example, predictions of dose rates at waste canister surfaces and in backfill material have been as high as 52 Gy h\(^{-1}\) and 72 Gy h\(^{-1}\) (Stroes-Gascoyne et al., 1994; Allard and Calas, 2009). Consequently, radiation
toxicity may govern the importance of microbially controlled processes in these environments and hence, there is a requirement to deliver fundamental physiological information on the impact of ionizing radiation on Fe(III)-reducing bacteria such as *Shewanella oneidensis*.

Many early studies of radiation sensitivity considered DNA as the principal target of radiation due to modification of DNA by both direct (energy deposition into the molecule from the radiation track) and indirect (reactive oxygen species from the radiolysis of water) mechanisms (Ghosal et al., 2005). Hence, it was thought that the biological impacts of ionizing radiation arose from DNA damage (Ward, 1994) and thus, damage to DNA has become a hallmark indicator of the effects of ionizing radiation (Daly et al., 2007).

However, *Shewanella spp.* sustain a similar amount of damage to DNA as many other species, yet are considerably more sensitive to radiation than *Escherichia coli* or *Deinococcus radiodurans* (Daly et al., 2004; Ghosal et al., 2005). Furthermore, the genome of *S. oneidensis* MR-1 encodes a conventional set of DNA repair mechanisms which are strongly induced after irradiation (Qiu et al., 2006). This suggests that the impact of ionizing radiation is likely more complex than just DNA damage and the cellular response may in fact arise from a large array of potential cellular targets (Qiu et al., 2006). For instance, it has been reported that proteins are likely the initial target of damage by reactive oxygen species (Du and Gebicki, 2004) and protein oxidation has been quantifiably related to bacterial sensitivity to ionizing radiation (Daly et al., 2007; Krisko and Radman, 2010).

In addition to proteins, ionizing radiation has also been shown to damage lipids, for example via fragmentation and peroxidation (Shadyro et al., 2002). Indeed, as lipids and lipoproteins are major components of biological membranes, radiation induced reactions to these molecules could alter the integrity and function of membranes, for instance, by changes in viscosity and permeability (Stark, 1991). Indeed, membrane composition and fluidity may be important to radiation sensitivity where DNA is associated with the membrane (Montaudon et al., 1987). Furthermore, in the case of *S. oneidensis*, many of the respiratory cytochromes and respiratory chain components are associated with the outer membrane (Ehrlich, 2002). Therefore,
oxidative damage to the membrane could restrict this species’ ability to use alternative electron acceptors, such as Fe(III) or radionuclides, whilst not necessarily being lethal. In addition, the c-type cytochromes themselves may be a significant source of superoxide, which in turn may yield a suite of reactive oxygen species (Imlay, 2003). It has therefore been proposed that energy metabolism during recovery of *S. oneidensis* from irradiation, could underpin its sensitivity (Daly et al., 2004). Thus, paradoxically, whilst many cytochromes give *S. oneidensis* respiratory versatility, this may predispose its metabolism to oxidative stress and sensitivity to radiation (Ghosal et al., 2005).

To conclude, it is evident that the mechanisms of cell killing by radiation are complex and remain poorly defined. Many previous studies have tried to identify the molecular targets which result in radiation sensitivity; however, there is a paucity of information regarding the whole cell response. A global cell analysis of the impact of a range of doses would allow an assessment of whether the phenotypic response to radiation is predictable. Furthermore, quantification of changes to the levels of specific biomolecules would provide useful information required to assess the physiological status and metabolic capabilities of irradiated *S. oneidensis*. Here, we profiled the whole cell metabolism of *S. oneidensis* MR-1 exposed to acute X-radiation doses (12 to 95 Gy) via Fourier transform infrared spectroscopy (FT-IR) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and report on specific changes to biomolecules which underpin the phenotypic response of this organism to radiation.

### 4.3. Materials and Methods

#### 4.3.1. Growth of cells

All cultures of *S. oneidensis* MR-1 were grown aerobically in a fully defined, pre-sterilized, liquid minimal medium (pH 7.4) based on that described previously by Myers and Nealson (1988): 9 mM (NH₄)₂SO₄; 5.7 mM K₂HPO₄; 3.3 mM KH₂PO₄; 2.2 mM NaHCO₃; 1 mM MgSO₄·7H₂O; 0.49 mM CaCl₂·2H₂O; 67.2 µM Na₂EDTA; 56.6 µM H₃BO₃; 10 µM NaCl; 5.4 µM FeSO₄·7H₂O; 5 µM CoCl₂·6H₂O; 5 µM NiCl₂·6H₂O; 3.9 µM Na₂MoO₄·2H₂O; 1.5 µM Na₂SeO₄; 1.3 µM MnCO₃; 1 µM ZnCl₂; 0.2 µM CuSO₄·5H₂O; 20 mg l⁻¹ L-arginine HCl; 20 mg l⁻¹ L-glutamate; 20 mg l⁻¹ L-serine; 100 mM sodium DL-lactate (carbon source and electron donor); 20
mM fumarate (electron acceptor). Six sterile Erlenmeyer flasks containing the defined medium were then inoculated with a late log/early stationary phase culture to give an optical density (OD) of 0.2 at 600 nm (approximately $5 \times 10^8$ cells ml$^{-1}$).

### 4.3.2. Irradiation

Half of the inocula were irradiated at ambient room temperature to a dose of 12 Gy using a Faxitron CP-160 Cabinet X-radiator (160kV; 6 mA; tungsten target). It should be noted that X-radiation is a good analogue for gamma radiation, offering “hard” x-rays with energy comparable to that of photons at the longer wavelength of the gamma spectrum. The remaining 3 replicates formed the non-irradiated control cultures and were lead shielded inside the irradiator to control for any potential temperature changes. After irradiation, all cultures were incubated at 30 °C and shaken at 130 rpm. This procedure and subsequent analysis was repeated with batch cultures for the range of doses: 12, 24, 48, 72 and 95 Gy. Prior to irradiation, the dose rate was determined as 0.79 Gy min$^{-1}$ using Fricke dosimetry as described previously (Fricke and Hart, 1935; Weiss et al., 1956; Spinks and Woods, 1964).

### 4.3.3. Quantification of growth

Biomass in each biological replicate was determined at regular time intervals with 1 ml samples extracted for analysis of optical density at 600 nm (OD$_{600}$) using a UV/Vis spectrophotometer (Jenway, UK).

### 4.3.4. Viability

Cell viability in all replicates was determined immediately after irradiation via serial dilution in phosphate buffered saline solution and subsequent plating on to minimal medium agar plates followed by incubation for two days at 30°C. The number of colony forming units was then determined over the range of dose from 12 to 143 Gy.

### 4.3.5. Analysis of metabolism by FT-IR Spectroscopy

The metabolic fingerprints of control and irradiated cells were recorded by FT-IR spectroscopy. Aliquots from each biological replicate were collected immediately after irradiation (lag phase), during mid exponential phase and at the maximum biomass yield of stationary phase. Samples were then centrifuged at 4 °C at 12,000g for 20 min after which the supernatant was removed and the cell pellet was washed.
twice with sterile 0.9% NaCl solution prior to being stored at -80 °C. Upon analysis, samples were thawed and resuspended into three separate samples in sterile 0.9% NaCl solution to an OD$_{600}$ of 5. A 96 well Si sample plate was washed thoroughly with 2-propanol and deionized water and allowed to dry at room temperature prior to use. 20 µL of each bacterial sample was then applied evenly in triplicate onto the plate (so called technical replicates) prior to drying at 55°C in an oven for 10 min. All FT-IR spectroscopy analysis was conducted using an Equinox 55 infrared spectrometer equipped with a high throughput motorized microplate module, HTS-XT™ (Bruker Optics, Coventry, UK). A deuterated triglycine sulfate (DTGS) detector was employed for absorbance measurements of the samples to be acquired. Thus, 9 spectra from each biological repeat were collected over the wavelength range of 4000 to 600 cm$^{-1}$ using the Opus software (Bruker Optics). Spectra were acquired at a resolution of 4 cm$^{-1}$ with 64 spectra co-added and averaged to improve the signal-to-noise ratio. The collection time for each spectrum was approximately 1 min.

4.3.6. Data processing
The ASCII data files were imported into MATLAB 2008a (The MathWorks Inc., Natwick, US). FT-IR spectra were normalized using extended multiplicative signal correction (EMSC) (Martens and Stark, 1991) and signals arising from CO$_2$ in the regions 2400-2275 cm$^{-1}$ and <700 cm$^{-1}$ were removed from the spectra and filled with a smoothed trend (Winder et al., 2006) before exportation to R version 2.9.2 for further analysis (R Foundation for Statistical Computing, Vienna, Austria). Prior to multivariate statistical analyses, the data were autoscaled by transforming the intensities for each wavenumber such that the mean was equal to zero and the standard deviation equal to 1 (Goodacre et al., 2007).

4.3.7. Partial least-squares regression
Supervised multivariate classification of treatments using partial least-squares regression (PLSR) was used to model the relationship between dose and metabolic fingerprints for all growth phases. The model was calibrated with FT-IR spectra from a “training” data set and used to predict the known doses on a separate test set. The objective of the PLS model is to predict the classification of the new samples and in doing so, any separation between classes is displayed in score plots of the
principle components (Wold et al., 2001). Each model was cross-validated and an $R^2$ value generated, as described by Correa et al. (2012), in order to measure prediction performance.

4.3.8. Discriminant analysis
Principal component analysis (PCA) was used to reduce the dimensionality of the FT-IR data from multiple absorbance measurements down to 5 principal components (PCs). The unsupervised method was performed on FT-IR data from all growth phases at each dose, such that the 5 PCs extracted represented the following percentages of the total variance in the FT-IR spectra of each treatment: 12 Gy = 96%; 24 Gy = 99%; 48 Gy = 98%; 72 Gy = 95%; 95 Gy = 97%. The PCs extracted were then used for discriminant function analysis (DFA, and hence PC-DFA), as described previously (Goodacre et al., 1998; Timmins et al., 1998). DFA is a supervised method, whereby discrimination between groups is based on a priori knowledge of the experimental class structure. The algorithm finds linear combinations of the variables (canonical variates of the PCs fed into the algorithm) which maximize the ratio of between-group variance to within-group variance (Chatfield and Collins, 1980; Correa et al., 2012). In order to quantify separation between control samples and treated samples at each growth phase, Euclidean distances between group centres were calculated from the scores plots of the first 3 DFs.

4.3.9. Quantification of spectral peak areas
The peak intensities of FT-IR spectral features characteristic of key biomolecules were quantified using a function written in-house for R version 2.9.2 (R Foundation for Statistical Computing, Vienna, Austria). A linear baseline trend was subtracted from each peak individually and the maximum absorbance of the peak area was calculated. These peak areas gave quantitative information regarding proteins, as determined from the peak intensity of the amide I/II region between 1479 to 1775 cm$^{-1}$ and amine region from 988 to 1187 cm$^{-1}$. Similarly, changes to the lipid content of cells were quantified by measurement of the peak intensity of the CH$_2$/CH$_3$ asymmetric stretch at 2885 to 2945 cm$^{-1}$ and aliphatic CH vibrations between 2949 and 2993 cm$^{-1}$. 

110
4.3.10. MALDI-TOF-MS

Quantification of proteins in irradiated *S. oneidensis* MR-1 was achieved using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). As for FT-IR spectroscopy, an aliquot was collected from each biological replicate at lag phase (immediately after irradiation), exponential phase and stationary phase. Prior to analysis, samples were washed twice in sterile deionized water and resuspended in 50 µL of deionized water to an OD$_{600nm}$ of 5. Each 50 µL sample was then diluted with 125 µL of a deionized water solution containing 50% v/v acetonitrile and 0.1% v/v trifluoroacetic acid. 5 µL of each sample was then mixed with 5 µL of a matrix solution comprising 10 mg sinapinic acid (Sigma-Aldrich, UK) dissolved in 1 mL of 50% v/v acetonitrile and 0.1% v/v trifluoroacetic acid solution. 2 µL of this mixture was then spotted in triplicate at random locations on a MALDI-MS stainless steel target and dried for 1 h at room temperature.

Samples were analyzed using a MALDI-TOF mass spectrometer (AXIMA-CRF™ plus; Shimadzu Biotech, Manchester, UK) equipped with a nitrogen pulsed 337 nm UV laser and positive ion source operated in the linear mode. A laser power of 90 mV was used and each spot was analysed using a random raster of 300 profiles with each profile containing data from five laser shots. Spectra were acquired over the mass range 1000-12000 Da with a resolution of 4000 FWHM.

Prior to analysis, mass spectra were imported into MATLAB 2008a (The MathWorks Inc., Natwick, US). Baseline correction was performed using asymmetric least squares (Peng et al., 2010) and spectra were normalized by dividing each spectrum by the square root of the sum of the squares of the spectrum (Brereton, 2003). PC-DFA was performed on the MALDI-TOF mass spectra as described earlier for FT-IR spectroscopy. Thirty PCs were extracted during PCA which represented 99% of the total variance in spectra from both the 12 Gy and 95 Gy treatments (including respective control treatments). As before, these PCs were passed to the DFA algorithm and Euclidean distances between control and irradiated cluster centres of each growth phase were computed.

Prior to assignment of mass peaks, mass drift in spectra was corrected using a script written in-house. Peaks were re-aligned using a median averaging function with a
maximum allowed shift of 500 Da. Subtraction spectra were then obtained by subtracting the mean spectrum of the control samples from the mean of the irradiated samples. The masses of peaks showing discrimination were tentatively assigned to proteins by reference to the UniProt Knowledgebase (Swiss-Prot and TrEMBL) (UniProt, 2013) accessed via the ExPASy Bioinformatics Resource Portal at http://web.expasy.org/tagident (Artimo et al., 2012) (accessed July 2013).

4.3.11. Fe(III) reduction by irradiated S. oneidensis

*S. oneidensis* MR-1 was grown aerobically in tryptic soy broth in Erlenmeyer flasks at 30 °C and shaken at 130 rpm. Late log - early stationary phase biomass was harvested by centrifugation at 4920g for 20 minutes at 4 °C and, then, washed twice in sterile 30 mM sodium bicarbonate buffer. These cell suspensions were then irradiated with 50 Gy X-radiation and cell viability was determined as described earlier. After irradiation, cell suspensions were sparged for 10 minutes in an 80:20 gas mix of N₂:CO₂. Aliquots of the irradiated cell suspension (0.2 ml) were then added to 10 mL anaerobic 30 mM bicarbonate buffer containing 20 mM lactate and 50 mM amorphous FeOOH. Riboflavin (10 µM) was added as an electron shuttle where necessary. Triplicate experiments, along with sterile controls, were incubated in the dark at 30°C.

Each experimental bottle was shaken and then sampled periodically (anaerobically and aseptically) for 0.5 N HCl extractable Fe(II) with Fe concentrations determined by ferrozine assay with the absorbance at 562.0 nm measured using a UV/Vis spectrophotometer (Jenway) (Lovley and Phillips, 1986a; 1986b).

4.4. Results and Discussion

4.4.1. Growth and survival of S. oneidensis after irradiation

Prior to analysis of metabolism, the impact of acute doses of X-radiation on the growth and viability of *S. oneidensis* MR-1 was assessed. The dose yielding 10% CFU survival (D₁₀) was ~84 Gy and D₂₀ (20% survival) was ~57 Gy (Figure S2). These values are higher than those observed in previous studies using a $^{60}$Co gamma source: D₁₀ = 70 Gy (Daly et al., 2007) and D₂₀ = 40 Gy (Qiu et al., 2006). This is perhaps not surprising as Co-60 gamma photons have a typical energy of 1.3 MeV.
compared to a maximum X-ray energy of 160 keV generated from the irradiator used in the present study. Despite this, the survival rates observed here are still indicative of the lethal effects of ionizing radiation and X-radiation is a good analogue for studying these effects.

The most marked effect of irradiation on the growth of the cultures was an extended lag phase, which increased up to ~7.5 h with a dose of 95 Gy (Figure 1 and Figure S1). As ionizing radiation is potentially lethal, an extension of the lag phase in irradiated cultures is likely explained by a reduction in the initial active biomass immediately after irradiation (Figure S2). However, despite receiving doses which yielded less than 10% survival (Figure S2), ionizing radiation had no significant effect on total biomass yield (Figure S1), suggesting that cultures were able to recover.

**Figure 1.** Mean time difference in lag phase duration between irradiated cultures and respective controls (measured at mid exponential phase). Error bars depict 95% confidence intervals from three biological replicates.

4.4.2. **Post irradiation metabolism**

To assess the impact of radiation on the metabolism of *S. oneidensis*, samples were collected from control and irradiated cultures immediately after irradiation (lag phase), at mid exponential phase and at stationary phase and were analyzed by FT-IR spectroscopy. The FT-IR spectra of control and irradiated cultures (12 to 95 Gy)
were typical of metabolic fingerprints reported previously for *S. oneidensis* (Figure 4A) (Wang et al., 2010a). Inspection of these data by eye is limited as the spectra are qualitatively very similar. Therefore, to observe overall trends in the data and assess the importance of both dose and growth phase on the phenotype of irradiated cultures, PC-DFA was performed on FT-IR data from all growth phases for each separate dose. The Euclidean distances between the cluster centres of control and irradiated samples at each growth phase were measured and are shown in Figure 2A. Separation between control and irradiated sample clusters was greatest for lag phase cultures at all doses. Euclidean cluster distances decreased for all further growth phases with a slight increase in distance between the control and irradiated clusters of stationary phase cultures for some doses (12 and 72 Gy). This suggests that changes to the metabolic fingerprint of irradiated cultures are greatest during the lag phase, before the cells have time to recover.

**Figure 2.** Euclidean distances between PC-DFA clusters of control and irradiated cultures at the three growth phases sampled using (A) FT-IR spectra and (B) MALDI mass spectra. PCs 1 to 5 (FT-IR) and 1 to 30 (MALDI-MS) were used by the DFA algorithm with *a priori* knowledge of machine replicates, i.e. 1 class per sample point and treatment, giving 6 classes in total for each dose.
PLS regression was employed to assess further the importance of dose on the metabolism of cultures in later growth phases and to determine whether an irradiated phenotype developed. PLSR is a supervised classification technique whereby a statistical model was supplied with information about the treatment of each sample (i.e. control or irradiated). PLSR was performed on the data from all doses and the principal component scores of the lag phase data are shown (Figure 3). At doses of 12, 24, and 48 Gy, the samples of control and irradiated lag phase cultures did not display a strong degree of clustering and separation between control and irradiated samples was not observed. For samples exposed to 72 Gy and 95 Gy of X-radiation on the other hand, there was evidence of separation between control and irradiated samples, suggesting that at higher doses, there is a pronounced metabolic response to radiation. As increased energy flux at higher doses increases the frequency of ionization and damage events (both directly, via energy deposition into biomolecules, and indirectly, via the production of reactive species by water radiolysis), such dose dependent perturbation of metabolism would be expected. However, the validation plots shown in Figure S3 indicate that for all doses, except 48 Gy and 72 Gy, the $R^2$ values of the test samples are much lower than the model training samples, even when the number of principal components used in the model was increased. When viewed in the context of the PC-DFA data, this suggests that the changes to metabolism at lag phase may be significant but are not necessarily predictable. This is perhaps not surprising as radiation damage is indiscriminate and damage may be inflicted to a large array of cellular targets.

To further assess how the recovery time after irradiation (i.e. growth phase) affected the metabolic fingerprints of cultures, PLSR was also performed on the FT-IR data from exponential and stationary phase samples. Similar to the lag phase cultures, separation between samples from control and irradiated exponential phase cultures was only observed after 72 and 95 Gy (Figure S4). This response was not as marked as for the lag phase cultures, and again, this is somewhat reflected in the validation plots of Figure S4. On the other hand, samples from stationary phase cultures showed a stronger degree of clustering and separation of these clusters based on their treatment (control or irradiated). Again, this effect was most evident for cultures treated with 48, 72 and 95 Gy (Figure S5) and the ability of the PLSR model to predict sample classification was best for cultures treated with 24, 72 and 95 Gy.
These observations suggest that the irradiated phenotype was preserved throughout subsequent generations of the culture despite irradiated cultures showing recovery of biomass to the same levels as non-irradiated controls (Figure S1). Qiu et al. (2006) reported significant alteration to the regulation of a variety of genes (including metabolism related genes) measured throughout a 1 hour recovery period of S. oneidensis exposed to 40 Gy irradiation. Thus, changes to the metabolism of S. oneidensis in exponential and stationary phases observed in this study may be related to radiation induced gene regulation. Furthermore, these results suggest that such effects may be maintained throughout several doublings of the population.
Figure 3. Principal component scores from partial least squares regression performed on FT-IR data of control and irradiated cultures at lag phase. Solid black circles represent control samples and crosses represent irradiated samples. The nine replicates of each treatment are formed from three experimental replicates from each biological replicate.

4.4.3. Dose related phenotypes estimated from FT-IR spectra.

To determine if the lag phase phenotypic response of *S. oneidensis* was characterised by changes to specific biomolecules, the ratios of intensities of specific IR regions were plotted against dose (Figure 4).
Figure 4. (A) FT-IR spectra of cells of lag phase *S. oneidensis* MR-1 cultures exposed to X-radiation. These spectra are offset so that the spectral features are clearly visible. The bars at the top of the panel relate to the following biochemical regions: CH, aliphatic CH vibrations; CH\(_{2/3}\), CH\(_2/CH_3\) asymmetric stretch; Ad, amide I and II; Am, amine. Quantitative analysis of highest peak intensities in absorbance bands of lag phase FT-IR spectra: (B) Amide I + II / amine ratio, ratio of amide I and II peak intensity to amine peak intensity; (C) Protein / lipid ratio, ratio of summed amide and amine region peak intensities to summed peak intensities of CH region and CH\(_2/CH_3\) asymmetric stretches. Data points show the mean of 9 measurements and error bars depict the standard error of the mean.

Initially, the protein/lipid ratios were calculated by dividing the intensities of spectral regions associated with proteins (amide and amine regions) by regions associated with lipids (aliphatic CH vibration and CH\(_2/CH_3\) asymmetric stretch) (Figure 4A and
As the amount of proteins and lipids play an important role in maintaining function and fluidity, protein/lipid ratios can be related to membrane integrity. These analyses showed that the protein/lipid ratio decreased in cultures immediately after irradiation and this effect showed a degree of dose dependence. This decrease was related to an increase in the CH$_3$/CH$_2$ asymmetric stretch intensity as a result of irradiation (data not shown). This effect increased with dose and was particularly significant at doses of 48, 72 and 95 Gy (P < 1×10$^{-5}$; n = 9). Similarly, increases in the aliphatic CH vibration were also observed immediately after irradiation and showed dose dependence above 24 Gy (P < 0.003 for doses 48, 72 and 95 Gy; n = 9). Significant oxidation of membrane lipids via addition of hydroxyl radicals may result from radiolysis of fatty acids (Shadyro et al., 2004). Furthermore, these oxidation reactions may occur with the various C moieties in the fatty acid chains and thus, changes to the CH, CH$_2$ and CH$_3$ levels may be related to hydrogen abstraction (Stark, 1991). However, an increase in all three of these bond groups would unlikely be generated by oxidation reactions. Thus, these changes may not be related to specific damage processes but rather to an increase in lipid metabolism throughout irradiation, although in a previous study, genes known to be involved in lipid metabolism were down regulated in *S. oneidensis* as a result of radiation (Qiu et al., 2006). Whilst the exact reason for our observations remains unclear, as these groups are associated with membrane phospholipids, such radiation induced increases could potentially alter membrane composition.

The radiation induced decrease in the protein/lipid ratio is also strongly associated with a decrease in the amine vibration intensity; evident in Figure 4B by an increase in the amide/amine ratio. The amine vibration peak intensity decreased in all irradiated cultures and by as much as 17% and 24% in cultures irradiated with 48 and 95 Gy, respectively (P < 0.001 for both doses; n = 9). Amine groups have been shown to incur reaction with oxygen free radicals generated during radiolysis, with oxidation leading to the formation of α-ketoacids, aldehydes or carboxylic acids (Stadtman, 1993). In all these mechanisms, loss of the amine group was observed and this may account for the decrease in the amine vibration observed here. Furthermore, carboxyl groups also absorb in a similar IR region (Wang et al., 2010b) and this amino acid moiety has also been shown to be susceptible to free radical mediated oxidation (Stadtman and Levine, 2003). These reactions may be related to
extensive protein oxidation observed in *S. oneidensis* (Daly et al., 2007) and as this can modify the redox potential of the cell, these results have implications for downstream metabolism as well as reduced protein turnover (Stadtman, 1993; Stadtman and Levine, 2003).

![Figure 5](image)

**Figure 5.** Mean MALDI-MS spectra of *S. oneidensis* MR-1 exposed to (A) 12 Gy X-radiation and (B) 95 Gy X-radiation. Spectra are offset to so that spectral features are clearly visible. Asterisks (*) show mass peaks in irradiated spectra that are discriminant with respect to batch controls, as observed in subtraction spectra (mean irradiated spectrum minus mean control spectrum) (Figure S6.). The mass range has been limited in the figure to only include peaks which are discriminant and to which tentative annotations can be assigned, displayed in Table 1.

### 4.4.4. Radiation induced changes to proteins

Whole cell samples were analysed using MALDI-TOF-MS to examine further the potential influence of radiation on proteins of *S. oneidensis* and to determine whether ionizing radiation targets specific proteins. Mass spectra of irradiated samples (12 and 95 Gy) and batch controls are shown in Figure 5 and peaks which appeared discriminant in subtraction spectra (Figure S6) are highlighted. Annotations of peaks are documented in Table 1 along with tentative assignments to proteins.
Table 1. Annotations of protein peaks in irradiated *S. oneidensis* from UniprotKB/Swiss-Prot and UniProtKB/TrEMBL protein sequence databases.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Discriminant peak m/z (Da)</th>
<th>Peak range (Da)</th>
<th>Concentrations relative to control</th>
<th>Protein annotations</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 Gy</td>
<td>6551</td>
<td>6547-6554</td>
<td>+</td>
<td>No match</td>
</tr>
<tr>
<td></td>
<td>7126</td>
<td>7123-7128</td>
<td>+</td>
<td>7125 Da carbon storage regulator homolog (csrA) inferred from homology (Locus SO3426).</td>
</tr>
<tr>
<td></td>
<td>7170</td>
<td>7168-7173</td>
<td>+</td>
<td>7170 Da 50S ribosomal protein L29 (rpmC) inferred from homology (Locus SO0239).</td>
</tr>
<tr>
<td></td>
<td>7269</td>
<td>7266-7274</td>
<td>+</td>
<td>7269 Da Uncharacterized protein (predicted; Locus SO0886).</td>
</tr>
<tr>
<td></td>
<td>7392</td>
<td>7392-7393</td>
<td>-</td>
<td>7392 Da Uncharacterized protein (predicted; Locus SO3548).</td>
</tr>
<tr>
<td></td>
<td>7604</td>
<td>7601-7607</td>
<td>+</td>
<td>7603 Da Lambda phage uncharacterized protein (predicted; Locus SO4795).</td>
</tr>
<tr>
<td></td>
<td>8903</td>
<td>8899-8905</td>
<td>+</td>
<td>8901 Da Protein with c-terminal DUF1078 domain (predicted; Locus SO4782).</td>
</tr>
<tr>
<td>95 Gy</td>
<td>6552</td>
<td>6547-6555</td>
<td>-</td>
<td>No match</td>
</tr>
<tr>
<td></td>
<td>7170</td>
<td>7167-7173</td>
<td>-</td>
<td>7170 Da 50S ribosomal protein L29 (rpmC) inferred from homology (Locus SO0239).</td>
</tr>
<tr>
<td></td>
<td>7270</td>
<td>7266-7274</td>
<td>+</td>
<td>7269 Da Uncharacterized protein (predicted; Locus SO0886).</td>
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<tr>
<td></td>
<td>7366</td>
<td>7361-7370</td>
<td>-</td>
<td>7365 Da Uncharacterized protein (predicted; Locus SO4740).</td>
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<td>7368 Da Uncharacterized Lambda phage protein (predicted; Locus SO3010).</td>
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<td></td>
<td></td>
<td></td>
<td>7368 Da DUF3012 domain-containing lipoprotein (predicted; Locus SO0515).</td>
</tr>
<tr>
<td></td>
<td>7392</td>
<td>7392-7394</td>
<td>-</td>
<td>7392 Da Uncharacterized protein (predicted; Locus SO3548).</td>
</tr>
<tr>
<td></td>
<td>7406</td>
<td>Peak inferred from Figure S6</td>
<td>+</td>
<td>7406 Da Cold shock protein (Csp family) inferred from homology (Locus SO1648).</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7405 Da sulphur carrier protein ThiS (predicted; Locus SO2442).</td>
</tr>
<tr>
<td></td>
<td>7603</td>
<td>7597-7613</td>
<td>-</td>
<td>7603 Da Lambda phage uncharacterized protein (predicted; Locus SO4795).</td>
</tr>
<tr>
<td></td>
<td>8903</td>
<td>8898-8903</td>
<td>-</td>
<td>8901 Da Protein with c-terminal DUF1078 domain (predicted; Locus SO4782).</td>
</tr>
</tbody>
</table>
Many of the proteins are predicted from the *S. oneidensis* genome (Heidelberg et al., 2002), and thus, do not take into account mass variability arising during translation and from processes such as post translational modification. Indeed, it is also possible that radiation may modify the protein mass via fragmentation, as a result of peptide bond cleavage (Stadtman, 1993; Berlett and Stadtman, 1997; Stadtman and Levine, 2003) and by ionization, which may generate subtle variations in the molecular mass. Hence, although Table 1 indicates the mass range in which the maximum peak intensities of each sample were observed, care is given when discussing these results with regard to protein identification.

Samples exposed to 12 Gy X-radiation generally displayed peaks with a greater intensity than respective controls (Figure S6 and Table 1). These increases in protein levels as a result of irradiation may be related to the up-regulation of genes associated with amino acid transport and metabolism, and protein turnover, post translational modification and chaperones (Qiu et al., 2006). Up-regulation of proteins has also been observed in irradiated *D. radiodurans*, of which, some had functions related to protein turnover (Lu et al., 2009).

In addition to peak analysis, PC-DFA was applied to the data in order to assess dose dependent changes and establish whether differences in the proteome were maintained throughout growth. Euclidean PC-DFA distances show that at low dose (12 Gy), separation between control and irradiated clusters was greatest immediately after irradiation and decreasing with growth phase (Figure 2). These data suggest recovery of cultures from low dose irradiation and the up-regulation of proteins at lag phase may be related to this recovery. However, whilst a strong response of genes related to DNA repair, oxidative stress and the scavenging of reactive oxygen species has been observed during transcriptomic analysis of *S. oneidensis* recovering from irradiation (Qiu et al., 2006), Table 1 indicates that up-regulated proteins do not appear related to repair mechanisms. Of the proteins that showed a change in concentration, most were uncharacterized. An uncharacterized protein encoded in the *S. oneidensis* lambda phage was increased and the induction of phage genes has been observed previously in both the UV and ionizing radiation response (Qiu et al., 2005; Qiu et al., 2006). Indeed, the induction of phage genes and the subsequent prophage lytic cycle has been inferred to contribute to the sensitivity of this
organism to ionizing radiation (Qiu et al., 2006). In addition, a carbon storage regulator homolog (csrA) was observed to increase, along with a 50S ribosomal protein L29 (rpmC). The reason for this response is unclear, though it may be related to a general response in metabolism and protein turnover (Qiu et al., 2006) and these changes may contribute to the recovery of cultures at lower doses.

Cultures exposed to 95 Gy X-radiation showed a reduction in the levels of most proteins (Figures 5 and S6, Table 1). Unlike at 12 Gy, 95 Gy resulted in a reduction of peaks which could be assigned to a 50S ribosomal L29 (rpmC) (7170 Da) and an uncharacterized lambda phage protein (7603 Da). Peaks at 7366, 7392 and 8903 Da also showed a decrease as a result of radiation, however, their function is uncharacterized. This maybe the result of down regulation of genes related to protein metabolism (Qiu et al., 2006), however, at this higher dose, it is perhaps more likely a result of protein damage arising from reactions such as oxidation and carbonylation (Daly et al., 2007; Krisko and Radman, 2010). In contrast to the reduction observed in most proteins after 95 Gy irradiation, increased levels of a protein at 7406 Da was observed. This mass can be tentatively assigned to a cold shock protein (Csp family) or a sulphur carrier protein (ThiS). Cold shock proteins may serve as RNA chaperones and gene regulators and have various physiological roles in response to a variety of stresses (Graumann and Marahiel, 1998; Phadtare et al., 1999). The up-regulation of this protein may be a reaction to cellular radiation stress which may share physiological triggers with cold shock. In addition, cold shock proteins have also been implicated in regulation of membrane fluidity (Ramos et al., 2001) and thus, the up-regulation of this protein could also be related to the increase in lipid related bonds revealed by FT-IR spectra. A protein with mass 7270 Da also increased, although its function is uncharacterized.

In contrast to the 12 Gy treatment, the clusters of control and 95 Gy treated samples showed greatest Euclidean PC-DFA distances between stationary phase samples (Figure 2). These data suggest that, whilst the proteome of a 12 Gy treated culture appears to show recovery, at 95 Gy, alteration to the proteome may be preserved, or indeed exacerbated through successive generations of the culture. This response is likely a result of changes in gene expression which may be persistent throughout the exponential growth of cultures irradiated to a high dose.
It is evident that radiation induced changes to the levels of protein in *S. oneidensis* are dose dependent and this dose dependence also strongly influences the phenotype in latter growth phases. In agreement with transcriptomic analysis of irradiated *S. oneidensis* (Qiu et al., 2006), we observed a strong response of proteins to radiation; however, this response, as analysed by MALDI-TOF-MS, does not appear coherent.

![Figure 6](image-url)

**Figure 6.** Fe(III) reduction by *S. oneidensis* MR-1 with and without exposure to 50 Gy X-radiation. Error bars depict the standard error of the mean of triplicate experiments. Rf = riboflavin.

As *S. oneidensis* is characterised by its respiratory versatility, particularly its ability to respire Fe(III) and other alternative electron acceptors, the ability of the irradiated phenotype to reduce amorphous Fe(III) oxyhydroxide was assessed. Systems containing irradiated (50 Gy X-radiation) biomass displayed more than double the levels of Fe(III) reduction after 15 days compared to non-irradiated controls (Figure 6) despite the presence of only 2.3% active biomass with respect to controls. The reason for this is unclear, although it could be related to a general up-regulation of metabolism immediately after irradiation, or physical damage to the cell structure facilitating extracellular electron transfer.
4.5. Conclusions
In summary, these results suggest that ionizing radiation imparts a control on the viability and growth of aerobic cultures of *S. oneidensis* over a range of doses. Modification to the protein and lipid content of cells characterises the metabolism immediately after irradiation and multivariate statistical analyses reveal the development of an irradiated phenotype throughout multiple generations despite biomass recovery. Both fatality and metabolic changes are likely contributors to observed growth effects and this may have implications for growth in subsurface environments, where doubling times are large.

As protein and lipid levels are fundamental to the integrity and functionality of membranes, ionizing radiation may have significant implications for the long-term metabolism and functionality of *S. oneidensis* during bioremediation applications. Furthermore, such biochemical changes may also be pivotal to the control *S. oneidensis* may exact on the chemistry of key electron acceptors, such as Fe(III) and radionuclides such as U(VI), in the near field environment of a geological nuclear repository. Further work is required to characterise fully the impact of radiation on the respiratory capabilities of *S. oneidensis*, particularly the ability of the irradiated phenotype to respire alternative electron acceptors, such as redox active metals and radionuclides.

4.6. Acknowledgements
This work was funded by a BBSRC PhD Studentship awarded to A. Brown and CASE award from the Nuclear Decommissioning Authority. The use of the Faxitron X-ray machine granted by Dr Kaye Williams, School of Pharmacy & Pharmaceutical Sciences and the assistance of Joana Senra and Amy Adlard in conducting the irradiations is gratefully acknowledged.
4.7. Supplementary information

**Figure S1.** Growth profiles of *S. oneidensis* MR-1 after exposure to 12, 24, 48, 72 and 95 Gy X-radiation. Data points show mean of triplicate batch cultures and error bars depict 95% confidence intervals.
Figure S2. Survival of *S. oneidensis* MR-1 exposed to acute doses of X-radiation. Error bars depict standard error of the mean CFU mL$^{-1}$. 
Figure S3. Principal component scores from partial least squares regression performed on FT-IR data of control and irradiated cultures at lag phase (left panel) and validation plots for each model (right panel). Solid black circles represent control samples and crosses represent irradiated samples. The nine replicates of each treatment are formed from three experimental replicates from each biological replicate.
Figure S4. Principal component scores from partial least squares regression performed on FT-IR data of control and irradiated cultures at exponential phase (left panel) and validation plots for each model (right panel). Solid black circles represent control samples and crosses represent irradiated samples. The nine replicates of each treatment are formed from three experimental replicates from each biological replicate.
Figure S5. Principal component scores from partial least squares regression performed on FT-IR data of control and irradiated cultures at stationary phase (left panel) and validation plots for each model (right panel). Solid black circles represent control samples and crosses represent irradiated samples. The nine replicates of each treatment are formed from three experimental replicates from each biological replicate.
Figure S6. MALDI-MS subtraction spectra of *S. oneidensis* MR-1 exposed to 12 Gy (top panel) and 95 Gy X-radiation (bottom panel). Spectra show the result of the mean irradiated spectrum minus the mean control spectrum with labels indicating the masses of peaks that show a deviation away from zero. The mass range has been limited in the figure to only include peaks which show a difference and to which tentative annotations can be assigned, displayed in Table 1.
4.8. References


Chapter 5

Research Paper

The impact of ionizing radiation on the bioavailability of Fe(III) minerals for microbial respiration
5. The impact of ionizing radiation on the bioavailability of Fe(III) minerals for microbial respiration

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5.1. Abstract
Conservation of energy by Fe(III)-reducing species such as \textit{Shewanella oneidensis} potentially controls the redox potential of environments relevant to the geological disposal of nuclear waste and radwaste contaminated land. As such environments are likely to receive large radiation doses, characterization of radiation damage to the mineralogy and the resultant impact upon microbial respiration of iron is essential as radiation induced changes to the iron mineralogy may impact upon microbial respiration and, subsequently, alter the stability of the radionuclide inventory. In the present work, Mössbauer spectroscopy and electron microscopy indicate that irradiation (1 MGY gamma) of 2-line ferrihydrite can lead to conversion to a more crystalline phase, one similar to akaganeite. The room temperature Mössbauer spectrum of irradiated hematite shows the emergence of a paramagnetic Fe(III) phase. Spectrophotometric determination of Fe(II) reveals a radiation induced increase in the rate and extent of ferrihydrite and hematite reduction by \textit{S. oneidensis} in the presence of an electron shuttle (riboflavin). Post-reduction sequential extractions of iron in combination with spectroscopic assays for ferrous iron, indicate that this additional Fe(II) is partitioned into solid phases extractable with 1 M hydroxylamine-HCl and 1 M sodium acetate in ferrihydrite systems, and a
phase extractable with 1 M hydroxylamine-HCl in hematite systems. This study suggests that changes in these minerals induced by radiation may lead to an increase in bioavailability of Fe(III) for respiration by Fe(III)-reducing bacteria.

5.2. Introduction

Fe(III)-reducing bacteria such as *Shewanella oneidensis* couple the oxidation of organic matter to the reduction of a range of redox active metals and radionuclides. This may occur both directly, via enzymatic reduction of redox active metal contaminants and radionuclides or, indirectly, via the reduction of bioavailable Fe(III) and subsequent abiotic electron transfer from Fe(II) to metals and radionuclides (Lloyd and Macaskie, 1996; Lloyd et al., 2000; Hansel et al., 2003; Wilkins et al., 2006). Hence, the biogeochemical cycling of iron can impart a critical control on the redox potential of environments relevant to the geological disposal of nuclear waste and radwaste contaminated land. For instance, the precipitation of problematic radionuclides such as mobile Tc(VII) and U(VI) can be achieved by their reduction to insoluble Tc(IV) and U(IV) species (Wildung et al., 2000; Williams et al., 2011). Similarly, toxic and mobile contaminants associated with nuclear wastes such as chromium can also be removed from solution by the reduction of Cr(VI) to the less toxic Cr(III) (Hansel et al., 2003; Fredrickson et al., 2004). Thus, dissimilatory iron-reducing species such as *Shewanella* sp. and *Geobacter* sp. have been considered for the remediation of complex wastes, including contaminant metals and radionuclides and also toxic organic compounds that can be degraded by these organisms (Lloyd, 2003).

However, remediation of these environments presents a challenge as many sites contaminated with radionuclides exhibit large fluxes of ionizing radiation (Fredrickson et al., 2004). Similarly, the concept of a geological repository for the long-term storage of intermediate level nuclear wastes requires the encapsulation of highly radioactive wastes. Dose rates are expected to be variable due to the dynamics of radioactive decay of a range of fission products and longer-lived actinides; however, absorbed doses over the life of a repository will be significant (Allard and Calas, 2009).
Proposed barrier materials for the encapsulation of intermediate level waste include iron rich materials, such as steel canisters, which will likely receive significant doses of ionizing radiation. Indeed, the waste form itself may contain large quantities of steel waste. Subsequent interactions between radiolysis products and steel may generate a range of iron oxides, including Fe$_2$O$_3$ and FeOOH (Zhang et al., 2007; Daub et al., 2011). These products would also be subject to a radiation flux. Furthermore, iron oxides may also be common in the rocks surrounding a geological repository and may also be irradiated after a failure of the encapsulant.

Due to the redox sensitivity of iron, it is possible to observe a range of radiation-induced oxidation and reduction reactions depending on the initial oxidation state and coordination chemistry of the iron. For example, Ladriere (1998) notes that both oxidation and reduction of the iron is visible in the Mössbauer spectra of gamma irradiated iron-containing compounds. In this case it is the formation of radicals in the first coordination sphere of the iron that induces any redox transformation. In aqueous systems, however, oxidizing and reducing species generated by the gamma radiolysis of water, such as $`$OH, e$^{-}_{aq}$, H$, $HO$, H$_2$, H$_2$O$_2$, H$^+$ are likely to dominate the redox potentials at the surface of iron-bearing compounds and, hence, facilitate their corrosion (Zhang et al., 2007; Daub et al., 2011).

The oxidation state of iron in part governs crystal structure, surface area and reactivity (Cornell and Schwertmann, 2003) and these parameters act as constraints on the thermodynamics of microbial iron reduction (Roden, 2003; Hansel et al., 2004; Roden, 2006; Cutting et al., 2009). Consequently, the radiation driven alteration of iron oxides, including any potential changes in crystal structure and oxidation state may impact upon microbial respiration of Fe(III)-bearing phases. Perturbations to the biogeochemical cycling of this iron may subsequently affect the solubility and mobility of radionuclides; hence, characterization of radiation damage to a range of environmentally relevant iron oxides, and the resultant impact upon microbial respiration of iron, is essential in the preparation of a geological disposal safety case.

In this study, suspensions of ferrihydrite and hematite were irradiated with 1 MGy of gamma radiation and any alteration was characterized using transmission electron
microscopy (TEM), including selected area electron diffraction (SAED), and Mössbauer spectroscopy. Ferrozine assay was used to evaluate the post-irradiation availability of these Fe(III) minerals for reduction by *S. oneidensis*. This is the first study in which an attempt is made to document the radiation-induced activation of iron oxides for microbial Fe(III)-reduction.

5.3. Experimental Section

5.3.1. Mineral synthesis and irradiation

Hematite and 2-line ferrihydrite were synthesised by methods described previously by Schwertmann and Cornell (1991). The structures of the synthesised minerals were confirmed via X-ray diffraction (XRD) using a Bruker D8 Advance instrument. Samples consisting of a suspension of the mineral with 1 mL of 480 mM Fe were then placed into ampoules and flame sealed, autoclaved and treated, as appropriate, with 1 MGy gamma radiation in a Shepherd ⁶⁰Co source.

5.3.2. Mineralogical characterisation

Post-irradiation samples of hematite and ferrihydrite were characterised using transmission electron microscopy (TEM) also involving selected area electron diffraction (SAED). Samples were prepared by suspending in ethanol prior to drop-casting onto carbon support films (Agar Scientific). Bright field TEM images were acquired using a Philips microscope fitted with a 200 kV Field Emission Gun and Gatan Imaging Filter (GIF200). SAED patterns for all samples were acquired using an appropriate aperture and d-spacings were measured and compared to reference iron oxides (see individual references and Downs and Hall-Wallace (2003)).

For Mössbauer spectroscopy, irradiated minerals were dried inside a desiccator within an anoxic cabinet. Where a volume of powder was insufficient for short analysis times, powders were mixed with boron nitride. Dried powders were then sealed anaerobically inside polyethylene mounts using epoxy sealant. Mössbauer spectra were collected at room temperature using a ⁵⁷Co source. Iron foil was used for calibration and all isomer shifts are reported with iron foil as the reference material. Data were acquired and processed using the *Recoil* Program (University of Ottawa).
5.3.3. Microbial Fe(III) reduction

*S. oneidensis* MR-1 was grown aerobically in tryptic soy broth in Erlenmeyer flasks at 30 °C and shaken at 130 rpm. This starting culture was then used to inoculate a fully defined, sterile, nitrogen sparged minimal medium based on that of Myers and Nealson (1988), using 100 mM lactate and 20 mM fumarate as the electron donor and acceptor respectively. After 24 hours incubation at 30 °C, late log - early stationary phase biomass was harvested by centrifugation at 4920g for 20 minutes at 4 °C and, then, washed twice in sterile 30 mM sodium bicarbonate buffer in an 80:20 gas mix of N₂:CO₂. Aliquots of the buffered cell suspension (0.2 ml) were added to 9 mL anaerobic 30 mM bicarbonate buffer containing 50 mM lactate as electron donor. 1 mL aliquots of Fe mineral suspension were added to produce a final Fe concentration of 10 mM. Riboflavin (10 µM) was added as an electron shuttle where necessary. Triplicate experiments, along with sterile controls, were incubated in the dark at 30°C.

Each experimental bottle was shaken and then sampled periodically (anaerobically and aseptically) for 0.5 N HCl extractable Fe(II) with Fe concentrations determined by ferrozine assay with the absorbance at 562.0 nm measured using a UV/Vis spectrophotometer (Jenway) (Lovley and Phillips, 1986a; 1986b). 24 hour 0.5 N and 3 N HCl digests with the addition of 0.25 N hydroxylamine-HCl were utilised to determine total Fe concentrations in ferrihydrite and hematite experiments, respectively.

The fate of microbially reduced Fe was determined by sequential extractions based on procedures described previously by Tessier et al. (1979), Keith-Roach et al. (2003) and Poulton and Canfield (2005). Fe(II) in solution was determined by centrifugation at 3000g and spectrophotometric measurement of Fe(II) using ferrozine assay. The first of several operationally defined fractions was adsorbed Fe(II), determined by a 2 hour extraction in 1 M magnesium chloride (pH 7). A ‘carbonate’ fraction was determined by 24 hour extraction in 1 M sodium acetate adjusted to pH 4.5 with acetic acid. An ‘easily reducible’ fraction was defined by a 24 hour extraction in 1 M hydroxylamine–HCl in 25% v/v acetic acid, and a ‘reducible’ fraction was determined by a 2 hour extraction in 0.33 M sodium dithionite solution buffered to pH 4.8 using 0.35 M acetic acid and 0.2 M sodium
citrate. A ‘magnetite’ extraction was achieved by a 6 hour digest in a 0.2 M ammonium oxalate solution with 0.17 M oxalic acid (pH 3.2). Finally, the ‘residual’ fraction was subject to a concentrated nitric acid digest. Fe concentrations in all extracts were determined using ferrozine assay.

5.4. Results and discussion
5.4.1. Mineralogical characterisation of irradiated Fe(III) oxides
To identify any structural changes that could influence the bioavailability of the irradiated Fe(III) phases, samples were examined using TEM. The TEM images of non-irradiated ferrihydrite displayed a morphology typical of amorphous 2-line ferrihydrite (Figure 2a) (Janney et al., 2000b) and SAED patterns contained reflections attributable to 2-line ferrihydrite (Figure 2b; see Figure 1a for proposed structure of 2-line ferrihydrite) (Schwertmann and Cornell, 1991; Manceau and Drits, 1993; Janney et al., 2000a).

Figure 1. (A) Schematic representation of the 2-line ferrihydrite structure determined by electron nanodiffraction. Image from Janney et al. (2000a). The box indicates a unit cell of dimensions $x = 0.52$ nm, $y = 0.3$ nm, $z = 0.94$ nm. Black spheres represent oxygen atoms and Fe atoms are inside the shaded octahedra. (B) The structure of akaganeite determined by Post (1989). Green spheres represent chlorine atoms and Fe atoms are inside shaded tetrahedra and octahedra.
Irradiated ferrihydrite displayed a mixture of poorly crystalline iron phases with the emergence of reflections attributable to 6-line ferrihydrite (Manceau and Drits, 1993; Jansen et al., 2002; Michel et al., 2007) and akaganeite (Figure 2d; see Figure 1b for akaganeite structure) (Post, 1989). However, the morphology of the crystallites did not appear different from the non-irradiated sample, and there was no distinct increase in the lattice interference pattern (from alternating high and low electron density) that might be expected with an increase in crystallinity towards 6-line ferrihydrite (Janney et al., 2000b). In addition, irradiated ferrihydrite did show a much lower degree of aggregation (Figure 2c); the reason for this is unclear.

Yakabuskie et al. (2011) found that the structure of γ-FeOOH did not change with prolonged irradiation (134 kGy ⁶⁰Co γ), although an increase in particle size as a function of irradiation time was reported. In our study, crystallites of ferrihydrite showed a possible increase in acicularity after irradiation (Figure 2c). However, this phenomenon was subtle and was observed to some extent in all the ferrihydrite samples (control and treated). Janney et al. (2000b) attributed acicularity to the presence of goethite in a ferrihydrite sample (an artefact of the synthesis procedure); however, SAED patterns of the irradiated ferrihydrite showed no evidence of goethite.
Figure 2. Transmission electron micrographs of (a) non-irradiated ferrihydrite and (c) irradiated ferrihydrite. The corresponding SAED pattern for non-irradiated ferrihydrite is shown in (b) with indexed lines for 2-line ferrihydrite. The corresponding SAED pattern for irradiated ferrihydrite is shown in (d) with measured interplanar spacings for irradiated suspensions in the bottom left segment and remaining segments displaying previously reported indexed patterns for selected Fe(III)-(oxy)hydroxides.

The Mössbauer spectrum of non-irradiated ferrihydrite was fitted with two Lorentzian doublets, as would be expected for 2-line ferrihydrite (Figure 3) (Murad and Schwertmann, 1980). The fitting gave an isomer shift of 0.37 mm/s and quadrupole splitting of 0.53 mm/s for the first component (comprising 62.2% of the
fit) and an isomer shift of 0.38 mm/s and a quadrupole splitting of 0.88 mm/s for the second component (37.8% of the fit). These values are in good agreement with previously reported Mössbauer parameters for poorly crystalline (2-line) ferrihydrite (Murad and Schwertmann, 1980; Vandenberghe et al., 1990).

![Mössbauer Spectra](image)

**Figure 3.** Mössbauer spectra of non-irradiated (top) and irradiated (bottom) ferrihydrite showing calculated fits (black curve) and component fits (red and blue curves). Respective $\chi^2 = 1.27$ and 1.51.

The Mössbauer spectrum obtained after irradiation of this material was best fitted with two Lorentzian doublets (Figure 3), with an isomer shift of 0.37 mm/s and quadrupole splitting of 0.54 mm/s for the first doublet (comprising 66.4% of the fit) and an isomer shift of 0.38 mm/s and quadrupole splitting of 0.93 mm/s for the second component (33.6% of the fit). Vandenberghe et al. (1990) suggest that the spectrum of a so-called “well crystalline” ferrihydrite exhibits a larger contribution from the first doublet, than for a more poorly crystalline ferrihydrite. Indeed, this is what we observe in the spectrum of the irradiated ferrihydrite compared to the non-irradiated ferrihydrite. This supports our previous observations that ferrihydrite underwent an increase in crystallinity upon irradiation.
Table 1. Mössbauer parameters of all samples and parameters previously reported for akaganeite.

<table>
<thead>
<tr>
<th>Oxide and treatment</th>
<th>Lorentzian component</th>
<th>Quadrupole splitting $\Delta E_Q \text{ mm s}^{-1}$</th>
<th>Isomer shift $\delta \text{ mm s}^{-1}$</th>
<th>Site populations %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrihydrite</td>
<td>Doublet 1</td>
<td>0.53</td>
<td>0.37</td>
<td>62 %</td>
</tr>
<tr>
<td></td>
<td>Doublet 2</td>
<td>0.88</td>
<td>0.38</td>
<td>38 %</td>
</tr>
<tr>
<td>Irradiated ferrihydrite</td>
<td>Doublet 1</td>
<td>0.54</td>
<td>0.37</td>
<td>66 %</td>
</tr>
<tr>
<td></td>
<td>Doublet 2</td>
<td>0.93</td>
<td>0.38</td>
<td>34 %</td>
</tr>
<tr>
<td>Akaganeite (Cornell and Schwertmann, 2003)</td>
<td>1st component</td>
<td>0.55</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2nd component</td>
<td>0.95</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>Hematite</td>
<td>Doublet 1</td>
<td>0.35</td>
<td>0.21</td>
<td>2 %</td>
</tr>
<tr>
<td></td>
<td>Sextet 1</td>
<td>-0.11</td>
<td>0.39</td>
<td>65 %</td>
</tr>
<tr>
<td></td>
<td>Sextet 2</td>
<td>-0.12</td>
<td>0.37</td>
<td>33 %</td>
</tr>
<tr>
<td>Irradiated hematite</td>
<td>Doublet 1</td>
<td>0.27</td>
<td>0.21</td>
<td>45 %</td>
</tr>
<tr>
<td></td>
<td>Sextet 1</td>
<td>-0.20</td>
<td>0.39</td>
<td>55 %</td>
</tr>
</tbody>
</table>

Non-irradiated ferrihydrite showed a degree of asymmetry in the recorded Mössbauer spectrum (Figure 3). Vandenberghe et al. (1990) state that this is typical of structural disorder - a defining characteristic of an amorphous material. Hence, an increase in symmetry would suggest increased structural ordering post irradiation (Figure 3). Interestingly, the parameters reported for irradiated ferrihydrite are closer to those reported previously for room temperature measurements of akaganeite than for either 2-line or 6-line ferrihydrite (Table 1) (Cornell and Schwertmann, 2003), though the authors add the caveat that both ferrihydrite and akaganeite are characterized by similar doublets in their Mössbauer spectra (Childs and Johnston, 1980; Vandenberghe et al., 1990). However, the SAED pattern of irradiated ferrihydrite does display a reflection at 5.71 Å which may be attributable to the more crystalline akaganeite (Figure 2d) (Post, 1989). Transformation to akaganeite would require chloride to be present in solution. Indeed, ferrihydrite was prepared by hydrolysis of a FeCl₃ solution, and whilst the precipitate was washed six times via centrifugation and confirmed as 2-line ferrihydrite by XRD, trace amounts
of chloride may have been present in solution and therefore partial transformation to akaganeite cannot be ruled out.

Figure 4. Transmission electron micrographs of non-irradiated (a) and irradiated (c) hematite. Corresponding SAED patterns show indexed lines for hematite in (b) and (d) respectively.

Non-irradiated hematite displays a morphology typical of a very crystalline iron oxide (Figure 4a) (Schwertmann and Cornell, 1991), and the associated SAED pattern contains reflections attributable to the interplanar spacings reported previously (Figure 4b) (Blake et al., 1966). Similarly, the Mössbauer spectrum was constrained by the fitting of two sextets with parameters typical of hematite (Figure 5 and Table 1) (Cornell and Schwertmann, 2003). Whilst the Mössbauer spectrum
of hematite may be expected to contain only one sextet (as hematite contains only one Fe(III) lattice site), previous studies have constrained hematite spectra better with two sextets (Musić et al., 1997; Jacob and Abdul Khadar, 2010). These studies base their observations on a study by Shinjo et al. (1983) which observed that the surface magnetic hyperfine field was smaller than in the bulk. Jacob and Abdul Khadar (2010) suggest that it is this difference in site environments of surface and core Fe in nanoparticulate hematite that gives rise to the two spectral components.

![Mössbauer spectra of non-irradiated (top) and irradiated (bottom) hematite showing calculated fits (black curve) and component fits (red and blue curves). Respective reduced $\chi^2 = 214.92$ and 2.45.](image)

**Figure 5.** Mössbauer spectra of non-irradiated (top) and irradiated (bottom) hematite showing calculated fits (black curve) and component fits (red and blue curves). Respective reduced $\chi^2 = 214.92$ and 2.45.

Unlike irradiated ferrihydrite, the SAED pattern for irradiated hematite remains similar to the starting material (Figure 4d). Furthermore, no lattice defects were apparent in TEM images (Figure 4c) and lattice interference patterns were well intersected by crystal edges, i.e. no amorphisation was apparent at these fringes and no surface depositions of amorphous oxides were visible which could have enhanced the bioavailability of Fe(III) (Cutting et al., 2009).
Daub et al. (2011) observed a mixture of goethite and magnetite upon gamma irradiation of carbon steel, and, although goethite may be considered more bioavailable than hematite, no evidence for these phases were observed in TEM images or SAED patterns. Radiation-induced particle size increase in $\gamma$-FeOOH reported by Yakabuskie et al. (2011) appears to be more important for amorphous iron phases, as no alteration in hematite particle sizes was noted after irradiation in our study (non-irradiated hematite mean particle size = 78.09 ± 2.14 nm (n=132); irradiated hematite mean particle size = 78.01 ± 2.05 nm (n=119)).

Despite the apparent lack of evidence for radiation damage or alteration to hematite in photomicrographs or diffraction patterns, the Mössbauer spectrum of irradiated hematite showed the emergence of a very distinct, paramagnetic Fe(III) component (room temperature doublet) with a very low isomer shift ($0.21 \text{ mm s}^{-1}$) and quadrupole splitting ($0.27 \text{ mm s}^{-1}$) comprising 45% of the peak areas (Figure 5 and Table 1). Whilst it is difficult to define this paramagnetic phase, the emergence of this doublet is associated with enhanced Fe(III)-reduction in irradiated hematite systems (discussed below). Thus, given the constraints on microbial reduction of iron oxides (Cutting et al., 2009), it is suggested that this new phase is a poorly crystalline iron oxide.

5.4.2. Microbial reduction of irradiated oxides

Ferrozine assay results indicated that, as expected, non-irradiated ferrihydrite was readily reduced by S. oneidensis as evidenced by a ~50% increase in Fe(II) after 30 days incubation. Reduction rates were significantly increased in the presence of riboflavin as an electron shuttling compound (Figure 6) (von Canstein et al., 2008). Despite an increase in crystallinity as observed in SAED patterns and Mössbauer spectra, irradiated ferrihydrite underwent significantly higher levels of iron reduction (~80%). This result is somewhat surprising, as crystallinity has been shown to be a major limiting factor in dissimilatory iron reduction by bacteria, with crystalline phases generally expected to be more recalcitrant to microbial reduction (Cutting et al., 2009).

This observation may be related to the conversion of 2-line ferrihydrite to a phase similar to akaganeite during irradiation, as observed via Mössbauer parameters and
SAED patterns. A study by Cutting et al. (2009) showed akaganeite is reduced at double the rate of poorly crystalline ferrihydrite, but only in the presence of an electron shuttle. Indeed, we also observed that a doubling in the rate of microbial reduction of irradiated ferrihydrite only occurred with the addition of riboflavin (Figure 6).

**Figure 6.** Microbial Fe(III)-reduction in media containing irradiated (red) and non-irradiated (blue) ferrihydrite. Error bars depict the standard error of the mean.

In addition, Cutting et al. (2009) suggest that aggregation imparts a large control on iron reduction via a subsequent decrease in effective surface area and, thus, surface site availability. Hence, a decrease in ferrihydrite particle aggregation as a result of irradiation may also explain the high levels of Fe(III) reduction observed.

Figure 7 indicates that non-irradiated hematite is largely recalcitrant to reduction by *S. oneidensis* as expected (Cutting et al., 2009), though limited levels of iron reduction were observed. However, as with irradiated ferrihydrite, irradiation of hematite yielded a three-fold increase in microbially reduced iron (Figure 7). The production of Fe(II) occurred only with the addition of an electron shuttle, suggesting that any radiation induced changes to the mineral are structural, i.e. ‘activated’ sites are not available for enzymatic reduction. Indeed, this increase is unlikely to be a function of the particle size of irradiated crystals, as no change in particle size was noted by measurement of crystal dimensions in TEM images.
Nevertheless, this does not preclude the possibility of subtle changes in surface-accessible sites not revealed by TEM.

Figure 7. Microbial Fe(III)-reduction in media containing irradiated (red) and non-irradiated (blue) hematite. Error bars depict the standard error of the mean.

5.4.3. Fate of microbially reduced iron

In order to understand the lability of the Fe(II) in irradiated and inoculated treatments, the fate of microbially reduced iron in all treatments was investigated using sequential extractions coupled to UV/Vis spectroscopic analyses for Fe(II). In all experiments, ammonium oxalate and residual extractions yielded less than 0.7% of the total Fe, and have therefore been removed from supplementary information (SI) Figures S1 and S2. We should also stress that it may be challenging to make direct comparisons between data obtained with these synthetic nano-mineral phases and the more complex sediment systems studied previously (Tessier et al., 1979; Keith-Roach et al., 2003; Poulton and Canfield, 2005). We therefore use these operationally defined extractions to draw conclusions about the properties, such as crystallinity of post-reduction iron mineral phases, based upon the increasing strength of reagents required to extract the iron.

After 70 days incubation, microbially-reduced iron from non-irradiated ferrihydrite was partitioned into phases extractable with 1 M sodium acetate, 1 M hydroxylamine-HCl and 0.3 M sodium dithionite (SI Figure S1). After irradiation,
iron extracted by the first two reagents increased in the presence of *S. oneidensis* and riboflavin. A 1 M sodium acetate extract has previously been shown to extract iron in a carbonate phase (Poulton and Canfield, 2005). The incorporation of bioreduced iron into a carbonate phase has been observed previously (Kukkadapu et al., 2005) and is likely given the use of a bicarbonate buffer. Further, an increase in Fe(II) associated with a solid phase extractable with 1 M hydroxylamine-HCl also suggests that the increase in biogenic Fe(II) due to irradiation results in the incorporation of Fe(II) into a solid phase.

Sequential extractions of post-reduction hematite indicate that most of the iron remains in a fraction extractable with 0.3 M sodium dithionite after 70 days incubation (SI Figure S2) as would be expected for hematite (Poulton and Canfield, 2005). In irradiated systems, however, the increase in Fe(II) as a result of microbial reduction is mainly incorporated into a fraction extractable with 1 M hydroxylamine-HCl. Whilst the exact mineral phase in which the Fe(II) resides remains undefined, it is evident that this Fe(II) is associated with a solid phase.

5.5. Conclusions
These results show that Fe(III) minerals are susceptible to radiation damage which can alter their availability for microbial reduction. At present, safety case models for radwaste geological disposal facilities lack detailed information on key microbial processes. Iron-based materials will be prevalent in the waste-forms, barrier systems and building structures that dominate many repository designs, e.g. for intermediate level waste in the UK (Nirex, 2003), and after a period of corrosion, have the potential to influence biogeochemical evolution of the facility. The microbial reduction of Fe(III) minerals may be enhanced by radiation damage and, here, it could be promoted further by the production of electron donors such as hydrogen and organic compounds formed by the radiolysis of water and polymers, respectively. Stimulation of dissimilatory Fe(III)-reduction may promote direct enzymatic or indirect (e.g. via biogenic Fe(II)) reduction of priority radionuclides, such as U(VI) and Tc(VII), and could, therefore, play an important role in controlling radionuclide migration. Thus, enhanced radiation inputs could provide the basis of a novel ecosystem capable of exerting important controls on the biogeochemical evolution of a radwaste geological disposal facility.
5.6. Acknowledgements

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5.7. Supplementary information

SI Figure S1. Sequential extractions of iron in ferrihydrite-containing systems. Error bars indicate the standard error of the mean.
SI Figure S2. Sequential extractions of iron in hematite-containing systems. Error bars indicate the standard error of the mean.

5.8. References


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Chapter 6

Research Paper

Stimulation of microbial Fe(III)-reducing communities by gamma radiation
6. Stimulation of microbial Fe(III)-reducing communities by gamma radiation

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6.1. Abstract

Microbial communities have the potential to control the biogeochemical fate of priority radionuclides in contaminated land and also in geodisposal scenarios. However, there have been few studies on the impact of ionizing radiation on microbial communities in sediment systems. Here, sediments representative of those surrounding the Sellafield nuclear facility were irradiated with 0.5 and 30 Gy h⁻¹ gamma radiation for 8 weeks in order to assess the impact of chronic gamma-irradiation on sediment microbial communities and the biogeochemical processes they control, both during irradiation and post-irradiation. In sediments containing added electron donor (acetate and lactate), NO₃⁻ and Fe(III)-reduction occurred in all systems, although less Fe(III) was reduced in 30 Gy h⁻¹ treatments. Treatment with 30 Gy h⁻¹ led to an increase in the solubilisation of SO₄²⁻, although this had no significant effect on microbial SO₄²⁻ reduction. Ion chromatography revealed that these systems were dominated by fermentation processes, whilst pyrosequencing indicated that the 30 Gy h⁻¹ treatment resulted in the stimulation of a community dominated by two Clostridial species, including a strain closely related to C. bowmanii. In systems containing no added electron donor, irradiation with both dose rates did not restrict NO₃⁻, Fe(III) or SO₄²⁻ reduction. Rather, Fe(III)-reduction was stimulated in the 0.5 Gy h⁻¹ treated systems. In irradiated systems, there was a relative increase in the proportion of bacteria capable of Fe(III)-reduction, with Geothrix fermentans and Geobacter sp. identified in 0.5 Gy h⁻¹ and 30 Gy h⁻¹ treatments respectively. These results indicate that, despite significant total absorbed doses, biogeochemical processes will likely not be restricted by radiation dose rates expected in environments relevant to land contaminated with radioactive waste and
geological disposal of nuclear waste. Indeed, these data suggest electron accepting processes in a deep geological repository may be stimulated by radiation and low dose gamma radiation could be the basis of a novel ecosystem in the deep biosphere. These results have important implications to the stability of a radwaste inventory.

6.2. Introduction

In many countries including the UK, the current policy for the long term disposal of intermediate-level nuclear waste is deep geological disposal. The present UK concept is a geological disposal facility (GDF) in which the waste forms will be packaged in grouted stainless steel containers and then backfilled with cement (Nirex, 2003). The stability of the radionuclide inventory over geological time is of paramount importance to the safety case for such facilities. However, the vicinity of the GDF will not be a sterile environment and microbial activity in the surrounding geosphere could have important implications for the evolution of biogeochemical processes, including microbial gas generation and utilization, microbial corrosion of waste forms and the mobility of radionuclides (Lloyd, 2003). In addition, there will be elevated concentrations of potential electron donors in and around the repository, including organics from the degradation of cellulose in the waste (Glaus and Van Loon, 2008) and also molecular hydrogen from the radiolysis of water and the anaerobic corrosion of steel drums (Libert et al., 2011). Indeed, the availability of alternative electron acceptors will likely not be limited, as nitrate is common in many nuclear waste materials (Lloyd and Renshaw, 2005), and Fe(III) will be present in significant quantities from the aerobic corrosion of waste components and engineered infrastructure during the operational phase of the GDF.

The stimulation of an Fe(III)-reducing community due to an increase in electron donors and acceptors is of particular interest as this may promote the reduction and precipitation of redox-active radionuclides via the production of biogenic Fe(II)-bearing phases (Lloyd, 2003; Lloyd and Renshaw, 2005). Indeed, many key Fe(III)-reducing species may also possess cytochromes and hydrogenases capable of directly reducing radionuclides, such as Tc(VII), Np(V) and U(VI) (Lloyd et al., 2000a; Lloyd et al., 2000b). As these processes could enhance the stability of the radionuclide inventory, the microbial ecology and potential for Fe(III)-reduction in geodisposal environments has been the focus of recent research.
However, these environments, and the microbially driven processes that occur within them, may be subject to significant radiation doses. Firm values for total absorbed doses and dose rates are difficult to predict as they are likely to be highly heterogeneous and dependent on the activity of the waste, the radiation type, decay dynamics and the absorbing materials of the waste form. For example, in the case of a GDF, the maximum dose rate suggested by Canadian researchers is predicted to be 52 Gy h\(^{-1}\) at the surface of a waste container (Stroes-Gascoyne et al., 1994). Similarly, Alard and Calas (2009) suggest that dose rates in silicate clays used for backfill material may be in the order of 72 Gy h\(^{-1}\) over the first 1000 years of the repository lifetime. Significant radiation fluxes may also be associated with near surface sites contaminated by radionuclides, for example, activities up to 0.37 GBq kg\(^{-1}\) have been measured at contaminated DOE sites (Riley et al., 1992; Fredrickson et al., 2004). Again, it is difficult to predict how activities such as this relate to dose rates and total absorbed doses, however, as a reference, it has been calculated by particle track calculation and Monte Carlo simulation that activities of 8.1 MBq kg\(^{-1}\) \(^{90}\)Sr and 9.6 MBq kg\(^{-1}\) \(^{137}\)Cs in Chernobyl soils equate to dose rates of 51.7 Gy y\(^{-1}\) and 14.8 Gy y\(^{-1}\) respectively (Niedree et al., 2013).

Ionizing radiation is potentially lethal to organisms as the energies involved are sufficient to cause strand breaks in DNA. Furthermore, the additional oxidative stress associated with cytoplasmic water radiolysis (Ghosal et al., 2005) is able to disrupt the metabolism of other essential biomolecules, such as proteins (Du and Gebicki, 2004; Daly et al., 2007). Therefore, as microbial metabolism in nuclear environments is pertinent to waste form integrity, there has long been a focus on determining radiation sensitivity in environmentally important species. For example, the extreme radiation resistance of \textit{Deinococcus radiodurans}, through to the sensitivity of subsurface bacterial species such as Fe(III)-reducing \textit{Shewanella} sp. has been assessed (Daly et al., 2004; Ghosal et al., 2005). However, many of these studies were conducted with pure cultures at high acute doses, and whilst acute dose laboratory studies may predict canister vicinities to be sterile (Stroes-Gascoyne et al., 1994; Lucht and Stroes-Gascoyne, 1996), survival may actually be possible under dose rates more relevant to nuclear environments. For example, under a chronic dose rate of \(~2\) Gy h\(^{-1}\), microorganisms isolated from a spent nuclear fuel pond were
capable of surviving total absorbed doses five times greater than tolerated in acute
dose experiments (>426 Gy h\(^{-1}\)) (Bruhn et al., 1999; Bruhn et al., 2009). Furthermore, microbes from the indigenous endolithic community of a proposed repository were capable of surviving low gamma doses in a viable but non-culturable state (Pitonzo et al., 1999a), such that resuscitation may be possible when environmental conditions become more favourable (Pitonzo et al., 1999b). This highlights the importance of gathering low dose rate data, particularly as lower dose rates may allow species to respond via up-regulating repair mechanisms (Qiu et al., 2006) or even adapting over geological timescales, relevant to radwaste disposal scenarios. Similarly, the survival data from pure culture studies are perhaps not applicable to relatively nutrient limited sediments where there is competition from different species of the community and where radiation is perhaps not the only selective stress. Indeed, Bruhn et al. (2009) showed that the survival of the usually radioresistant \textit{D. radiodurans} in a mixed culture was somewhat limited, probably as a result of competition with \textit{Pseudomonas} sp. However, this study was conducted in a rich Tryptic Soy Broth medium that is far from representative of \textit{in situ} GDF conditions.

Whilst it is important to examine the radiation tolerance of microbial community members, radiation may also impact upon the extracellular environment, which may consequently influence the capacity for microbial processes. For instance, the radiolysis of water generates molecular hydrogen which may be used as an electron donor for a range of microbial electron accepting processes (Pedersen, 1997; Lloyd, 2003; Galès et al., 2004; Lin et al., 2005). Furthermore, radiation has been shown to break down natural organic matter in soils (Bank et al., 2008) and dissolved organic carbon (DOC) has been observed to increase (Schaller et al., 2011). This radiolytic degradation of organic matter may enhance the bioavailability of organic carbon for microbial metabolism.

The oxidation state of potential electron acceptors may also be altered by ionizing radiation. For instance, irradiation led to Fe(III)-reduction in a range of materials, including clays and goethite (Ladriere, 1998; Gournis et al., 2000; Plotze et al., 2003; Bank et al., 2008). On the other hand, irradiation induced oxidation of Fe in steel and aqueous Fe(II) solutions led to the generation of the Fe(II)/(III) oxides
lepidocrocite, maghemite and magnetite (Daub et al., 2011; Yakabuskie et al., 2011). Such changes to the oxidation state of Fe may have important implications for the bioavailability of Fe(III) for microbial respiration.

Furthermore, even when no radiation induced oxidation/reduction is observed, Fe(III) in both ferrihydrite and hematite may be made more available for microbial reduction via alteration to the crystalline structure (Brown et al., unpublished; Chapter 5). As Fe is likely to be a significant component of waste packaging and repository infrastructure, such radiation effects could have important implications to deep subsurface microbial communities. With regards to other electron acceptors, many studies have observed a decrease in the concentration of nitrate in irradiated soils (McNamara et al., 2003). On the other hand, sulphate concentrations increased in a soil by 17% after 30 kGy gamma-irradiation, albeit this was attributed to releases from lysed cells (Marschner, 1993).

It is, therefore, evident that radiation may impact upon both cellular physiology and availability of growth substrates; i.e. electron donors, acceptors and presumably nutrients (McNamara et al., 2003). However, despite the potential consequences to the evolution of biogeochemical processes in nuclear environments, there is a lack of information on the combined effects of all these processes, and the resultant impact on microbial metabolism at low dose rates. Here, we address the impact of low-dose chronic gamma-irradiation upon a sediment microbial community and the biogeochemical processes controlled by this community both during irradiation and throughout a subsequent recovery stage. In addition, Fe(II) concentrations were probed to assess the ability of an irradiated community to carry out Fe(III) reduction. To the authors’ knowledge, this has been conducted using the lowest dose rate over the longest irradiation period of any comparable study to date. Two dose rates were employed: 30 Gy h\(^{-1}\) is representative of dose rates at radwaste canister surfaces and 0.5 Gy h\(^{-1}\) was used to simulate dose rates further afield, or indeed, after radiation levels have decayed and the repository vicinity repopulated. This is in sharp contrast to the acute levels used in other pure culture studies.
6.3. Methods

6.3.1. Sediment collection

Sediment samples were taken from a location representative of the Quaternary, unconsolidated alluvial flood-plain deposits which underlie the UK Sellafield reprocessing site, called Sellafield sediment herein. The sediments largely comprise coarse grained sands and gravels with a clay component and a comprehensive description of the chemical composition is given in Law et al. (2010). These deposits are laterally continuous toward the Irish Sea and are underlain by the Triassic Sherwood Sandstone at a depth of 10 to 40 m beneath the Sellafield site (Nirex, 1997). These superficial deposits act as a minor aquifer and the regional hydrogeological model supposes groundwater recharge in the Cumbrian Fells to the east with a south-westerly flow and discharge occurring in the adjacent river, beach and Irish Sea. Thus, these sediments represent the strata in which plumes of radionuclide contaminated groundwater from historical leaks inside the Sellafield site occur (McKenzie and Armstrong-Pope, 2010).

Sediment samples were collected from the shallow sub-surface at a locality ~2 km from the Sellafield site, in the Calder Valley, Cumbria (Law et al., 2010; Thorpe et al., 2012a; Thorpe et al., 2012b). Samples were transferred into sterile containers, sealed, and stored in the dark at 4 °C prior to use.

6.3.2. Sediment microcosms

To assess the impact of gamma radiation on the indigenous microorganisms of the Sellafield sediment, microcosms were prepared in sterile serum bottles by the addition of a sterile synthetic groundwater representative of the Sellafield region (Wilkins et al., 2007) to samples of Sellafield sediment (10 ± 0.1 g Sellafield sediment; 100 ± 1 mL pH 7 buffered groundwater). After buffering, the final pH of microcosms was approximately 6.4. Sodium lactate (7 mM) and sodium acetate (7mM) were added as electron donors where necessary to give a range of systems as shown in Table 1. Triplicate microcosms were then sealed with butyl rubber stoppers prior to irradiation.

Microcosms containing no added electron donor were also irradiated prior to addition of a Geobacter sulfurreducens culture to investigate the effect of radiation
on Fe(III)-reduction in the Sellafield sediments, whilst controlling the impact of radiation toxicity on the indigenous microorganisms. Three needles (1.2 mm Ø) were passed through the butyl rubber bottle stoppers to prevent overpressurisation by gas production during irradiation. After irradiation, needles were removed from Sellafield sediment microcosms, re-sealed and purged with an N₂-CO₂ (80:20) gas mix to drive the sediments anoxic to support microbial Fe(III) reduction. Slurries of *G. sulfurreducens* (100 µL) were added, where necessary, to give a final optical density (absorbance at 600 nm) of 0.04, approximately $1 \times 10^7$ cells mL⁻¹. Cultures were initially prepared by growing *G. sulfurreducens* at 30 °C in a fully defined anaerobic medium, as described previously (Lloyd et al., 2003). Sodium acetate (20 mM) and fumarate (40 mM) were added as electron donor and acceptor, respectively. After 24 h, late log/early stationary phase cultures were harvested anaerobically by centrifugation at 4920g for 20 min under N₂-CO₂ (80:20) and washed twice with sterile nitrogen purged 30 mM sodium bicarbonate (pH 7.2).

**Table 1.** Initial microcosm compositions and treatments.

<table>
<thead>
<tr>
<th>System</th>
<th>Dose rate (Gy h⁻¹)</th>
<th>Total absorbed dose (kGy)</th>
<th>Added lactate (mM)</th>
<th>Added acetate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sellafield sediment + electron donor</td>
<td>Non-irradiated</td>
<td>0</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0.5 ± 10%</td>
<td>0.6 ± 10%</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>30 ± 10%</td>
<td>38.6 ± 10%</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Sellafield sediment</td>
<td>Non-irradiated</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5 ± 10%</td>
<td>0.6 ± 10%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>30 ± 10%</td>
<td>38.6 ± 10%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sellafield sediment + <em>G. sulfurreducens</em></td>
<td>Non-irradiated (no added <em>G. sulf.</em>)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Non-irradiated</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5 ± 10%</td>
<td>0.6 ± 10%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>30 ± 10%</td>
<td>38.6 ± 10%</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**6.3.3. Irradiations**

Microcosm irradiations were carried out at Cell 5, AMEC, Harwell, UK. Co-60 gamma (1.25 MeV) was supplied to two separate sets of microcosms in the dark at dose rates of 0.5 Gy h⁻¹ ± 10% and 30 Gy h⁻¹ ± 10% over a 56 day period. Total absorbed doses are shown in Table 1 and where samples were taken after 28 days,
the total absorbed dose was 19.2 kGy ± 10%. Dose measurements were made with instrumentation traceable to national standards. The temperature inside the cell was 18 ± 1 °C and external control experiments were maintained at the same temperature. After irradiation, all microcosms were returned to the University of Manchester Geomicrobiology laboratory and incubated in the dark at 19 °C.

6.3.4. Geochemical analyses
Experiments were sampled periodically for geochemical analyses and microbial community analysis using aseptic techniques under anoxic conditions. Microbial Fe(III)-reduction was monitored in all microcosms by spectrophotometric determination of Fe(II) using the ferrozine assay (Stookey, 1970). Biogenic Fe(II) was determined by digestion of 100 µL of sediment slurry in 5 mL 0.5 N HCl for 1 h; and total bioavailable Fe was determined by digestion of 100 µL of sediment slurry in 5 mL 0.25 N HCl and 0.25 N hydroxylamine-HCl, followed by the ferrozine assay (Lovley and Phillips, 1986).

Sediment slurry (2 mL) from each replicate was centrifuged at 3000g for 3 minutes to provide separate sediment and porewater samples for microbiological characterisation and ion chromatography, respectively. Samples were stored at -20 °C prior to analysis.

6.3.5. Ion chromatography
Chloride, nitrate, nitrite, sulphate, phosphate and organic acids were measured using a Dionex IC5000 system with a Dionex Capillary AS11-HC 4µ column. 0.4 µL samples were injected into a potassium hydroxide mobile phase with a flow rate of 0.015 mL min⁻¹ and a gradient of 1 mM – 36 mM KOH over 40 minutes.

6.3.6. 16S amplicon pyrosequencing and data analysis
DNA was isolated from microcosm samples (200 µl slurry) using the MoBio PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's instructions. PCR of the V1-V2 hypervariable region of the bacterial 16S rRNA gene was performed using universal bacterial primers 27F (Lane, 1991) and 338R (Hamady et al., 2008), synthesised by IDTdna (Integrated DNA Technologies, BVBA, Leuven, Belgium). As these primers are specific to
bacterial DNA, no archaeal or eukaryotic DNA sequences were amplified or sequenced.

The fusion forward primer (5′-CCATCTCATCCCTGCGTGTCTCC GACTCAGXXXXXXAGAGTTTGATGTMGGCTCAG-3′) contained the 454 Life Sciences “Lib-L Primer A”, a 4 base “key” sequence (TCAG), a unique eight-base barcode “MID” sequence for each sample (XXXXXXX), and bacterial primer 27F. The reverse fusion primer (5′-CCTATCCCCTGTGCTCAGTCTCAGTGCTGCCTCCGGAGT-3′) contained the 454 Life Sciences “Lib-L Primer B”, a 4 base “key” sequence (TCAG), and bacterial primer 338R. The PCR amplification was performed in 50 μl volume reactions using 0.5 μl (2.5 units) Fast Start High Fidelity DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), 1.8 mM MgCl₂, 200 μM of each dNTP, 0.4 μM of each forward and reverse fusion primers, and 2 μL of DNA template. The PCR conditions included an initial denaturing step at 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, and a final elongation step at 72°C for 5 min.

PCR products were loaded in an agarose gel, and following gel electrophoresis, bands of the correct fragment size (approximately 410 bp) were excised, cleaned up using a QIAquick gel extraction kit (QIAGEN, GmbH, Hilden, Germany), and eluted in 30 μl of DNAse free H₂O. The cleaned up PCR products from this study (22 samples in total) were quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA), and pooled so that the mixture contained equal amounts of DNA from each sample. The emulsion emPCR and the pyrosequencing run were performed at the University of Manchester sequencing facility, using a 454 Life Sciences GS Junior system (Roche).

The 454 pyrosequencing reads were analysed using Qiime 1.6.0 release (Caporaso et al., 2010). Low quality reads (mean quality score less than 25) and short sequences (less than 300 bp) were discarded, and both forward and reverse primers were removed from further analysis. De-noising and chimera removal was performed during OTU picking (at 97% sequence similarity) with usearch (Edgar, 2010) in Qiime, and a representative sequence for each OTU was identified. Taxonomic
classification of all reads was performed in Qiime using the Ribosomal Database Project (RDP) at 80% confidence threshold (Edgar, 2010), while the closest GenBank match for the OTUs that contained the highest number of reads (the representative sequence for each OTU was used) was identified by Blastn nucleotide search.

6.4. Results and Discussion

6.4.1. Biogeochemistry of irradiated microcosms containing added electron donor

To assess the impact of chronic gamma irradiation on the biogeochemical processes in the Sellafield sediment, a range of microcosms were prepared with and without added electron donor and irradiated for 56 days at 0.5 and 30 Gy h$^{-1}$. In control and irradiated microcosms spiked with 7 mM lactate and acetate, electron acceptor usage progressed in the order nitrate > Fe(III) > sulphate during the irradiation period (Figure 1). Treatment with 30 Gy h$^{-1}$ gamma radiation did not appear to affect the reduction of nitrate, which was removed completely from porewaters after 28 days in both treated and control microcosms. However, 0.5 N HCl extractable Fe(II) concentrations in microcosms after treatment with 30 Gy h$^{-1}$ for 56 days were ~0.5 mM compared to ~2 mM in non-irradiated controls, suggesting that Fe(III)-reduction was inhibited in the 30 Gy h$^{-1}$ irradiated systems. After the irradiation period had finished, 0.5 N HCl extractable Fe(II) increased gradually to ~1.2 mM in these microcosms, suggesting that Fe(III)-reduction was possible, even after the maximum radiation dose applied. However, 0.5 N HCl extractable Fe(II) concentrations did not recover to the levels in non-irradiated microcosms over the 119 day recovery period that Fe(II) was monitored. This observation may be a result of radiation induced changes in the Fe(III) mineralogy, for example, the abiotic reduction of Fe(III) in goethite by radiation has been observed previously in gamma irradiated sediments (Bank et al., 2008). Such processes could limit the total amount of Fe(III) available for microbial reduction. On the other hand, it is perhaps more likely that this result is a product of decreased viability of Fe(III)-reducing microorganisms arising from a total absorbed dose of 38.6 kGy. Indeed, Fe(III) reduction continued throughout the recovery period, albeit at a slower rate.
Sulphate concentrations in the porewaters of the non-irradiated microcosms decreased after 29 days, consistent with the onset of microbial sulphate reduction. However, complete removal from solution was not observed and no further reduction occurred after 105 days. Conversely, in systems irradiated with 30 Gy h\(^{-1}\), sulphate concentrations doubled during the 56 day irradiation period from \(\sim0.35\) mM to \(\sim0.7\) mM, with the majority of this increase occurring during the first 28 days of irradiation. Marschner (1993) also reported sulphate increases of 17% after 30 kGy gamma irradiations due to release from dead microbial biomass. On the other hand, Ishii et al. (2011) suggested that for a rice paddy sediment irradiated with 1 Gy day\(^{-1}\), increases may be a result of radiation induced activation of mineralization processes. In addition, sulphate may be generated via the oxidation of sulphide bearing minerals by radiolytically produced oxidants, such as hydrogen peroxide (Lin et al., 2006; Leticariu et al., 2010).

Sulphate was removed from solution between days 57 and 105, suggesting that microbial sulphate reduction occurred despite treatment with a total dose of 38.6 kGy. However, after a 90 day recovery period, sulphate reduction did not recover to the same level as noted in control systems, indicating that although radiation increased the concentration of dissolved sulphate, its usage as an electron acceptor was not enhanced during the incubation period.

Treatment with 0.5 Gy h\(^{-1}\) gamma radiation did not have a dramatic impact on the amount of nitrate, Fe(III) or sulphate reduction noted (Figure 1). Indeed, the reduction of nitrate and Fe(III) occurred during the 58 day irradiation period alongside that of control systems. These data suggest that irradiation at this lower dose rate did not have a significant effect on the microbial communities which control electron acceptor turnover.
Figure 1. Concentrations of nitrate, 0.5 N HCl extractable Fe(II) and sulphate in microcosms containing added lactate (7 mM) and acetate (7 mM). Dashed line indicates removal of microcosms from the irradiation cell. Error bars represent the standard error of the mean of triplicate measurements and where not visible, error bars are within the symbol size.

Lactate concentrations in all spiked systems decreased throughout the irradiation period, resulting in the complete removal from solution after 56 days in irradiated systems and 28 days in control microcosms (Figure 2). This suggests lactate was likely used as a carbon source or electron donor for the electron accepting processes described earlier. Lactate removal was not as rapid in 30 Gy h\(^{-1}\) treated systems and this may be related to a reduction in microbial activity associated with radiation toxicity at this higher dose rate.
Acetate concentrations did not change significantly in all systems during the irradiation period, however, it was completely removed from solution in control systems and 0.5 Gy h\(^{-1}\) treated microcosms after the 48 day recovery period. This is consistent with the use of acetate as an electron donor, as observed in previous studies with this sediment type (Thorpe et al., 2012a), albeit after more thermodynamically favourable processes had consumed other electron donors, such as lactate. However, this was not observed in microcosms treated with 30 Gy h\(^{-1}\), perhaps suggesting a loss of viability in microbial populations at this higher level of radiation.

Propionate appeared in both control systems and systems irradiated with 0.5 Gy h\(^{-1}\) and increased throughout the irradiation period to a concentration of ~3.5 mM. After 56 days, when experiments were removed from the radiation source, propionate concentrations decreased throughout the recovery period at approximately the same rate as the control experiments, resulting in the almost complete removal from solution by the end of the monitoring period. Propionate was not detected in 30 Gy h\(^{-1}\) treated systems at any sampling point. The appearance and removal of propionate is consistent with its production via fermentation of lactate (Seeliger et al., 2002) and subsequent use as an electron donor.
**Figure 2.** Concentrations of lactate, acetate, propionate and formate in microcosms containing added lactate (7 mM) and acetate (7 mM). Malate equivalent represents the concentration of an organic acid which elutes with a very similar retention time to malate on the ion chromatography system used in these experiments. The exact identity of the molecule remains undetermined and the quantities are therefore calculated using the molar mass of malate. The dashed line indicates removal of microcosms from the irradiation cell. Error bars represent the standard error of the mean of triplicate measurements and where not visible, error bars are within the symbol size.
A slight increase in formate concentrations to ~50 µM was observed in non-irradiated microcosms and 0.5 Gy h⁻¹ treatments during the recovery period after 147 days. In 30 Gy h⁻¹ treatments, on the other hand, formate appeared during the latter half of irradiation to a concentration of ~120 µM. In addition to formate, a large increase in an unknown molecule was also observed in the 30 Gy h⁻¹ treated microcosms. The exact identity of the molecule is undetermined due to difficulties in separation of the following anions which have similar retention times on the ion chromatography system used for these analyses: glutarate; succinate; malate; malonate; maleate. The quantities of this anion were calculated using the molar mass of malate and concentrations are therefore expressed as malate equivalents. The analyte was only observed in 30 Gy h⁻¹ treated systems, with a significant increase during the recovery period to ~12 mM. Whilst DOC has previously been observed to increase as a result of sediment gamma irradiation (Schaller et al., 2011), the significant production of this analyte during the recovery period and formate during the latter half of the irradiation period, suggests they are likely fermentation products. Furthermore, their subsequent removal from solution is consistent with their use as electron donors.

6.4.2. Microbial ecology of Sellafield sediments

Analysis of the microbial ecology of the oxic starting sediment revealed a relatively diverse community with 16 phyla detected through pyrosequencing of 16S rRNA gene amplicons. However, communities were dominated by species representing the Acidobacteria (47%) and Proteobacteria (32%), consistent with previous studies conducted on Sellafield-type sediments (Law et al., 2010; Thorpe et al., 2012a). Of the most dominant individual species, an uncharacterized Acidobacteria sp. and a bacterium of the Bradyrhizobiaceae family (Proteobacteria) represented 5% and 4% of the complex microbial community, respectively.

6.4.3. Microbial ecology of Sellafield sediments with added electron donor

After 147 days, the microbial community of non-irradiated sediment microcosms containing added lactate and acetate showed a decrease in the contributions of Acidobacteria (21%) and Proteobacteria (17%) (Figure 3). However, the most marked shift was an increase in organisms affiliated with the Bacteroidetes (29% of
the community) and Firmicutes (22%; of which, 97% were affiliated with Clostridia). Of the Bacteroidetes, these included uncultured Prolixibacter sp. (7% of the total microbial community), two uncultured Bacteroidetes bacteria (4% and 3%) and an organism affiliated with Paludibacter propionicigenes (2%). The Prolixibacter genus comprises facultative anaerobes capable of sugar fermentation (Holmes et al., 2007), with P. propionicigenes an anaerobic propionate producing strain which can utilize a range of sugars to produce acetate and propionate as major fermentation products (Imachi et al., 2006). In addition, organisms affiliated with the Clostridial group (Firmicutes) catalyse a mixed acid fermentation under anoxic conditions (Moat et al., 2002). Thus, the relative increase in Clostridia and Paludibacter species is likely related to the significant production of propionate observed during the first 56 days (Figure 2) (Seeliger et al., 2002). Furthermore, Clostridia, such as Pelotomaculum spp. (10% of the community), includes species capable of oxidizing propionate (Imachi et al., 2007). The increase in such species may be related to the decrease in propionate observed after 56 days (Figure 2).

**Figure 3.** Microbial community analysis of microcosms containing added lactate (7 mM) and acetate (7 mM).
Species of the known Fe(III)-reducing genus *Geobacter* showed a slight increase to represent 1% of the community in control systems. This is likely a result of the increase in Fe(III)-reduction observed during the first 56 days (Figure 1). Although Fe(III)-reduction is clearly a key electron-accepting process in these sediments, *Geobacter* spp. or other known Fe(III)-reducing bacteria are not dominant components of this community, probably due to the domination of fermentative processes as a result of the addition of significant organic carbon concentrations.

Similar community shifts were also observed in 0.5 Gy h\(^{-1}\) treatments. An organism affiliated with the Bradyrhizobiaceae was also represented at a similar proportion of the community as in non-irradiated controls (4%). In contrast to control systems, bacteria of the phylum Firmicutes were not as well represented in the 0.5 Gy h\(^{-1}\) treatment (13%). However, an organism closely related to a member of the genus *Pelotomaculum* (97% match) was again the main representative of this class (3%) and this may be related to the similar levels of propionate observed in these two treatments. The Proteobacteria appeared slightly enriched in this treatment (24%) compared to the control sample (17%), with *Geobacter* spp. comprising 8% of this group. Thus, significant Fe(III)-reduction by this genus was likely more important in these systems. A Betaproteobacterium closely related to species of the genus *Janthinobacterium* also represented a significant proportion of the community at 4%.

Species of this genus were well represented in a previous study using similar sediments containing added nitrate, and it was suggested that Betaproteobacteria such as this may be involved in the reduction of nitrate (Geissler et al., 2011). Thus, the appearance of this genus in sediments treated with 0.5 Gy h\(^{-1}\) may be related to nitrate reduction observed early on in the irradiation period.

In the 30 Gy h\(^{-1}\) treatment, after 147 days there was a marked loss of diversity with a strong shift toward species of the Firmicutes phylum (91%) (Figure 3). Two close relatives to known Clostridial species were the main components of this phylum. The first, an uncultured Clostridiacea bacterium, represented 83% of the total community. This species is most closely related to an organism isolated from a sulphate-reducing enrichment of sediments from an acid mine lake (95% match) (Meier et al., 2012). The second, an organism most closely related to a novel *Clostridium bowmanii* species (98% match) originally isolated from a microbial mat
in the McMurdo Dry Valley region of Antarctica (Spring et al., 2003), represented 8% of the total community. Members of the Clostridial family catalyse a mixed acid fermentation, with *C. bowmanii* capable of generating butyrate; acetate; formate; ethanol and lactate (Spring et al., 2003). As such, it is possible that these species may be involved in fermentation processes, including acetate and formate production, observed throughout the incubation period (Figure 2). In addition, most of the species within this family are able to form endospores, which may allow cells to survive a range of environmental stresses (Wiegel et al., 2006). As such, it is likely that both these species represent radioresistant members of the Sellafield sediment community. Thus, in environments with significant radiation fluxes, in conjunction with available fermentable substrates such as lactate, species from the Clostridiaceae family could predominate.

6.4.4. **Biogeochemistry in microcosms containing no added electron donor**

In addition to systems containing added carbon, the impact of radiation on sediment biogeochemistry and microbial communities was also assessed with microcosms containing no added electron donor (lactate and acetate). Radiation had no significant effect on the generation or reduction of sulphate. However, after irradiation, significant Fe(III)-reduction was observed in microcosms treated with 0.5 Gy h\(^{-1}\) gamma radiation, whereas Fe(III)-reduction in control and 30 Gy h\(^{-1}\) treated microcosms was not observed until day 105. Indeed, Fe(III)-reduction in the 0.5 Gy h\(^{-1}\) treated systems continued throughout the incubation period at an enhanced rate. No increase in 0.5 N HCl extractable Fe(II) was observed in control or irradiated microcosms during the 56 day irradiation period as the absence of added electron donor likely precluded microbial Fe(III)-reduction during this initial period.

In contrast to systems containing added lactate and acetate, nitrate concentrations in systems treated with 30 Gy h\(^{-1}\) after 28 days were slightly lower (~70 µM) than in non-irradiated microcosms (~120 µM) (Figure 4). General removal of nitrate in both systems is likely related to the activity of denitrifying bacteria. However, the increased removal in 30 Gy h\(^{-1}\) treated systems is consistent with the abiotic removal of nitrate in previous studies of gamma sterilisation of sediments (McNamara et al., 2003). The reasons for this are unclear; however, radiolysis studies have shown that
the abiotic decomposition of nitrate is possible, leading to nitrite and oxygen formation (Daniels and Wigg, 1969). It is not possible to say whether this process also occurred in 0.5 Gy h\(^{-1}\) treatments as nitrate measurements were not taken throughout the irradiation of these systems. However, these results suggest that radiolysis of nitrate may promote the removal of nitrate in irradiated sediments. In turn, this may have resulted in the early onset of Fe(III)-reduction observed in 0.5 Gy h\(^{-1}\) treated microcosms, due to a decreased competition for the alternative electron acceptor. It is not clear why this enhanced Fe(III)-reduction was not observed in 30 Gy h\(^{-1}\) treated systems; however, this may be precluded by increased radiation toxicity associated with a higher dose.

**Figure 4.** Concentrations of nitrate, 0.5 N HCl extractable Fe(II) and sulphate in microcosms containing no added electron donor. The dashed line indicates the removal of the microcosms from the irradiation cell. Error bars represent the standard error of the mean of triplicate measurements and where not visible, error bars are within the symbol size.
Figure 5. Concentrations of lactate, acetate and formate in microcosms containing no added electron donor. The dashed line indicates the removal of microcosms from the irradiation cell. Error bars represent the standard error of the mean of triplicate measurements and where not visible, error bars are within the symbol size.

Formate was generated in all treatments and controls (~50 µM) throughout the recovery period and is likely a product of fermentation (Figure 5). Acetate, on the other hand, was not observed in control systems or in 0.5 Gy h⁻¹ treatments; however, ~0.2 mM acetate was produced in 30 Gy h⁻¹ treated microcosms during the latter half of irradiation. This continued throughout the recovery period until 105 days, and by the end of the incubation period, acetate had largely been removed from solution. The production of acetate during the latter part of irradiation only and its subsequent removal suggests its production by microbial fermentation, followed by its oxidation as an electron donor. It is possible that these processes are occurring in the non-irradiated systems and the 0.5 Gy h⁻¹ treated systems, however, acetate may be metabolised as quickly as it is created. Thus, the detection of acetate in the 30 Gy h⁻¹ treated systems may be a result of radiation toxicity in acetate-oxidizing species.
It is unclear whether the enhanced reduction of Fe(III) in 0.5 Gy h\(^{-1}\) treated systems is related to an increase in the availability of organic electron donors, increases in the bioavailability of Fe(III), or a decrease in electron acceptor competition arising from enhanced nitrate removal.

6.4.5. **Microbial ecology of Sellafield sediments with no added electron donor**

To assess potential changes to the microbial community which may have lead to the enhanced Fe(III)-reduction observed in 0.5 Gy h\(^{-1}\) treated systems, community analysis was conducted on samples taken immediately after irradiation (T = 57; Figure 6). Both the non-irradiated and 0.5 Gy h\(^{-1}\) treated system showed slight enrichment of Proteobacteria species, including a representative of the Bradyrhizobiaceae (Alphaproteobacteria) (6% in controls and 8% in 0.5 Gy h\(^{-1}\) treatments). Controls were also enriched with a relative of a known *Janthinobacterium* sp. (Betaproteobacterium) (5%), although this was not observed in treated systems. However, 0.5 Gy h\(^{-1}\) treated microcosms did show a slight increase in an organism affiliated with *Rhodoferax* spp. (99% sequence similarity) (2% in treated versus <1% in controls). The closest known relative was originally identified in Arctic glacier melt water and has 98% sequence similarity to a *Rhodoferax ferrireducens* strain which exhibits dissimilatory Fe(III)-reduction (Finneran et al., 2003; Vardhan Reddy et al., 2009). This would be consistent with the enhanced Fe(III)-reduction observed in the 0.5 Gy h\(^{-1}\) treated system.

As with electron donor spiked systems, community analysis after 147 days of incubation of the non-irradiated system containing no added carbon revealed a relative increase in the Bacteroidetes (9%) and Firmicutes (3%) phyla (Figure 6). As with electron donor spiked systems, the Bacteroidetes phylum was strongly represented by *Prolixibacter* related species (6%). A significant increase in a relative of *Geothrix fermentans* (Acidobacteria) was also observed (from <1% in the T = 57 sample to 5% in the T = 147 sample) and, as a known Fe(III) reducing species (Coates et al., 1999), this increase is likely related to the Fe(III)-reduction observed in this system after 105 days.
Figure 6. Microbial community analysis of microcosms with no added electron donor. Microcosms were removed from the irradiation cell at T = 56.

Community analysis of the T = 147 0.5 Gy h⁻¹ treatment displayed a further relative increase in representatives of the Bacteroidetes (19%) and Firmicutes (7%) phyla. Unclassified species of the Bacteroidales order showed a significant increase, representing 17% of the total microbial community, with respect to the control sample (6%). Uncultured Prolixibacter spp. also showed an increase with respect to control samples and were well represented in this treatment at 10% of the total community. The increase in representatives of the Firmicutes phylum mainly arose from a general increase in Clostridia species, which may indicate an increase in fermentation activity or is perhaps related to spore formation and enhanced survival.

In addition, an organism most closely related to Geothrix fermentans (Acidobacteria) (~98% sequence similarity) showed a significant increase with respect to the control sample, representing 22% of the total microbial community. Geobacter spp. were increasingly represented with respect to controls, with an organism most closely related to G. chapellei comprising 3% of the community, compared to the most populous in control samples: a G. bremensis relative (0.2% of the total community).
This increase in *Geothrix* and *Geobacter* spp. reflects the enhanced level of Fe(III)-reduction in the 0.5 Gy h\(^{-1}\) treatment.

In the 30 Gy h\(^{-1}\) treatment, further relative increases were observed in the Bacteroidetes, Firmicutes phyla (Figure 6). Unclassified species from the order Bacteroidales represented 37% of the total microbial community. This comprised two dominant species, the first (12% of the community) was most closely related to an uncultured bacterium isolated from moss pillars at an Antarctic lake (Nakai et al., 2012) and the second, an uncultured *Prolixibacter* sp. (11%). *Paludibacter* spp., also of the order Bacteroidales, represented 5% of the total community and species of the family Chitinophagaceae (Bacteroidetes phylum) comprised 7% of the total community. Two uncultured Sphingobacteria (Bacteroidetes phylum) represented 14% of the community.

Of the key Fe(III)-reducing species in the 30 Gy h\(^{-1}\) treatment, 18% of the total community was affiliated with known *Geobacter* species. However, unlike in the 0.5 Gy h\(^{-1}\) treatments, *Geothrix* species were not well represented, comprising <0.1% of the total microbial community. In addition, a close relative of the *Herbaspirillum frisingense* (Betaproteobacteria) comprised 5% of the total community (98% sequence similarity). This species is capable of nitrate reduction and nitrogen fixation and can oxidise a broad range of sugars and alcohols (Kirchhof et al., 2001). As with the 0.5 Gy h\(^{-1}\) treatment, the increase in the Firmicutes phylum mainly arose from a general increase in Clostridial species.

These results indicate that despite sediments receiving a total absorbed dose of nearly 40 kGy, Fe(III)-reduction was still possible in sediments without added electron donor. Furthermore, irradiation of these sediments resulted in significant increases in Fe(III)-reducing species compared to non-irradiated systems. This may suggest that, although Fe(III)-reduction was not enhanced in the 30 Gy h\(^{-1}\) treated systems, these sediments may be poised for Fe(III)-reduction.
6.4.6. Fe(III)-reduction in irradiated microcosms inoculated with *G. sulfurreducens*

To assess the potential for enhanced Fe(III)-reduction in the 30 Gy h\(^{-1}\) treated systems, irradiated and control microcosms were inoculated with cultures of *G. sulfurreducens* and 0.5 N HCl extractable Fe(II) was monitored (Figure 7). Both 0.5 and 30 Gy h\(^{-1}\) treated microcosms showed enhanced Fe(III)-reduction with respect to control systems, 21 days after inoculation. Fe(III)-reduction was observed in inoculated non-irradiated microcosms after 35 days and Fe(II) concentrations did approach those of irradiated systems after 92 days, albeit at a slower rate. These data indicate that, like 0.5 Gy h\(^{-1}\) treated systems, there is also potential for enhanced Fe(III)-reduction in 30 Gy h\(^{-1}\) treated systems (Figure 4); however, reduced viability of Fe(III)-reducing species in 30 Gy h\(^{-1}\) treatments likely precludes this.

**Figure 7.** 0.5 N HCl extractable Fe(II) concentrations in control and irradiated microcosms inoculated with *G. sulfurreducens*. Microcosms were removed from the irradiation cell and inoculated at T = 0. Error bars represent the standard error of the mean of triplicate measurements and where not visible, error bars are within the symbol size.
Radiation has previously been shown to release significant quantities of DOC into solution in a range of soils exposed to 25 kGy to 60 kGy (Lynch, 1982; Marschner and Bredow, 2002; Schaller et al., 2011). This could potentially increase the availability of carbon for use as a carbon source or electron donor. However, whilst irradiation at 30 Gy h$^{-1}$ did lead to increased organic carbon concentrations (Table 2), such µM increases are likely not sufficient to account for the observed Fe(III)-reduction. Moreover, no significant increases in organic acids were observed in systems treated with 0.5 Gy h$^{-1}$, nor were there significant radiation induced increases during the irradiation period of the non-inoculated microcosms.

Previous experiments have indicated that gamma radiation may lead to an increase in the availability of Fe(III)-oxides for microbial Fe(III)-reduction (Brown et al., unpublished). It may be possible that the enhanced Fe(III)-reduction observed here may be related to this phenomenon. Whilst the previous study observed this effect after acute irradiation to 1 MGy, our results may suggest that a similar, more subtle process may also occur at lower doses.

On the other hand, the enhanced Fe(III)-reduction may also be related to the removal of nitrate by radiolysis as for the irradiation of sediments containing no added G. sulfurreducens or electron donors. Nitrate concentrations in irradiated microcosms (0.13 mM in 30 Gy h$^{-1}$ treatments and ~0.3 mM in 0.5 Gy h$^{-1}$ treatments) were significantly lower immediately after irradiation than in non-irradiated systems (~0.5 mM). Again, these results are consistent with radiation enhanced removal of nitrate and the early onset of Fe(III)-reduction, as discussed previously.
Table 2. Concentrations of bioavailable Fe, inorganic anions and organic acid salts in Sellafield sediment microcosms immediately after irradiation and prior to addition of *G. sulfurreducens*. Errors indicate the standard error of the mean of triplicate measurements.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bioavailable Fe mM</th>
<th>NO$_3$\textsuperscript{-} mM</th>
<th>NO$_2$\textsuperscript{-} mM</th>
<th>SO$_4$\textsuperscript{2-} mM</th>
<th>Lactate (\mu\text{M})</th>
<th>Acetate (\mu\text{M})</th>
<th>Propionate (\mu\text{M})</th>
<th>Butyrate (\mu\text{M})</th>
<th>Formate (\mu\text{M})</th>
<th>Fumarate\textsuperscript{a} (\mu\text{M})</th>
<th>Oxalate\textsuperscript{a} (\mu\text{M})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-irradiated</td>
<td>0.85 ± 0.02</td>
<td>0.73 ± 0.05</td>
<td>n.d.</td>
<td>0.60 ± 0.02</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>0.5 Gy h\textsuperscript{-1} + <em>G. sulf.</em></td>
<td>0.74 ± 0.02</td>
<td>0.53 ± 0.02</td>
<td>n.d.</td>
<td>0.40 ± 0.01</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>30 Gy h\textsuperscript{-1} + <em>G. sulf.</em></td>
<td>0.81 ± 0.03</td>
<td>0.13 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.42 ± 0.01</td>
<td>3.5 ± 3.0</td>
<td>57.2 ± 70.0</td>
<td>3.9 ± 0.4</td>
<td>1.1 ± 0.9</td>
<td>32.7 ± 18.1</td>
<td>45.5 ± 13.7</td>
<td>59.5 ± 17.9</td>
</tr>
</tbody>
</table>

n.d. = not detected at 50x dilution.

\* Fumarate and oxalate both have a very similar retention time on the chromatography system used in these experiments. Concentrations have been calculated for each based upon their respective molecular mass, though it is not possible to ascertain which molecule is responsible for the quantities detected.
6.4.7. **Implications to the geodisposal of nuclear waste**

This study highlights the impact of gamma radiation on the biogeochemistry and microbial ecology of Sellafield-type sediments. Dose rates representative of nuclear waste canister surfaces in the near field of a geological disposal facility were used, whilst a recovery period simulated conditions after significant radioactive decay has occurred.

Previous studies have suggested that microbial activity will be suppressed in these environments. For instance, studies of survival of microorganisms from clay buffer material suggest that typically only 10% of the population survives after ~1.6 kGy (Stroes-Gascoyne et al., 1994) and that dose rate may not have a significant impact on the viability of microbial populations (Lucht and Stroes-Gascoyne, 1996). On the other hand, irradiation of an endolithic community associated with a proposed high-level nuclear waste repository indicated that indigenous microbes were capable of surviving, but in a non-culturable state, which may allow resuscitation when environmental conditions become favourable (Pitonzo et al., 1999a; Pitonzo et al., 1999b).

In contrast, the results presented here indicate that a sediment community can survive long-term gamma irradiation and individuals remain active in biogeochemical processes, particularly Fe(III)-reduction. We have shown this to be the case for doses of up to ~38 kGy using a lower, environmentally relevant dose rate of 30 Gy h⁻¹. Indeed, dose rate had a strong influence on the community structure in systems with and without added carbon. This demonstrates the importance of acquiring low dose rate data, particularly as lower dose rates may allow species to respond via up-regulating repair mechanisms (Qiu et al., 2006) or indeed adapting over the geological timescales involved.

Radiation led to significant shifts in the microbial communities, with fermentative bacteria, such as Clostridia, dominant in systems with added carbon. Such changes may be important in environments where there is an excess of carbon substrates, such as in cellulosic waste forms (Glaus and Van Loon, 2008). Despite this loss of diversity, these results suggest that Fe(III) reduction will still be an important electron accepting process in such sediments. Furthermore, in environments with
lower electron donor concentrations, an Fe(III)-reducing community may be promoted by radiation. This may occur both directly, by radiation induced changes to the bioavailability of Fe(III); or indirectly, by radiation induced removal of other electron acceptors, such as nitrate, which may lead to the early onset of microbial Fe(III) reduction. Regardless, a relative increase in Fe(III)-reducing species was also observed in irradiated systems which did not display enhanced Fe(III)-reduction. These results have positive implications to the geodisposal of radioactive waste, whereby the stimulation of an Fe(III)-reducing community by radiation may enhance the reduction and subsequent precipitation of radionuclides by direct enzymatic or indirect (e.g. biogenic Fe(II)-mediated) mechanisms. Furthermore, the oxidation of molecular hydrogen by the radiolysis of water coupled to the enhanced reduction of alternative electron acceptors by low dose gamma radiation could provide the basis of a novel ecosystem in the deep biosphere. Further work is required to assess how these altered communities may affect the mobility of key radionuclides.

6.5. Acknowledgements
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6.6. References


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Chapter 7

Research Paper

The morphological and physiological effects of ionizing radiation on the freshwater alga *Haematococcus pluvialis*
7. The morphological and physiological effects of ionizing radiation on the freshwater alga *Haematococcus pluvialis*

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7.1. Abstract

This paper describes the morphological and physiological response of the common freshwater chlorophyte *Haematococcus pluvialis* to ionizing radiation. This algal species has been shown previously to dominate the microbial community in an outdoor spent nuclear fuel pond, in which significant radiation fluxes are present. In order to understand the morphological and physiological response of this algal species to ionizing radiation, cell cultures were treated with periodic doses of X-radiation (total absorbed dose 80 Gy) over a 5-day incubation period. Cell counts initially revealed enhanced growth of irradiated cultures followed by a significant loss of viability after 5 days, whilst light microscopy indicated significant production of the anti-oxidant astaxanthin with increasing dose. However, Fourier transform infrared spectroscopy suggested that radiation did not have a widespread impact on the metabolic fingerprint of *H. pluvialis*, despite receiving doses sufficient to cause cell death. This study suggests that the production of astaxanthin-rich encysted cells may be related to the preservation of the *H. pluvialis* phenotype. *H. pluvialis* may be able to survive in environments with significant radiation fluxes, such as spent
nuclear fuel ponds, via the production of pigments with antioxidant properties which protect against the oxidative stress associated with ionizing radiation.

7.2. Introduction

Ionizing radiation is ubiquitous on Earth, from natural sources, such as cosmic rays and naturally occurring radionuclides, to the anthropogenic sources of nuclear weapons testing and accidental releases, such as the 2011 Fukushima incident. However, of particular concern is radiation from high level waste material from nuclear plant operation and fuel reprocessing (Lloyd, 2005), spent nuclear fuel, and associated contaminated infrastructure and materials of lower and intermediate activity. Such legacy sites and nuclear facilities containing radionuclides have significant ionizing radiation fluxes.

Sporadic reports suggest that microorganisms have the ability to colonise radioactive environments at a number of nuclear facilities (Booth, 1987; Mal'tsev et al., 1996; Stroes-Gascoyne et al., 1997; Santo Domingo et al., 1998; Mironenko et al., 2000; Romanovskaya et al., 2002; Sarró et al., 2003; Chicote et al., 2004; Fredrickson et al., 2004; Chicote et al., 2005; Lloyd and Renshaw, 2005; Nedelkova et al., 2007; Sarró et al., 2007; Evans et al., unpublished). Microbes can have both advantageous and disadvantageous roles in a nuclear establishment. They have the potential to impact on plant operation and the physical structure of stored materials e.g. through promoting microbially-induced corrosion and biofilm formation. Some species also have the ability to take up radionuclides into the cell using biological transport mechanisms, or accumulate radionuclides at the cell wall surface via physico-chemical processes (biosorption), or facilitate sorption to biominerals which are precipitated by microorganisms (Gadd, 1996; Warren and Ferris, 1998; Lloyd and Macaskie, 2000; Lloyd and Lovley, 2001; Gadd, 2010).

The sensitivity of microorganisms to ionizing radiation varies greatly between species and can be further modified by additional environmental factors such as temperature and redox conditions (Gazsó et al., 1997). The bacterium Deinococcus radiodurans is the most radioresistant microorganism known and is capable of surviving single doses of up to 25 kGy (Minton, 1994; Battista, 1997; Daly et al., 2004; Cox and Battista, 2005; Slade and Radman, 2011). Algal cultures, however,
may typically exhibit 50% survival at a dose of between 30 to 6000 Gy (LD<sub>50</sub>) (Farhi, 2008), with some species having a LD<sub>50</sub> of 10 000 Gy (Rivasseau et al., 2013). A more recent study isolated an autotrophic eukaryote from a spent nuclear fuel storage pool, *Coccomyxa actinabirotis* sp. nov., that can withstand and recover from ionizing radiation doses of up to 20,000 Gy (Rivasseau et al., 2013). This microalga was also found to accumulate radionuclides, including <sup>238</sup>U, <sup>137</sup>Cs, <sup>110m</sup>Ag, <sup>60</sup>Co, <sup>54</sup>Mn, <sup>65</sup>Zn, and <sup>14</sup>C and its use for bio-decontamination of radionuclides in a nuclear fuel storage pool was proposed (Rivasseau et al., 2013).

Ionizing radiation, such as alpha, beta and gamma radiation, results in a variety of different chemical effects in biological molecules, such as ionization and oxidation reactions which may impact upon cellular physiology. These effects may arise from both direct energy deposition in biomolecules and the action of radicals and reactive oxygen species (ROS) generated by water radiolysis (Spinks and Woods, 1964; Ghosal et al., 2005; Daly et al., 2007). Recent studies have suggested that proteins are particularly susceptible to oxidation by reactive oxygen species (Du and Gebicki, 2004). Indeed, protein oxidation has been quantifiably related to cellular sensitivity to ionizing radiation (Daly et al., 2007). Such damage to proteins may prevent the repair of single and double strand DNA breaks by enzymes, which may ultimately lead to cell death (Daly, 2012).

In response to the harmful effects of ionizing radiation, many species exhibit a variety of defence and repair mechanisms. These include the increased production of stress proteins, the production of ROS endogenous scavengers (e.g. glutathione, superoxide dismutase (SOD), catalase and peroxidase) and enzymatic mechanisms of repair; nucleotide excision repair and base excision repair, to reduce and reverse oxidative damage (Ghosal et al., 2005). In addition to these ubiquitous defence mechanisms, carotenoid pigment production by microorganisms, such as algae, acts to quench and scavenge ROS, particularly singlet oxygen (¹O₂), hydrogen peroxide and peroxyl radicals (ROO•) (Hirayama et al., 1994; Kobayashi et al., 1997; Tatsuzawa et al., 2000; Stahl and Sies, 2003; Slade and Radman, 2011).

This paper pays particular attention to the ionizing radiation resistance of the carotenoid-producing, freshwater alga *Haematococcus pluvialis*. This organism has
previously been identified as the dominant eukaryotic species in an outdoor spent nuclear fuel storage pond, in Sellafield, Cumbria (Evans et al., unpublished). *H. pluvialis* is usually associated with small ephemeral pools where they are often subjected to high UV radiation fluxes (Vernon, 1957), rather than a large, flushed body of oligotrophic water such as nuclear storage pond. It is hypothesized that tolerance to the high radiation flux and large radionuclide inventory in the spent fuel storage pond at Sellafield nuclear facility could result in selection for this organism. This species has indicated its potential for bioaccumulation of radionuclides, however, very little is known about its ability to survive radiation fluxes typical of a spent nuclear fuel storage pond (Evans et al., unpublished).

*H. pluvialis* is characterised by its production of the red keto-carotenoid pigment astaxanthin (3,3’-dihydroxy-β,β-carotene-4,4’-dione) which belongs to the xanthophyll subclass of carotenoids (Borowitzka et al., 1991; Kobayashi et al., 1991). Astaxanthin is normally present at low concentrations within the cell, but is accumulated rapidly when environmental conditions become unfavourable for normal cell growth (Boussiba and Vonshak, 1991). The astaxanthin molecule has an extended shape, with polar ionone rings at the ends and a non-polar zone of conjugated carbon-carbon bonds in the middle. These polar ionone rings have been found to be effective for quenching free radicals or other oxidants (Goto et al., 2001; Kidd, 2011), whilst the conjugated middle section also serves to remove high energy electrons from free radicals (Pashkow et al., 2008; Kidd, 2011). As this molecular structure may provide an efficient defence against oxidative stress, the production of astaxanthin may allow this species to withstand the radiation stress posed by the nuclear fuel storage pond.

This study aims to characterise the impact of ionizing radiation on the morphology and physiology of *H. pluvialis*. Here, cultures of *H. pluvialis* were irradiated with daily acute doses of X-radiation which are representative of radiation fluxes delivered to organisms in a flushed spent nuclear fuel pond. Morphological changes were probed using light microscopy, whilst a global cellular analysis of the metabolism of irradiated *H. pluvialis* was achieved via Fourier transform infrared spectroscopy (FT-IR). We attempt to relate observed phenotypic changes to the occurrence of this microorganism in an environment atypical for this species.
7.3. Materials and methods

7.3.1. Organism and culture conditions

Unialgal cultures of *H. pluvialis* (strain *H. pluvialis* Flotow (1844) CCAP 34/7 1) were obtained from the Culture Collection of Algae and Protozoa, UK. Axenic *H. pluvialis* cultures were grown phototrophically in pre-sterilised Jaworski’s media, according to the guidelines from the Culture Collection of Algae and Protozoa (CCAP) – Catalogue of Strains 1988 (adapted for freshwater algae). Stock cultures were grown in 500 mL Erlenmeyer flasks fitted with cotton wool plugs and placed on a rotary shaker at 100 rpm in a controlled incubation room at 24 ± 1 °C. The photon flux density (PFD) was maintained at 80 μmol m⁻² s⁻¹ with a 16:8 h light–dark cycle (supplied by cool fluorescent daylight lamps).

7.3.2. Irradiation of cultures

Three 20 mL biological replicates were prepared by inoculating fresh Jaworski’s media with stationary phase stock cultures which had been incubated for 10 days (OD₆₂₅nm 0.17; approximately 7.2×10⁴ cells mL⁻¹). These cultures were irradiated with 16 Gy X-radiation at ambient room temperature using a Faxitron CP-160 Cabinet X-radiator (160 kV; 6 mA; tungsten target). A further 3 biological replicates formed the non-irradiated control cultures. These were placed inside the irradiator to control temperature and were shielded by an appropriate thickness of lead. After irradiation, all cultures were incubated at 24 ± 1 °C and shaken at 100 rpm in a light incubator with a photon flux density of 80 μmol m⁻² s⁻¹ with a 16:8 h light–dark cycle. Irradiations, and subsequent analysis, were repeated each day for five days, giving a total absorbed dose of 80 Gy. The dose rate was determined as 0.8 Gy min⁻¹ prior to irradiation using Fricke dosimetry (Fricke and Morse, 1927) as described by Weiss et al. (1956).

7.3.3. Algal cell number and viability

Cell concentration and viability was determined immediately after irradiation each day via 5 replicate cell counts using a Neubauer haemocytometer and a light microscope; Zeiss Axio Imager A1 (Carl Zeiss Microimaging GmbH, Germany). To determine cell viability, cells were stained using a fresh solution of Evans blue dye (200 mg Evans blue (Sigma-Aldrich) in 10 mL phosphate buffered saline (PBS) solution) which was then added to the samples to give a final concentration of 0.1%
v/v (Sigee et al., 2007). The percentage of encysted cells was also determined by cell analysis under the light microscope. Encysted cells were identified by their larger size (>10µm), lack of flagella (non motile), increased cell wall thickness and orange-red colour (increased astaxanthin accumulation). Additionally, cultures were photographed daily using a Zeiss microscope camera connector and a digital camera (Olympus B071).

7.3.4. Analysis of metabolism by FT-IR spectroscopy
The metabolic fingerprints of control and irradiated cells were recorded by FT-IR spectroscopy. Aliquots were taken from each biological replicate immediately after irradiation each day. Samples were then centrifuged at 3000g for 5 minutes, after which, the supernatant was removed and the cell pellet was washed twice with sterile 0.9% NaCl solution prior to being centrifuged, flash frozen and stored at -80 °C. Upon analysis, samples were homogenised, by freeze-thawing three times, and then resuspended in sterile 0.9% NaCl solution to an OD of 1. A 96 well Si sample plate was washed thoroughly with 2-propanol and deionised water and allowed to dry at room temperature prior to use. 20 µL of each algal sample was applied evenly in triplicate onto the plate at random locations (so called technical replicates) prior to drying at 55°C in an oven for 10 min. All FT-IR spectroscopy analysis was conducted using an Equinox 55 infrared spectrometer equipped with a high throughput motorized microplate module, HTS-XT™ (Bruker Optics, Coventry, UK). A deuterated triglycine sulfate (DTGS) detector was employed for absorbance measurements of the samples to be acquired. Thus, 3 spectra from each biological replicate were collected over the wavelength range of 4000 to 600 cm⁻¹ using the Opus software (Bruker Optics). Spectra were acquired at a resolution of 4 cm⁻¹ with 64 spectra co-added and averaged to improve the signal-to-noise ratio. The collection time for each spectrum was approximately 1 min.

7.3.5. Multivariate statistical analyses
Prior to statistical analysis, spectra were visually inspected and outlying spectra which deviated from the natural variability were removed from the data set. Features arising from CO₂ at 2400-2275 cm⁻¹ and below 700 cm⁻¹ were removed from the spectra and a smoothed trend applied. An extended multiplicative scatter correction (EMSC) method was used to normalise spectra. This method was originally
developed to reduce the effects of light scattering by particles and is particularly effective at removing noise and unavoidable intensity shifts from the spectra (Martens and Stark, 1991; Correa et al., 2012). Prior to principal component – discriminant function analysis (PC-DFA) and hierarchical cluster analysis (HCA), spectra were scaled using an auto-scaling process, such that for all spectra, the intensities at each wavenumber had a mean of zero and a standard deviation of one. Unsupervised principle component analysis (PCA) was performed on the data to reduce data dimensionality (Goodacre et al., 2007). Principal components (PCs) were then extracted during this analysis and discriminant function analysis (DFA) was applied to these PCs (i.e. PC-DFA). DFA is a supervised method, whereby discrimination between groups is based on a priori knowledge of experimental class structure. The algorithm acts to maximise between-group variance and minimise within-group variance. In this experiment, classes were assigned based on treatment and day of experiment. Discriminant function scores from PC-DFA were then used for HCA. The HCA algorithm uses the mean of the scores of each group to construct a dendrogram based on Euclidean distance between groups.

7.4. Results and discussion

7.4.1. *H. pluvialis* cell enumeration, viability and encystations

Analysis of total cell numbers in the control and irradiated cultures of *H. pluvialis* indicated that irradiation did not have a deleterious impact on cell proliferation, but in fact appeared to stimulate growth over four consecutive days of irradiation treatment (Figure 1A). This was also evident by the large amount of motile flagellated zooids in the irradiated culture after three consecutive days of irradiation. However, after five days of consecutive irradiation, total cell counts declined significantly to $7.8 \times 10^4 \pm 2.0 \times 10^4$ cells mL$^{-1}$ compared to control cultures ($1.6 \times 10^5 \pm 0.43 \times 10^5$ cells mL$^{-1}$). Figure 1B shows percentage cell viability in the cultures over the 5 days, which remained similar between the two treatments throughout, dropping to $80\% \pm 11\%$ in the irradiated culture and, $90\% \pm 5\%$ in the control at the end of the experiment. There was also a significant amount of cell debris and “ghost” cells identified in the X-treated culture by day 5 compared to the control culture (Figure 2C).
Figure 1. The effect of ionizing radiation on the growth, survival and morphology of *H. pluvialis* cultures determined immediately after irradiation on each day. (A) Cell numbers (cells mL\(^{-1}\)). (B) Percentage of viable cells in X-irradiated and control cultures. (C) Percentage of encysted cells in X-irradiated and control cultures.

Analysis of cell morphology revealed an increase in encystation during the incubation period of the irradiated culture compared to control culture (Figure 1C). By day 5, the percentage of encysted cells in irradiated cultures was 50% ± 16% compared to the control culture which had only 17% ± 8%. Microscopy images also showed that, by day 3, X-irradiated cultures were dominated by green immotile palmelloid cells (Figure 2A), and by day 5 the majority of cells appeared larger, had thicker cell walls and appeared to have increased astaxanthin accumulation, visible as red pigmentation (Figure 2B). This was largely absent in non-irradiated control cultures (Figure 2D).
Figure 2. (A) X-treated culture after three consecutive irradiations (over three days) showing palmelloid green cells. (B) X-treated culture after five consecutive irradiations over five days showing encysted cells and ghost/nonviable cells. (C) X-treated culture showing high concentrations of ghost cells and cell debris after 5 consecutive days of X-radiation. (D) Control culture after five consecutive days showing small palmelloid green cells. Image B and C are both from the same sample; however, large coagulations of cell debris and ghost cells appeared to accumulate in conjunction with encysted cells in selected areas. Scale bar represents 50 µm.

7.4.2. Analysis of *H. pluvialis* fingerprints by FT-IR spectroscopy

To quantify the impact of ionizing radiation on the metabolic fingerprints of *H. pluvialis*, infrared spectra were collected from both control and irradiated cultures immediately after each daily dose for 5 days (Figure 3). FT-IR spectra from each treatment were qualitatively similar, making it difficult to discriminate between spectral features by visual inspection alone. Therefore, in order to detect the development of an irradiated phenotype, cluster analysis was performed using PC-DFA. Figure 4A indicates that the replicates of each treatment form tight clusters in the scores plot of discriminant functions 1 and 2, suggesting that the metabolic fingerprints of cells from each treatment class were reproducible. In addition, the samples from both control and irradiated cultures taken on each treatment day cluster
tightly together, such that day five control samples (C5), for example, appear most closely related to day five irradiated samples (X5) in the HCA dendrogram (Figure 5). Likewise, there were no strong patterns of separation between clusters of control and treated samples in the scores plot of discriminant functions 1 and 3 (Figure 4B). Whilst there is some evidence of discrimination between respective irradiated and control clusters, Euclidean distances were not quantifiably related to dose (Figure 5).

![Figure 3](image)

**Figure 3.** Raw mean FT-IR spectra obtained from control (C) and irradiated (X) cultures throughout the five day experiment (day of treatment = 1, 2, 3, 4, 5). The spectra are offset in the y-axis for ease of visualisation.

Collectively, these data indicate that the metabolic fingerprints of cells from both the control and irradiated cultures on each day are quantitatively very similar, with differences between the metabolic fingerprints mainly arising from the growth phase of the culture, rather than irradiation. Hence, it appears that total absorbed doses of up to 80 Gy had no discernible effect on the metabolic profiles detected by FT-IR spectroscopy, despite significant astaxanthin production as a result of irradiation.
(evidenced via cyst formation and pigmentation; Figure 2). It is perhaps surprising that the development of this phenotype was not revealed by FT-IR spectroscopy as this technique has been used previously to demonstrate molecular diversity within species (Sigee et al., 2002) and to discriminate between different algal species (Dean and Sigee, 2006; Sigee et al., 2007). It may be that the metabolic fingerprint of *H. pluvialis* is dominated by biomolecules such as proteins or lipids, which may mask any subtle changes to other biochemical components, such as carotenoids. Indeed, the FT-IR spectrum of astaxanthin extracts exhibits absorbance at IR regions similar to those of lipids and proteins (data not shown).

![Figure 4](image-url)

**Figure 4.** PC-DFA score plots of FT-IR spectra from all treatments. Scores for discriminant function 1 (DF1) versus discriminant function 2 (DF2) are shown in (A), whilst the scores of DF1 versus DF3 are plotted in (B). 20 PCs were extracted from PCA and passed to the DFA algorithm.

Whilst direct spectroscopic determination of astaxanthin was hindered due to the difficulty in homogenising cysts for astaxanthin extraction, light microscopy illustrated a greater percentage of astaxanthin rich non-motile encysted cells with increased radiation dose. Carotenoids, such as astaxanthin, protect the photosynthetic apparatus from light-mediated stress by quenching triplet state
chlorophyll molecules, singlet oxygen molecules and other reactive oxygen species formed within the chloroplast via photooxidation (Young, 1991). The production of this molecule in irradiated cultures may allow *H. pluvialis* to quench and scavenge ROS generated by ionizing radiation. Indeed, astaxanthin production in *H. pluvialis* has also been observed during other types of environmental and metabolic stress, including high UV fluxes, which can also generate reactive oxygen species (ROS) that can damage DNA, proteins, and membranes (Borowitzka et al., 1991; Kobayashi et al., 1991). Furthermore, Kobayashi et al., (1997) suggested that *H. pluvialis* may also synthesise a suite of antioxidant enzymes in vegetative palmelloid cells, which do not show high concentrations of astaxanthin. The combination of both protection mechanisms may protect cells throughout the duration of the cell cycle, including during cell division and formation of zoospores. These protective mechanisms may contribute to this species survival and eventual dominance via algal blooms in a spent nuclear fuel storage pond (Evans *et al.* unpublished).

![Figure 5.](image)

**Figure 5.** HCA dendrogram constructed from Euclidean distances between DF clusters in PC-DFA score plots of FT-IR spectra. Each treatment category represents the mean from each treatment class.
It is perhaps not surprising that an organism such as *H. pluvialis* that produces the conjugated double bonded carotenoid, astaxanthin, as part of its usual repertoire of defence against excessive oxidative stress from high photon irradiance, also exhibits production of astaxanthin upon treatment with ionizing radiation. It is this pigment-mediated protection against oxidative stress which may be related to the preservation of the metabolic fingerprint of irradiated *H. pluvialis* noted here. In addition, it is possible that repair mechanisms may also have contributed to the preservation of the phenotype as observed by FT-IR spectroscopy. These could include enzymatic repair mechanisms, nucleotide excision repair and homologous recombination. Further work is clearly required to determine the broad spectrum of mechanisms which may potentially limit and reverse oxidative damage.

7.5. **Conclusion**

These results indicate that ionizing radiation had little impact on cell numbers and the metabolic profile of *H. pluvialis*. However survival strategies were apparent which included formation of encysted cells and the production of the molecular antioxidant astaxanthin, capable of quenching and scavenging toxic ROS that are associated with the oxidative stress induced by ionizing radiation (Kobayashi et al., 1997; Wang et al., 2004). These protection mechanisms can potentially explain how this organism survives in, and dominates the microbial community of, a spent nuclear fuel storage pond.

Further work is required to quantify astaxanthin production in irradiated cells in culture, as described here, but also *in situ* (in the spent nuclear fuel storage pond). A comparative investigation of radiation resistance in wild-type *H. pluvialis* isolated from a spent nuclear fuel pond or by using mutants that cannot produce pigmentation, may also help to determine other metabolic adaptations at species and community level in this and other nuclear environments.
7.6. Acknowledgements

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7.7. References


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Chapter 8

Conclusions

Conclusions and future directions
8. Conclusions and future directions

8.1. Conclusions

Microbial activity in environments relevant to the storage, disposal and bioremediation of radioactive waste could influence the evolution of biogeochemical conditions, waste stability and radionuclide mobility (Lloyd, 2003; Lloyd and Renshaw, 2005; Lloyd et al., 2005; Rizoulis et al., 2012). For example, microbiologically influenced corrosion (MIC) may lead to the degradation of waste containers and the infrastructure of a geological repository (King and Stroes-Gascoyne, 1997; West et al., 2002). In addition, repository over-pressurisation from the microbial generation of gases may lead to fracturing and enhanced radionuclide transport in both the gas phase and in groundwater (Nirex, 1994). Indeed, microbial activity may also mitigate over-pressurisation via utilisation of gases, such as hydrogen, as electron donors.

Furthermore, some microbes are capable of radionuclide bioaccumulation and sorption, via biofilm and ligand production (West et al., 2002), whilst microbial degradation of mobile organic-radiouclide complexes may restrict radionuclide mobility (Reed et al., 1999; Keith-Roach, 2008). Of particular importance is the microbial reduction of Fe(III), which may promote redox potentials which favour the reduction, and subsequent precipitation, of redox active radionuclides (Wildung et al., 2000; Law et al., 2010; Williams et al., 2011). For instance, Fe(III)-reducing species, such as Geobacter and Shewanella spp. may facilitate the precipitation of radionuclides via direct enzymatic reduction or indirectly, via the abiotic electron transfer from biogenic Fe(II) bearing phases to the radionuclide (Lloyd and Macaskie, 1996; Lloyd et al., 2000; Anderson et al., 2003; Hansel et al., 2003; Wilkins et al., 2006). In addition, these respiratory pathways present the opportunity for the use of such species in the bioremediation of radionuclide contaminated land (Heidelberg et al., 2002; Tiedje, 2002; Lloyd, 2003).

Despite the importance of microbial processes to the integrity of radionuclide inventories in storage, geodisposal and bioremediation scenarios, the characterisation of such processes has largely occurred with little consideration of ionizing radiation...
fluxes. Thus, the research presented in this thesis sought to investigate the range of ionizing radiation impacts on microorganisms pertinent to the storage, disposal and bioremediation of radioactive waste. To achieve this aim, objectives were formulated to (i) characterise the cellular physiology of microorganisms after irradiation with acute X-radiation doses; (ii) assess the impact of high dose gamma radiation on Fe(III) oxides and quantify changes to their availability as electron acceptors and (iii) determine whether these observations could be extended to low-dose rate sediment systems by probing microbial communities and biogeochemical changes.

8.1.1. Characterisation of the cellular physiology of irradiated microorganisms

The impact of acute doses of ionizing radiation on the physiological status of two microorganisms was characterized. The first of these organisms, *Shewanella oneidensis* MR-1, is a model subsurface Fe(III)-reducing organism capable of reducing a range of electron acceptors. The results from this study suggest that although acute doses of 12 to 95 Gy restrict viability and growth, a microbial population may recover to the same biomass levels as non-irradiated populations. Increases in lipid and protein content of cells characterized metabolism immediately after irradiation. As protein and lipid levels are fundamental to the integrity and functionality of membranes (where electron transfer proteins are predominantly localised), ionizing radiation may affect the ability of Fe(III)-reducing species, such as *S. oneidensis*, to reduce Fe(III) and radionuclides, such as U(VI), Tc(VII) and Np(V), in the near field environment of a geological repository. In addition, the long-term metabolism and functionality of *S. oneidensis* during bioremediation applications may also be affected. Indeed, the irradiated phenotype exhibits enhanced Fe(III)-reduction, although the reason for this remains unclear.

In addition to *S. oneidensis*, the radiation tolerance of an algal species identified in a spent nuclear fuel pond was also assessed. Cultures of *Haematococcus pluvialis* are capable of surviving daily doses of 14 Gy. FT-IR spectroscopy revealed the metabolism of the irradiated phenotype did not change significantly with successive treatments. Furthermore, this coincided with radiation induced production of the carotenoid astaxanthin. These findings suggest that *H. pluvialis* may be able to
survive in environments with significant radiation fluxes, such as spent nuclear fuel ponds, via the production of antioxidant pigments which likely protect against the oxidative stress initiated by ionizing radiation. This information is important to help inform biomass control strategies at Sellafield. As this species exhibits the potential for bioaccumulation of radionuclides (Evans et al., unpublished), these results also have implications for its involvement in bioremediation strategies.

8.1.2. Radiation enhanced reduction of Fe(III) oxides

In addition to the cellular environment, the impact of radiation on the extracellular environment was also assessed. Gamma radiation (1 MGy; equivalent to total absorbed doses after >$10^5$ years in a geological repository (Allard and Calas, 2009)), activated ferrihydrite and the usually recalcitrant hematite for reduction by *S. oneidensis*. Electron diffraction and Mössbauer spectroscopy revealed that for ferrihydrite, this was a result of a change in the structure toward a phase similar to akaganeite. Indeed, Fe(III) in this phase has previously been demonstrated to be reduced readily by *Geobacter sulfurreducens* in the presence of an electron mediator, such as riboflavin (Cutting et al., 2009). The reason for enhanced reduction of hematite is unclear, however, Mössbauer spectroscopy revealed the emergence of an uncharacterized paramagnetic component which suggested the generation of a poorly crystalline Fe(III) phase which may have been more bioavailable.

As iron-based materials will be prevalent in waste-forms, barrier systems and infrastructure of a geological disposal facility, corrosion of these materials will likely lead to an increase in the biogeochemical cycling of this Fe. These results suggest that dissimilatory Fe(III)-reduction in such environments may be enhanced by radiation damage. Indeed, this could be promoted further by the production of electron donors such as hydrogen and organic compounds formed by the radiolysis of water and organic matter, respectively.

8.1.3. The impact of low-dose rate gamma radiation on microbial communities and biogeochemical processes

The observations from experimental chapters 4 and 5 provided valuable information on how ionizing radiation may impact on the interaction between microorganisms and potential electron acceptors. Despite this, environments which exhibit radiation
fluxes will be more complex, with a range of electron donors and acceptors along with a potentially diverse microbial community. In addition, environmental dose rates will be much lower than those used in previous experiments (Figure 1, Chapter 2).

Accordingly, sediment microcosms were irradiated over an eight week period using dose rates representative of nuclear waste canister surfaces in the near field of a geological disposal facility. The observations suggest that a Sellafield-type sediment community may be able to survive long term gamma irradiation; though dose rate and availability of carbon sources will have a significant influence on community structure. Furthermore, despite significant total absorbed doses, biogeochemical processes may only be partially restricted by radiation dose rates expected in environments relevant to the bioremediation and geological disposal of nuclear waste. Moreover, electron accepting processes, such as Fe(III) reduction, may be stimulated in a deep geological repository by radiation. This will likely result in microbial communities with a significant cohort of Fe(III)-reducing species.

8.1.4. Overarching conclusions

It is challenging to relate the observations from acute dose studies to complex environmental systems, such as sediments, in which dose rates will likely be significantly lower. The simultaneous irradiation of a range of absorbing materials and molecules, which display a range of radiation effects, gives rise to complex interactions between microorganisms and their environment.

Collectively, the experiments of this thesis provide evidence for a range of impacts of ionizing radiation on microorganisms and these results have important implications to the long-term storage of nuclear waste and the geomicrobiology of nuclear environments. Perhaps of most prominence, is the conclusion that ionizing radiation may stimulate a Fe(III)-reducing community in environments relevant to the geodisposal of waste. This may occur via the irradiation of cellular metabolism, Fe(III) oxides and via complex biogeochemical interactions. Potentially, this may promote enhanced reduction of priority radionuclides, such as U(VI), Np(V) and Tc(VII), via direct enzymatic reduction or indirect electron transfer from biogenic Fe(II). As such processes may lead to the subsequent precipitation of U(IV), Np(IV)
and Tc(IV), this could play an important role in controlling radionuclide migration in a geological disposal facility. Furthermore, ionizing radiation fluxes could provide the basis of novel ecosystems capable of exerting important controls on the biogeochemical evolution of environments pertinent to the storage, geodisposal and bioremediation of radioactive waste.

### 8.2. Future directions

Whilst the research chapters of this thesis have gone some way in exploring ionizing radiation impacts on microorganisms, there are still many areas in which this research could be developed further. These are discussed below in the context of specific experiments and general research directions.

#### 8.2.1. The impact of ionizing radiation on microbial metabolism

Assessment of the *S. oneidensis* MR-1 proteome via MALDI-TOF-MS revealed that, although FT-IR spectroscopy indicated phenotypic recovery post irradiation, there were significant changes to proteins in stationary phase cultures irradiated with 95 Gy. However, peak assignments were only made for lag phase cultures and therefore, the production of subtraction spectra for the stationary phase cultures should be considered. Whilst MALDI-TOF-MS did reveal changes to specific proteins as a result of irradiation, the technique was limited in the number of proteins that could be detected. A comprehensive proteomic study, e.g. using liquid chromatography mass spectrometry (LC-MS), is perhaps warranted. This may provide further information on the recovery response of this organism and give insight into radiation effects in proteins associated with the respiratory capabilities of the irradiated culture.

In addition to proteins, FT-IR spectroscopy also revealed changes to the lipid content of irradiated *S. oneidensis*. A more targeted approach would allow quantification of specific radiation damage in lipids, allowing these impacts to be related to dose and membrane integrity. For example, analysis of fatty acid methyl esters (FAME) via gas chromatography mass spectrometry (GC-MS) may offer a more comprehensive study of changes to lipids and how this might relate to the respiratory capabilities of irradiated *S. oneidensis* (see below). Indeed, this technique has been used in a
previous study to relate metal toxicity to membrane integrity in *S. oneidensis* (Wang et al., 2010).

Irradiated *S. oneidensis* exhibited enhanced Fe(III)-reduction despite cultures suffering a significant loss of active biomass. The reasons for this were unclear, however, an assessment of the oxidation state of irradiated cytochromes or alterations in membrane structure may reveal whether this effect is directly related to the respiratory chain components. In addition to Fe(III)-reduction, an assessment of whether this enhanced reduction also occurs with radionuclides, such as U(VI) and Tc(VII), should also be pursued.

More generally, it would be beneficial to determine whether the conclusions from the study of *S. oneidensis* can be applied to other microorganisms relevant to the storage, disposal and bioremediation of radioactive waste. This may include other Fe(III)-reducing species, such as *Geobacter* spp., and sulphate reducing bacteria, which may facilitate the corrosion of radioactive waste canisters.

8.2.2. The impact of ionizing radiation on Fe-bearing minerals

As a result of the radiation enhanced Fe(III) reduction of ferrihydrite and hematite, it was hypothesised that the resultant Fe(II) may facilitate the reduction of radionuclides. Indeed, biogenic Fe(II) mediated reduction of radionuclides has been demonstrated previously (Lloyd et al., 2000; Wilkins et al., 2006). However, the reactivity of this phase will dictate the efficiency of radionuclide removal from solution. Therefore, the biogenic Fe(II) phases generated via radiation enhanced Fe(III)-reduction should be characterized, e.g. via TEM and XRD, and challenged with contaminants, such as Tc(VII).

Hematite and ferrihydrite represent extremes in crystallinity of Fe(III) oxides. As radiation increased the availability of both these phases for Fe(III)-reduction, it is hypothesised that this finding may be extended to other environmentally important Fe(III) oxides, such as lepidocrocite and goethite. In addition, the mixed Fe(II)/Fe(III) phase magnetite (Fe₃O₄) will be present in a geological disposal facility as it is a likely product of the anaerobic corrosion of steel (Smart et al., 2002;
Schütz et al., 2013). Therefore, the impact of radiation on the bioavailability of these phases should also be assessed.

Previous studies have demonstrated the radiation induced oxidation of Fe(II) in Fe(II) solutions and Fe(II) bearing minerals (Ladriere, 1998; Yakabuskie et al., 2011). Thus, the potential for microbial reduction of Fe(III) generated by the radiation driven oxidation of Fe(II) in environmentally relevant Fe(II) mineral phases, such as siderite and vivianite, should also be determined.

In addition to single phase Fe oxides, the impact of radiation on the bioavailability of Fe in other Fe bearing materials relevant to a geological repository may be considered. For instance, bentonite has been proposed as a backfill material for repository vaults. Radiation induced changes to the oxidation state of Fe in this material could be fundamental to the development of microbial communities at or near canister surfaces.

### 8.2.3. The impact of radiation on alternative electron donors and acceptors

The irradiation of sediment microcosms resulted in enhanced Fe(III)-reduction in systems containing no-added electron donor. Ion chromatography indicated that this effect may be related to loss of nitrate from solution. Thus, it would be appropriate to investigate the impact of radiation on other electron acceptors, such as nitrate, in pure culture experimental systems. In addition, sulphate concentrations were observed to increase in some systems as a result of irradiation. As sulphate may also be used as an electron acceptor, an investigation into its radiation driven generation may be warranted. Previous studies suggest this could be related to pyrite oxidation (Chivian et al., 2008; Lefticariu et al., 2010), however, the effect of this on rates of reduction and microbial communities remains undetermined.

In addition to the impact of radiation on electron accepting processes, the potential for radiation induced electron donor production has received little attention. Molecular hydrogen is a product of water radiolysis and has been proposed as a potential electron donor in the deep subsurface (Galès et al., 2004; Lin et al., 2005a; Lin et al., 2005b; Chivian et al., 2008; Lefticariu et al., 2010; Libert et al., 2011). Rates of reaction between radiolytically generated hydrogen and electron acceptors
over a range of environmentally relevant dose rates would provide useful information to determine whether radiolytic hydrogen will be an important electron donor in geodisposal environments.

Furthermore, the potential for carbon source and organic electron donor generation via the radiolytic degradation of natural organic matter and organic polymers in likely waste forms requires more attention. A few previous studies have suggested that dissolved organic carbon concentrations may increase as a result of sediment irradiation (Bank et al., 2008; Berns et al., 2008; Schaller et al., 2011); however hypotheses are limited to suggest that this is a result of radiolytic degradation of complex organic matter. Cellulose, which will be present in waste forms, has been shown to undergo radiolysis (Humphreys et al., 2010), although the implications to microbial processes are unclear. A mechanistic understanding of radiolytic degradation of organic matter would provide insight into the potential for increased bioavailability of organic electron donors in repository environments. Candidate molecules for irradiation studies include cellulose and the ubiquitous humic acid, which is also environmentally important as an extracellular electron ‘shuttle’ and electron donor (Lovley et al., 1999).

**8.2.4. The impact of ionizing radiation on algal cells**

The metabolic fingerprints of irradiated *H. pluvialis* cultures remained unchanged, despite receiving significant doses of ionizing radiation. Light microscopy suggested that this observation may have been related to the production of the antioxidant molecule astaxanthin associated with encysted cells. As this molecule may be fundamental to the survival of this organism in spent nuclear fuel storage pools, there is a clear requirement to quantifiably relate astaxanthin concentrations to radiation dose and physiological status.

Initial determination of astaxanthin in irradiated cells was precluded by difficulties in quenching cells. Furthermore, there was an absence of detectable unique astaxanthin vibrations in the IR spectrum; nor did the molecule exhibit vibrational transitions observed by Raman spectroscopy. High performance liquid chromatography (HPLC), on the other hand, could provide a suitable quantification technique and has
been utilised in previous studies (Brinda et al., 2004; Wang et al., 2004; Ranga et al., 2009).

In addition to the assessment of ionizing radiation impacts upon axenic cultures of *H. pluvialis*, a comparative study using a non-astaxanthin-producing mutant, or the wild-type strain isolated from a spent nuclear fuel pond, may provide additional information about novel survival strategies which has allowed the adaptation of this species to this ecological niche.

### 8.2.5. General approaches

In addition to specific experiments discussed above, there are several general approaches which would facilitate stepwise improvements in our understanding of the interaction of ionizing radiation with microorganisms. Foremost, there is a requirement to assess the impact of other ionizing radiation types on the microbial physiology and biogeochemical processes described throughout this thesis. The experiments documented here made use of gamma and X-radiation; however, few studies have explored the impact of alpha and beta radiation. The radiation chemistry of these different radiations may be similar, i.e. their effects all arise from ionizations in the absorbing materials; however, the energy transfer, and hence penetration, of these particles differs significantly. Thus it is conceivable that each radiation type will produce different effects over the scales that are required for the study of microbial systems.

The challenges in studying the effects of alpha and beta radiation primarily arise from the difficulty in supplying a dose. *In vitro* systems required for microbiological studies of this type will limit the penetrating dose of external sources. Therefore, microcosm systems spiked with sufficient activities of radionuclides could provide an *in situ* radiation source. Indeed, such a set-up would prove advantageous as it would allow the simultaneous analysis of radiation effects and the interactions between microbes and radionuclides. Such an investigation of microbial communities has already been conducted using $^{137}$Cs and $^{90}$Sr (Niedree et al., 2013); however, the high concentrations of radionuclides required for a more appropriate dose rate may ultimately result in metal toxicity. Thus, experiments of this nature would require the selection of a radionuclide with an appropriate decay mode and
half-life and a stable isotope that could be used for control experiments. This would assume no isotopic fractionation effects by microbial processes and would also require the effects of metal toxicity to be distinguishable from radiation toxicity.

In addition to these general approaches, it would be appropriate to consider the impact of high pH on the range of effects documented in this thesis. The current disposal concept for intermediate level waste in the UK involves the long term disposal of waste in a repository using a cementitious backfill (Nirex, 2005). Thus, there is a requirement to assess the impact of the dual stress of radiation and high pH.

8.3. References


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Appendix
Appendix

The bioavailability of Fe(III) for microbial respiration in irradiated hematite single crystals

The experiments documented below were completed as part of the research project; however, the findings did not produce a significant contribution to the understanding of the impacts of radiation on microorganisms. As such, this study was not considered for submission to a scientific journal and was, therefore, not included in the main body of this thesis. Despite this, the results presented here are relevant to the aims and objectives of this thesis and have, therefore, been documented in this appendix.

Introduction

The results of Chapter 5 indicated that gamma radiation increased the availability of Fe(III) oxide minerals for microbial reduction. This previous study was conducted using nanoparticulate oxides and it is therefore unclear whether a similar effect would be obtained for more crystalline Fe oxides. To assess this, single crystals of hematite were irradiated with gamma radiation. The use of single crystals also allowed the impact of radiation on the crystalline structure to be probed using techniques which are not suitable for powdered samples, such as scanning electron microscopy (SEM) and atomic force microscopy (AFM). This study aimed to use these techniques to provide a mechanistic understanding of radiation enhanced microbial Fe(III)-reduction at the molecular scale.

The Fe(III)-reducing species Geobacter sulfurreducens was used in these experiments as, unlike Shewanella oneidensis, this species is not known to produce electron shuttling compounds such as flavins (von Canstein et al., 2008). Thus, in order to utilise Fe(III) as an electron acceptor, cells would be required to make direct enzymatic contact with the crystalline surfaces. It was therefore hypothesised that the distribution of cells on crystalline surfaces may be related to radiation damage sites.
Materials and methods

Irradiation of hematite

Single crystals of hematite (5-10 mm³) were prepared by manually cleaving samples of natural rose hematite. Crystals were then flame sealed in ampoules containing 2 mL of sterile deionised water and irradiated, where necessary, with 1 MGy gamma radiation using a Shepherd ⁶⁰Co source.

Microbial Fe(III)-reduction

*Geobacter sulfurreducens* was grown anaerobically in the dark in a modified freshwater medium (Lloyd et al., 2003). Sodium acetate (25 mM) and sodium fumarate (40 mM) were supplied as the electron donor and acceptor, respectively. After 24 hours incubation at 30 °C, late log - early stationary phase biomass was harvested by centrifugation at 4920g for 20 minutes at 4 °C and, then, washed twice in sterile 30 mM sodium bicarbonate buffer (pH 7) under an 80:20 gas mix of N₂-CO₂. Aliquots of the buffered cell suspension (0.2 ml) were added to pre-sterilised anaerobic 30 mM bicarbonate buffer (10 mL) containing 10 mM lactate as electron donor. Irradiated crystals were then added to systems aseptically. Triplicate experiments, along with sterile controls, were incubated in the dark at 30°C.

Fe(II) concentrations in solution were measured periodically using Ferrozine assay. Briefly, 100 µL of solution was removed from systems and added to 900 µL 0.5 N HCl. Aliquots of these acidified solutions were added to Ferrozine solution and Fe²⁺ concentrations were determined by measuring absorbance at 562.0 nm using a UV/Vis spectrophotometer (Jenway) (Lovley and Phillips, 1986a; 1986b). Fe(II) associated with the crystalline phase was monitored by taking sacrificial digests of crystals throughout the experiment. Similarly, crystals were digested in 0.5 N HCl for 1 hour. 200 µL of this digest was then added to 2.3 mL of Ferrozine solution and the Fe²⁺ concentration measured against a standard curve. The mass of each crystal was determined prior to digestion and the Fe(II) concentrations in each experiment were normalised to the mass of each respective crystal.

Microscopy

At the end of the incubation period, microbial cells were fixed prior to analysis via environmental scanning electron microscopy (ESEM). Formalin was added to
experiments to give a final formaldehyde concentration of 2% v/v. Crystals were then removed from experimental vessels, dried, and analysed using a FEI/Philips XL30 FEG ESEM equipped with an EDAX Genesis EDX system. Crystals were also analysed using atomic force microscopy (AFM). AFM was performed using a Bruker Multimode AFM and images were processed using the WSXM processing software (Horcas et al., 2007).

**Spectroscopy**
After 25 days of incubation, Fe(II) concentrations on crystal surfaces were also probed by X-ray photoelectron spectroscopy (XPS) using a Kratos Axis Ultra spectrometer with a monochromated Al-Kα X-ray source. Prior to this, cells were washed in anoxic 5% sodium dodecyl sulphate solution (SDS) to remove organic carbon from mineral surfaces which would prevent absorption of X-rays. Similarly, unwashed crystals were also probed to ensure the SDS washing procedure had no effect on Fe(II) concentrations, which was confirmed by XPS (data not shown).

**Results and discussion**

![Figure 1](image_url)

**Figure 1.** (A) Fe(II) concentrations in solutions containing irradiated and non-irradiated single crystals of hematite (Hm). (B) Fe(II) associated with the solid crystalline phase in experiments containing irradiated and non-irradiated hematite. Where present, error bars depict the standard error of the mean of triplicate experiments.
Figure 2. AFM images representative of (a) non-irradiated hematite (sterile); (b) non-irradiated hematite incubated with *G. sulfurreducens*; (c) irradiated hematite (sterile) and (d) irradiated hematite incubated with *G. sulfurreducens*. Colour intensities indicate relative height in the *z*-dimension as indicated by the scale bars to the right of each panel.
Figure 3. ESEM images of (a) non-irradiated hematite; (b) non-irradiated hematite incubated with \textit{G. sulfurreducens}; (c) irradiated hematite and (d) irradiated hematite incubated with \textit{G. sulfurreducens}.

Figure 4. EDX spectra of (A) ‘non-amorphous’ surface site on irradiated hematite and (B) ‘amorphous’ surface site on irradiated hematite.
Figure 5. XPS spectra of Fe 2p from the cleaved surface of (a) non-irradiated hematite; (b) non-irradiated hematite incubated with *G. sulfurreducens*; (c) irradiated hematite and (d) irradiated hematite incubated with *G. sulfurreducens*.

After 12 days incubation, the addition of *G. sulfurreducens* had no significant impact on the amount of Fe(II) in the solutions of experiments containing non-irradiated hematite crystals (Figure 1A). Likewise, systems containing irradiated hematite did not show significant Fe(II) production upon addition of *G. sulfurreducens*.

Similarly, after 25 days, the addition of *G. sulfurreducens* in non-irradiated systems had no significant effect on Fe(II) associated with the solid crystalline phase (Figure 1B). Some data points suggested that transient increases in Fe(II) occurred, e.g. ~4.5 µmol g⁻¹ Hm after 7 days, however, these effects likely arise from differences between individual crystals used for each sacrificial time point. In general, these results suggest that Fe(III) in rose hematite is not available for microbial reduction, however, there may be low concentrations of Fe(II) associated with the surfaces, which may arise from small areas of a more amorphous and bioavailable Fe(III) oxide phase.
In irradiated systems containing *G. sulfurreducens*, there was a slight increase in Fe(II) associated with the crystalline phase throughout the first 14 days of incubation. However, this effect was observed to some extent in both sterile and inoculated systems and Fe(II) concentrations did not exceed those in non-irradiated control systems. Furthermore, after 25 days of incubation, there was no difference in Fe(II) concentrations between irradiated and non-irradiated systems. These results suggest that 1 MGy gamma radiation had no significant effect on the bioavailability of Fe(III) in single crystals of rose hematite.

Characterisation of irradiated hematite powders revealed that radiation enhanced Fe(III) reduction was related to changes in the crystallinity of Fe oxides (Chapter 5). Despite an absence of radiation enhanced Fe(III) reduction in the single crystal systems used here, the cleaved surfaces of crystals were probed to identify any changes in crystalline structure which may have restricted Fe(III) reduction. AFM and ESEM images of cleaved surfaces of sterile hematite revealed a rough texture, with microfractures and microcrystalline structures present on the surface of the crystals (Figures 2a and 3a). Similar textures were observed in systems with added *G. sulfurreducens* (Figure 3b). In addition, AFM indicated the presence of amorphous surface structures which were likely a result of organic matter from the microbial culture (Figure 2b). Indeed, 1 to 2 µm rods were observed which is typical of the morphology of *G. sulfurreducens* cells.

Similar textures were observed in the AFM images of the irradiated systems indicating that radiation had no discernible impact on the crystalline structure at the µm scales viewed by AFM (Figure 2c). Again, the introduction of *G. sulfurreducens* led to the presence of amorphous organic structures, a likely result of the secretion of extracellular polymeric substances by the bacteria (Figure 2d).

On irradiated crystals, ESEM revealed the presence of a secondary semi-amorphous phase, which consisted of rod shaped nodules of 3-10 µm in length (Figures 3c and 3d). Interestingly, these textures were only observed on crystal edges and ‘steps’ of the cleaved surface of irradiated crystals. The distribution was very heterogeneous and less than five of these areas were viewed on each 5 to 10 mm cleaved surface.
Energy dispersive X-ray (EDX) spectra were taken to determine the elemental composition of these regions compared to the composition of the typical cleaved surface. The spectrum of the cleaved surface displayed peaks attributable to iron and oxygen, as would be expected of Fe$_2$O$_3$ hematite, along with a titanium trace (Figure 4A). On the amorphous surface, EDX spectra revealed the emergence of a sulphur peak in addition to iron and oxygen, suggesting that this amorphous phase has sulphur associated with its structure (Figure 4B). Given the distribution of these amorphous textures and the unique chemical composition, it is likely that these textures are a secondary mineral phase associated with the original crystal structure, rather than a product of irradiation.

To further probe the oxidation state of the iron at the surface of the crystals, XPS spectra of the Fe 2p region of all treatments were obtained (Figure 5). The spectra were fitted using a multiplet fitting method to model the cleaved sample using 9 individual fit regions. All samples exhibited typical spectra for Fe$_2$O$_3$, with the addition of a shoulder peak at ~709 eV. A peak at this binding energy appears inherent in cleaved samples of this type and is likely a Fe$^{3+}$ intensity (Yamashita and Hayes, 2008).

The intensity of the fourth of the nine fits was used to model the Fe 2p satellite at ~715 eV, which is associated with Fe$^{2+}$ (Aronniemi et al., 2005; Aronniemi et al., 2007; Yamashita and Hayes, 2008). In all treatments, there is a constant, but negligibly small Fe$^{2+}$ satellite intensity above the background of the fit. On the other hand, the Fe$^{3+}$ satellite at 719 eV is consistently larger, suggesting that in all treatments, the majority of iron at the surface of the crystals is present as Fe(III). These data are in good agreement with the Ferrozine assay data which suggest that radiation did not lead to enhanced Fe(II) production by *G. sulfurreducens*.

Collectively, these data suggest that, unlike nanoparticulate Fe(III) oxides, 1 MGy gamma radiation does not increase the bioavailability of Fe(III) in highly crystalline hematite for microbial reduction. This may be because of a higher initial crystallinity of the rose hematite compared with synthetic nanoparticulate hematite or because of a smaller surface with which to incur reaction with radiolysis products.
References


