Contractor Report to RWM
The impact of ionizing radiation on microbial cells pertinent to the geological disposal of radioactive waste

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RWM Preface

Radioactive Waste Management (RWM) carries out Research and Development (R&D) in support of geological disposal of the UK’s higher activity waste. The work presented in this report forms part of our R&D programme and was carried out at the University of Manchester on our behalf. The work has been reviewed by RWM and by two independent peer reviewers. RWM accepts the data and conclusions in this report.
The impact of ionizing radiation on microbial cells pertinent to the geological disposal of radioactive waste

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Preface

This report highlights the main findings of a PhD studentship funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and Radioactive Waste Management. Further details can be found in the doctoral thesis of A. R. Brown (2014); published by the University of Manchester and made accessible via the University’s eScholar repository (eScholar ID: uk-ac-man-scw:216843). The project was supervised by Professors Jonathan Lloyd, Simon Pimblott and Royston Goodacre with particular support from Dr. Joe Small, National Nuclear Laboratory. References to publications that have arisen from the experiments described in this report and in the aforementioned thesis can be found in each section of the relevant work.

Abstract

Microorganisms control many processes pertinent to the stability of radioactive waste inventories in nuclear storage and disposal facilities. However, in such environments, 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. In addition to the cellular environment, the impact of radiation on the extracellular environment was also examined. Gamma radiation activated ferrihydrite and the usually recalcitrant hematite for enzymatic reduction by *S. oneidensis*. 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 initial experiments. Sediment microcosms irradiated over a two month period at chronic dose rates exhibited enhanced microbial Fe(III)-reduction despite receiving potentially lethal doses. This report provides evidence for a range of impacts of ionizing radiation on microorganisms, including the potential for radiation to provide energy sources that could underpin novel ecosystems. As such these results are relevant to the long-term storage and disposal of radioactive waste and the geomicrobiology of nuclear environments.
Executive Summary

Microorganisms control many processes pertinent to the stability of radioactive waste inventories in nuclear storage and disposal facilities. In addition, numerous subsurface bacteria, such as *Shewanella spp.* have the ability to couple the oxidation of molecular hydrogen or organic matter to the reduction of a range of metals, anions and radionuclides. For important radionuclides such as Tc(VII), U(VI) and Np(V), this biological reduction may lead to their precipitation, thus limiting their mobility in the near-field of a geological disposal facility. Such microbial metabolisms are therefore an important consideration for the geological disposal of radioactive waste.

However, in such environments, 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. 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 indicated an increase in lipid associated vibrations and decreases in vibrations tentatively assigned to nucleic acids, phosphate, saccharides and amines. MALDI-TOF-MS detected an increase in total protein expression in cultures exposed to 12 Gy, whilst at 95 Gy, a decrease in total protein levels was generally observed. These experiments suggested that significant alteration to the metabolism of *S. oneidensis* results from ionising radiation and that dose dependent changes to specific biomolecules characterise this response. Enhanced levels of poorly crystalline Fe(III) oxide reduction was also observed by irradiated *S. oneidensis*, though the mechanism underpinning this phenomenon is unclear.

In addition to the cellular environment, the impact of radiation on the extracellular environment was also assessed. 1 MGy gamma radiation activated two model Fe-oxides, ferrihydrite and the usually recalcitrant hematite, for enzymatic reduction by *S. oneidensis*. TEM, SAED and Mössbauer spectroscopy revealed that this was a result of radiation induced changes to crystallinity.

Environments exposed to radiation fluxes will be much more complex than the conditions used in model systems, 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 the experiments described previously. To explore this, a series of microcosms were created using well-characterised sediments taken from outside the Sellafield site boundary and were irradiated over a two-month period at 0.5 or 30 Gy h⁻¹ using Co-60 gamma.

Acetate and lactate amended sediment microcosms irradiated at both dose rates all displayed NO₃⁻ and Fe(III) reduction, though the rate of Fe(III) reduction was decreased in 30-Gy h⁻¹ treatments. Fermentation processes dominated these systems and 16S rRNA gene
pyrosequencing indicated that the 30-Gy h⁻¹ treatment resulted in a community dominated by two Clostridial species. Analysis of microcosms with no added electron donor indicated that irradiation with either dose rate did not restrict NO₃⁻, Fe(III), or SO₄²⁻ reduction. Rather, the rate and extent of Fe(III) reduction was increased in the 0.5 Gy h⁻¹-treated systems. Here, an increase in the proportion of bacteria capable of Fe(III) reduction was observed, with a Geothrix sp. and Geobacter spp. identified in the 0.5 and 30-Gy h⁻¹ treatments, respectively.

This report provides evidence for a range of impacts of ionizing radiation on microorganisms. These results suggest that biogeochemical processes may not be restricted by dose rates in environments relevant to the long-term storage and disposal of radioactive waste. Indeed, electron-accepting processes may even be stimulated by radiation.

This document is a summary report highlighting the findings of a BBSRC and RWM funded doctoral thesis. The aims of the thesis were exploratory within the context of geological disposal, rather than a targeted project utilising experimental parameters specific to UK ILW engineered disposal concepts. As such, the integration of specific data or further interpretation of results with respect to a GDF performance assessment is beyond the scope of this report. Whilst no formal recommendations can be made, this report endeavours to discuss findings in the context of geological disposal.
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1. Aims and objectives

The impact of ionizing radiation on microorganisms and the biogeochemical processes they catalyse may influence the chemical evolution of a geological disposal facility for radioactive waste. This report highlights the main findings of the described research project and further detail can be found in the doctoral thesis of A. R. Brown (2014). Specifically, the aim of the 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, an attempt was made to constrain the mechanisms by which radiation may influence microbial processes in these settings. These aims were pursued via the following objectives, which are summarised in Figure 1:

i) Characterise the metabolism and phenotypic response of an Fe(III)-reducing bacterium irradiated with acute doses of ionizing radiation.

ii) Assess the bioavailability of a range of irradiated Fe(III) oxides for microbial Fe(III) 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.

These aims are exploratory within the context of geological disposal, rather than a targeted set of experiments utilising experimental parameters specific to UK ILW engineered disposal concepts. As such, the integration of specific data or further interpretation of results with respect to a GDF performance assessment is beyond the scope of this report. Whilst no formal recommendations can be made, this report endeavours to discuss findings in the context of geological disposal.
Figure 1. A schematic summary of the interactions of radiation with microorganisms addressed by this report.1 (A) A schematic of the impact of ionizing radiation on (a) microbial metabolism; (b) Fe(III)-bearing minerals and (c) the resultant impact on the interaction between the two. (B) The interaction of ionizing radiation with complex environmental systems and indigenous microbial communities was also assessed, under more environmentally relevant dose rates (d).

2. Background

2.1 Microbial metabolism pertinent to radioactive waste disposal

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.2-5 For example, microbially influenced corrosion (MIC)
may lead to the degradation of waste containers and the infrastructure of a geological repository, e.g. rock bolts and metal structures.\textsuperscript{6,7} In addition, gas generation by microbial metabolism may further contribute to repository over-pressurisation potentially arising from gas production from metal corrosion and radiolysis. Such processes may lead to repository damage and enhanced radionuclide transport in both the gas phase and in groundwater.\textsuperscript{8} Conversely, microbial activity may also mitigate over-pressurisation via utilisation of gases, such as hydrogen, as electron donors. Indeed, any potential for over-pressurisation is ultimately dependent on the host geology and the disposal concept chosen.

Furthermore, some microbes are capable of radionuclide bioaccumulation and sorption, via biofilm and ligand production,\textsuperscript{7} whilst microbial degradation of mobile organic species may limit any enhanced radionuclide mobility arising from organic-radionuclide complexation.\textsuperscript{9–11} Detail of microbial activity and corrosion which provides further context to this report can be found in the following NDA/RWM reports.\textsuperscript{12,13}

Of particular importance is the microbial reduction of Fe(III), which may facilitate redox potentials which favour the reduction, and subsequent precipitation, of several redox active radionuclides, including Tc(VII), U(VI) and Np(V).\textsuperscript{14–16} For instance, Fe(III)-reducing species, such as \textit{Geobacter} and \textit{Shewanella spp.} may facilitate the precipitation of radionuclides via direct enzymatic reduction or indirectly, via abiotic electron transfer from biogenic Fe(II) bearing phases to radionuclides.\textsuperscript{17–21} Such reductive precipitation will likely restrict the mobility of radionuclides in the subsurface and as such, these respiratory pathways also present the opportunity for the use of such species in the bioremediation of radionuclide contaminated land.\textsuperscript{2,22,23}

\textbf{2.2 Radiation doses relevant to the geological disposal of radioactive waste}

Despite the possible role of microbial processes in the chemistry of some radionuclides in storage, geodisposal and bioremediation scenarios, the characterisation of such processes has largely occurred with little consideration of ionizing radiation fluxes. Such fluxes will be present in both geodisposal and radionuclide contaminated land scenarios, however, total absorbed doses and dose rates will cover a large range because of the different radionuclide inventories of waste packages, their spatial distribution and their evolution with time. For instance, doses will be governed by radionuclide concentrations, their respective half-lives, the decay mode and emission energy, distance from source and elapsed time. Much of the available data in the published literature focuses on dose rates surrounding high level waste (HLW) packages and spent fuel, and this is highlighted in Figure 2.

Estimates of total absorbed doses for HLW from the Savannah River Plant, South Carolina, are of the order 600 MGy beta/gamma after 10\textsuperscript{3} years, rising to 1 GGY after 10\textsuperscript{6} years (Figure 2).\textsuperscript{24,25} For alpha radiation, total absorbed doses of 90 MGy after 10\textsuperscript{3} years and 800 MGy after 10\textsuperscript{6} years have been estimated.\textsuperscript{24,25} These values are only indicative of doses in
the vitrified waste-forms themselves; alpha radiation will be absorbed by the waste and its container, whilst gamma doses away from the container surface will decrease with distance.

Dose rate simulations for a HLW container in Boom Clay gave dose rates up to 400 Gy h\(^{-1}\) at the clay-canister interface, decreasing to 25 Gy h\(^{-1}\) at 20 cm distance.\(^{26}\) This five-year simulation did not take into account decay rates after waste emplacement and as such, this figure may represent the upper limit of dose rates near canister surfaces. Much lower predictions for gamma radiation and neutron doses of 2 Gy h\(^{-1}\), decreasing by an order of magnitude over 200 years, have also been made, albeit for different wastes, engineered barriers and host geology (Figure 2).\(^{27,28}\) Despite this, total absorbed doses are still significant, with 0.7 MGy gamma and 140 MGy alpha predicted after 10\(^4\) years. Other predictions of dose rates at waste canister surfaces and in backfill material include 52 Gy h\(^{-1}\) and 71 Gy h\(^{-1}\).\(^{29,30}\) Lower estimates of 500 mGy h\(^{-1}\) have also been predicted, namely for the disposal of Swedish spent nuclear fuel.\(^{31}\)

With regard to UK disposal concepts for ILW, little to no information is available for dose rates surrounding waste packages in specific disposal scenarios. Rather, the focus has been on the requirement for shielding during handling and transportation, with the need being determined by the waste classification, e.g. ILW.\(^{32}\) In addition, concept summaries for HLW and spent fuel disposal in the UK highlight the need for shielding, but specific values for dose rates at waste package surfaces, i.e. the ‘near-field’, have not been defined.\(^{33,34}\) Equally, details of dose rates in the far-field are also not constrained for a UK disposal scenario. Despite this, a safety assessment of the SR-site, Sweden (for which key radionuclides are anticipated to be the same as in a UK spent fuel inventory), summed dose rates in the far-field remained below typical Swedish background radiation (1 mSv; annual effective dose) for a worst-case scenario in which canisters were breached at the time of emplacement.\(^{34}\)

It is therefore challenging to predict the coincidence of such doses with microbial populations, as this will largely depend on host geology, repository depth and the chosen engineered barrier and backfill system.\(^{13}\) These in turn influence the parameters that will dictate the development and composition of microbial communities, e.g. temperature, pressure, water availability, pH, carbon source availability, electron donor and acceptor concentrations. However, waste packages will likely not be sterile at the time of emplacement. In addition, ingress of groundwater over several thousand years may lead to inoculation of the near-field with microorganisms.\(^{35,36}\)
2.3 The interaction of ionizing radiation with microorganisms

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 radioactive waste disposal and bioremediation scenarios, previous research has focused on the sensitivity of species to radiation fluxes. DNA and proteins appear particularly susceptible to ionising radiation, 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 for microbe-metal-radionuclide interactions are not well defined.

Studies of the wider biological system, including the extracellular environment, collectively suggest that radiation may lead to the production of electron donors, either by the radiolysis of water (e.g. H₂) or via radiolysis of organic substrates. Similarly, alternative electron acceptors may also be generated or destroyed via radiation driven redox transformations. Selected processes are summarised in Figures 3 and 4 and highlight where information is lacking.

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 limited. However, it is possible that such radiation driven processes are likely to be most important in the immediate near-field of a GDF. Here, reactions (such as radiolysis) that generate reactive chemical species, alter environmental redox potentials and generate novel substrates will be most prevalent where radionuclide concentrations and associated dose rates are highest. Though the importance of these reactions to microbial activities depends on the incidence of microbial populations surrounding emplaced waste, which is poorly constrained.

It is of course possible that stable products of reactions involving radiation (i.e. not radical products of radiolysis), e.g. degraded organics, may be persistent in the environment and could potentially migrate to the far-field. Here, interactions with microorganisms may be more important due to increased microbial activity associated with more favourable conditions than in the engineered barriers of the near-field. Whilst such instances may occur in zones absent of radiation fluxes, they are nevertheless facilitated by ionizing radiation surrounding the waste. However, it should be stressed that there is a significant lack of detail regarding the potential for such reactions to occur, and their importance to evolution of biogeochemical conditions surrounding a GDF.

In light of the uncertainty regarding the impact of radiation on microbial processes pertinent to geological disposal, the project that this report summarises sought to characterise several possible interactions between radiation, bacteria and the substrates they metabolise. These
experiments are highlighted by the aims and objectives of Section 1, and are described in detail in the following sections. A focus has been placed on key Fe(III)-reducing species because of their well characterized ability to enzymatically reduced radionuclides and drive redox potentials that likely favour precipitation of priority radionuclides. In addition, the possibility of significant quantities of Fe (compared to other electron acceptors for microbial metabolism) in the engineered barrier system of a GDF, in the mineralogy of a potential host rock and indeed in the waste itself, further highlights the need to provide more detail on radiation effects on bacterial Fe metabolism.

Figure 3. The direct interaction of ionizing radiation with biologically important molecules. \(1,40,59,62–64\) Question marks represent unknown or uncharacterised mechanisms and/or the extent to which they contribute to the resultant effect.
Figure 4. The indirect interaction of ionizing radiation with biologically important molecules.\textsuperscript{1,41,54,62–73} Question marks represent unknown or uncharacterised mechanisms and/or the extent to which they contribute to the resultant effect.
3. Characterisation of the cellular physiology of an Fe(III)-reducing microorganism

Dissimilatory iron-reducing bacteria (DIRB), such as Shewanella spp. are capable of coupling the oxidation of organic matter to the reduction of a range of electron acceptors, including metals, anions and radionuclides.\textsuperscript{74–76} First, these characteristics may present the opportunity for the use of such species in bioremediation applications as a more versatile and cost-effective alternative to physicochemical methods of remediation.\textsuperscript{2,23} However, the utility of microorganisms in the remediation of highly radioactive environments will largely be governed by their ability to survive radiation stress, especially as many radionuclide contaminated sites may exhibit significant radiation fluxes.\textsuperscript{38,77–79} Second, as discussed previously, Fe(III)-reducing microorganisms may influence the redox potentials in the near field and in the disturbed zone around a GDF. As noted before, the environments in which such processes may occur may be subject to significant radiation fluxes and there is an interest in understanding the physiological status of Fe(III)-reducing organisms throughout and after irradiation.

To address both these points, the impact of acute doses of ionizing radiation on the metabolism of a model subsurface Fe(III)-reducing bacterium, Shewanella oneidensis MR-1, was characterised.\textsuperscript{80} This model species was selected as its metabolism has already been well characterised and its genome has been sequenced, permitting analysis of changes to (predicted) proteins after treatment with radiation.\textsuperscript{22,81–84} The whole cell metabolism of S. oneidensis was profiled via Fourier transform infrared (FT-IR) spectroscopy after exposure to acute X-radiation doses (12 to 95 Gy). Changes to the levels of specific biomolecules and proteins were quantified using FT-IR spectroscopy and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in order to determine how such changes underpin the response of this organism to radiation.

Prior to analysis of metabolism, the impact of X-radiation on the growth and viability of S. oneidensis was assessed. The dose yielding 10% survival was \textasciitilde 80 Gy which is similar to a previous study which observed 10% survival after 70 Gy from a \textsuperscript{60}Co gamma source.\textsuperscript{69} Thus, X-radiation is a good analogue for studying the effects of gamma radiation.

Growth curves revealed a dose-dependent increase in lag-phase duration (Figure 5), likely explained by a decrease in active biomass immediately after irradiation.\textsuperscript{80} Despite this, biomass yields recovered to the same levels as noted in non-irradiated controls across all doses measured, suggesting a microbial population may recover to the same biomass levels as seen in non-irradiated populations. However, it is unclear from this analysis what effect, if any, radiation induced changes to metabolism may contribute to the observed growth effects.
Figure 5. Growth, survival and extension of lag phase in X-irradiated cultures of *S. oneidensis* MR-1. (A) Growth profiles of aerobic cultures of *S. oneidensis* MR-1 (30°C) after exposure to 12, 24, 48, 72 and 95 Gy X-radiation (0.79 Gy min⁻¹). Irradiations began at t = 0. Data points show mean of triplicate batch cultures and error bars depict 95% confidence intervals. (B) 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. (C) Survival of *S. oneidensis* MR-1 exposed to acute doses of X-radiation. Cultures were irradiated in the growth medium described above, serially diluted in phosphate buffered saline and plated on to solid growth medium, Error bars depict standard error of the mean CFU ml⁻¹.
To assess this, samples were taken from cultures immediately after irradiation during the lag phase, at the mid-exponential growth phase and at the stationary growth phase (after the maximum yield of biomass was achieved) and were analysed via FT-IR spectroscopy. Principal component and discriminant function analysis (PC-DFA) of the metabolic fingerprints of irradiated cultures indicated that changes to the cultures are greatest immediately after irradiation, before cells have time to recover. Partial least-squares regression (PLSR) of the lag-phase data revealed an element of dose-dependence to metabolic changes, however, such changes are not easily predictable, suggesting that indiscriminate radiation damage may be inflicted on a large array of cellular targets. In addition, PLSR of exponential and stationary growth phase samples suggest that metabolic characteristics as a result of irradiation were preserved throughout subsequent generations of the culture despite irradiated cultures displaying recovery of biomass levels.

To assess the changes to key biomolecules immediately after irradiation that may characterise the irradiated phenotype, FT-IR spectral intensities characteristic of proteins and lipids in irradiated samples were quantified. Decreases in the protein/lipid ratio were evident after irradiation and exhibited dose dependence. This observation was primarily related to increases in lipid-associated intensities in the IR spectra of irradiated cultures, namely the CH vibration and CH₂/CH₃ asymmetric stretch. Previous studies of radiation damage in lipids suggest that this could be related to significant oxidation of lipids via hydroxyl radical addition or hydrogen abstraction. However, an increase in all three of these bonds is unlikely and thus these changes may not be related to specific oxidation reactions, rather, this observation may be related to increases in lipid metabolism throughout irradiation.

In order to characterise the extent of protein damage in irradiated S. oneidensis, MALDI-TOF-MS was used to examine changes in the levels of specific proteins. After irradiation with 12 Gy, the lowest dose used in this study, there appeared to be a general increase in proteins, namely a carbon storage regulator homolog and a 50S ribosomal subunit. The reason for this response is unclear, though it may be related to increased protein turnover and subsequent recovery at lower doses as suggested by PC-DFA applied to this data. In addition, expression of a protein encoded in the lambda phage (viral DNA) of S. oneidensis was increased. The induction of phage genes has been observed previously in the ionizing radiation response of this organism and the subsequent induction of the lytic cycle has been suggested as a contributor to the radiation sensitivity of this organism.

Unlike treatment with 12 Gy, irradiation with 95 Gy resulted in a general decrease in the levels of detectable proteins. These include a ribosomal protein subunit, as described above; an uncharacterised lambda phage protein and several uncharacterised proteins. Such decreases may be related to down regulation of genes associated with protein metabolism, however, at this higher dose, this could equally be related to protein damage via oxidation and carbonylation reactions for example.
In contrast to this general decrease in proteins after 95 Gy irradiation, increased levels of a putative cold shock protein or sulphur carrier protein were observed. Cold shock proteins act as RNA chaperones and gene regulators and have roles in the response to various environmental stresses and as such, the up-regulation of this protein may be a reaction to cellular radiation stress.

Furthermore, PC-DFA applied to MALDI-MS data suggested that, whilst the proteome of a culture irradiated with 12 Gy appeared to show recovery throughout the exponential and stationary growth phases, the 95 Gy treated culture displayed preserved or exacerbated alteration to protein levels. This observation may be related to initial high-levels of metabolic damage received at the higher dose, which perturbs metabolism throughout growth or due to gene expression that may be persistent throughout successive generations of the culture. Collectively, these observations indicate that radiation induced changes to protein levels in *S. oneidensis* exhibit dose dependence, which also impinges on the phenotype of latter growth phases.

![Figure 6. Fe(III) reduction by irradiated *S. oneidensis* MR-1.](image)

Cultures of *S. oneidensis* were grown aerobically in tryptic soy broth (30°C; 130 rpm) to late log–early stationary phase. Biomass was harvested and washed twice in sterile 30 mM sodium bicarbonate buffer prior to irradiation with 50 Gy X-radiation (rad). Immediately after irradiation, cell suspensions were driven anoxic with an 80:20 gas mix of N2:CO2 prior to inoculation into an anoxic medium containing 20 mM lactate as electron donor, 50 mM Fe(III) as poorly crystalline insoluble Fe(III) oxide and 30 mM sodium bicarbonate. The endogenous electron shuttle, riboflavin (Rf; 10 μM), which is known to be secreted by *S. oneidensis*, was added to media post-irradiation as an electron shuttle where necessary. Fe(II) concentrations were determined by ferrozine assay after extraction with 0.5 N HCl. Error bars depict standard error of the mean of triplicate experiments.
In order to characterise the respiratory capability of *S. oneidensis* post-irradiation, the reduction of amorphous Fe(III) oxyhydroxide (a model for solid phase Fe(III), to which extracellular electron transfer by *S. oneidensis* is well described)\(^90\) by a culture irradiated with 50 Gy X-radiation was assessed (Figure 6). Despite a significant reduction in active biomass, irradiated cultures exhibited a doubling in the extent of Fe(III) reduction.\(^80\) The reason for this is currently uncharacterised, though it may be related to the general up-regulation of metabolism immediately after irradiation, as described earlier, or due to physical damage to the cell structure, which may facilitate extracellular electron transfer.

In summary, these results suggest that ionizing radiation inhibits the viability and growth of *S. oneidensis* cultures. Observed growth effects are likely controlled by both viability and metabolic changes; for example, alteration to lipid and protein metabolism throughout irradiation, which may also be preserved throughout multiple generations despite biomass recovery. These metabolic changes may have implications for microbial population dynamics in subsurface environments where doubling times are very long, e.g. limited growth and metabolic changes may persist in the order of years to decades. Furthermore, as protein and lipid levels are fundamental to the integrity and functionality of membranes (where electron transfer proteins are predominantly localised), ionizing radiation may have significant implications for the long-term metabolism of Fe(III)-reducing bacteria, such as *S. oneidensis*. This may include their ability to reduce Fe(III) and radionuclides, such as U(VI), which may promote radionuclide precipitation and removal from groundwater in the near-field environment of a geological disposal facility.

4. Radiation enhanced reduction of Fe(III) oxides

In addition to the cellular environment, the impact of radiation on the availability of extracellular electron acceptors was assessed.\(^91\) The packaging of intermediate level wastes in the UK will feature iron rich container materials, such as stainless steel.\(^92\) Indeed, HLW and spent fuel disposal concepts in other countries may also feature a steel/iron container, e.g. the Swiss disposal concept.\(^93\) These containers will likely be subject to significant doses of ionizing radiation and subsequent reactions between radiolysis products and steel may generate a range of iron oxides, including Fe\(_2\)O\(_3\) and FeOOH.\(^41,94\) In addition, some waste-forms may contain large quantities of carbon steel and its corrosion products along with iron hydroxide floc from effluent treatment.\(^92\)

Radiation damage to these iron bearing materials could result in alterations to the availability of these phases for microbial Fe(III) reduction. To assess this, oxic ferrihydrite and hematite suspensions were irradiated with 1 MGy gamma radiation, equivalent to total absorbed doses after >10\(^5\) years in a HLW geological repository.\(^27,30\) Ferrihydrite is a poorly crystalline Fe(III)-(oxy)hydroxide that is considered readily available for microbial Fe(III) reduction and was used as an analogue for amorphous iron hydroxides that may be present
in waste-forms. Hematite ($\text{Fe}_2\text{O}_3$), on the other hand, is a crystalline iron oxide that is typically recalcitrant to reduction by Fe(III)-reducing species. As such, hematite was used as an analogue for more crystalline iron oxide corrosion products. Both these phases could be components of the evolving mineralogy of the near field of a GDF.

Radiation induced alterations to the mineralogy of these phases were probed using transmission electron microscopy (TEM) and selected area electron diffraction (SAED), along with Mössbauer spectroscopy. Changes to the bioavailability of Fe(III) in these phases was assessed by incubating irradiated mineral suspensions with cultures of $S. \text{oneidensis}$ and determining Fe(III) reduction via 0.5 N HCl extractions of the mineral suspensions, followed by spectrophotometric determination of Fe(II) using Ferrozine assay.

**Figure 7.** Transmission electron micrographs of (a) nonirradiated ferrihydrite and (c) irradiated ferrihydrite. The corresponding SAED pattern for nonirradiated 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.
Electron diffraction revealed that gamma radiation led to an increase in crystallinity of the ferrihydrite starting material (Figure 7). Specifically, reflections attributable to akaganeite (a Cl-bearing ferric hydroxide) were evident in SAED patterns and conversion to this phase was supported by increases in the quadrupole splitting of the Mössbauer spectrum of the irradiated material.

Ferrihydrite is considered readily available for microbial Fe(III) reduction, however, incubation of the irradiated material with *S. oneidensis* led to an increase in the rate and extent of Fe(II) production, despite an apparent increase in the crystallinity of this material as a result of irradiation. Fe(III) in akaganeite has previously been demonstrated to be reduced quicker than ferrihydrite by *Geobacter sulfurreducens* in the presence of an electron mediator. Indeed, we only observed this phenomenon in the presence of an added endogenous electron shuttle, riboflavin.

Powder X-ray diffraction (XRD) of the bioreduced solids from non-irradiated systems indicated that biogenic Fe$^{2+}$ was incorporated into magnetite and siderite as would be expected. Whilst in the irradiated systems, however, Fe$^{2+}$ was incorporated into magnetite, siderite and ferrous hydroxy carbonate. This latter phase has been observed previously as a stable transformation product of biogenic magnetite resulting from the bioreduction of a ferrihydrite and akaganeite mixed substrate. Indeed, the production of carbonate phases in general has been linked to increased rate and extent of Fe(III) reduction and is not surprising in this study given the use of a bicarbonate buffer. These observations are, therefore, consistent with the hypothesis that irradiation led to the alteration of ferrihydrite to a phase similar to akaganeite, followed by the enhanced rate and extent of Fe(III) reduction.

For hematite, Mössbauer spectroscopy revealed that irradiation led to emergence of a paramagnetic Fe(III) component that comprised approximately 45% of the material (Figure 8). Whilst this new phase was not further defined, its production was associated with a threefold increase in Fe(III) reduction during incubation with *S. oneidensis*. This, alongside the decrease in the contribution of this phase to the bioreduced solids, suggests that this phase was likely a poorly crystalline ferric oxide that was readily available for microbial Fe(III)-reduction. XRD and Mössbauer spectroscopy confirmed that the biogenic Fe$^{2+}$ was incorporated into siderite.
Mössbauer spectroscopy revealed the emergence of a poorly crystalline Fe(III) phase after irradiation. As iron-based materials will be prevalent in waste-forms, barrier systems and infrastructure of a geological disposal facility, corrosion of these materials may lead to an increase in the biogeochemical cycling of this Fe. These results suggest that microbial Fe(III)-reduction in such environments may be enhanced by radiation damage (Figure 9). Stimulation of Fe(III)-reducing microbial communities by radiation could promote the enzymatic or biogenic Fe(II) mediated reduction and subsequent precipitation of redox-sensitive radionuclides. Thus, such processes may play an important role in controlling radionuclide migration.

Figure 8. Mössbauer spectroscopy revealed the emergence of a poorly crystalline Fe(III) phase after irradiation.

Figure 9. Schematic summary showing enhanced microbial reduction of hematite after gamma irradiation.
5. The impact of low-dose rate gamma radiation on microbial communities and biogeochemical processes

The experiments described previously provide valuable information on how ionizing radiation may impact on the interaction between microorganisms and potential electron acceptors. Despite this, environments that will receive radiation fluxes will be more complex, with a range of electron donors and acceptors, complex mineral assemblages, along with a potentially diverse microbial community. In addition, dose rates in contaminated environments and in the near field of a geological disposal facility will be much lower than those used in previous experiments (Figure 2).

Accordingly, microcosms were constructed using sediments taken from outside the UK Sellafield reprocessing site boundary and using a synthetic groundwater representative of the site (Table 1). This material was selected due to the extensive experience of the University of Manchester Geomicrobiology research group in characterising the biogeochemistry and microbial communities of sediments from this area. The microcosms were irradiated over an eight-week period using dose rates representative of HLW canister surfaces in the near field of a geological disposal facility. Two dose rates were selected: 0.5 and 30 Gy h⁻¹, resulting in total absorbed doses of 0.6 and 38.6 kGy over the eight-week irradiation period. Whilst these dose rates may be higher than predicted for ILW, the total absorbed doses may still be relevant for ILW. For each treatment, two sets of sediment microcosms were prepared with and without added lactate and acetate (7 mM each) as electron donors and carbon sources, in order to simulate conditions with and without available organic carbon. Biogeochemical processes, i.e. electron donor and acceptor usage, were monitored during irradiation and throughout a subsequent 220-day recovery period, and samples were analysed for microbial phylogenetic diversity at selected time points.

Table 1. Initial microcosm compositions and treatments. Lactate and acetate were added, where required, to give the final concentrations shown below.

<table>
<thead>
<tr>
<th>Expt system</th>
<th>Mean dose rate (Gy h⁻¹)</th>
<th>Mean total absorbed dose (kGy)</th>
<th>Electron donor amendment³</th>
<th>Lactate (mM)</th>
<th>Acetate (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment + electron donor</td>
<td>Nonirradiated</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.6</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>38.6</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Sediment</td>
<td>Nonirradiated</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>38.6</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Sediment + G. sulfurreducens</td>
<td>Nonirradiated</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>38.6</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

³ Lactate and acetate were added, where required, to give the final concentrations shown.
⁴ ± 10% relative standard deviation.
In non-irradiated control and 0.5 Gy h\(^{-1}\) irradiated microcosms containing added lactate and acetate, electron acceptor usage proceeded in the order nitrate > Fe(III) > sulphate. Whilst irradiation with 0.5 Gy h\(^{-1}\) did not impact upon the rate nor extent of these processes, irradiation with 30 Gy h\(^{-1}\) did lead to restricted rates of Fe(III) reduction and sulphate reduction. This observation suggested that radiation may have led to decreased viability of Fe(III)-reducing microorganisms as a result of radiation toxicity. Despite this, Fe(II) generation and sulphate removal did recover to the same level as in control treatments after a 220 day recovery period, suggesting that some organisms capable of respiring these electron acceptors were able to survive, despite a total absorbed dose of 38.6 kGy.

**Figure 10.** Concentrations of lactate, acetate, propionate, formate, and malate in microcosms containing added lactate and acetate (final added concentrations of 7 mM each). The gray shaded area indicates the duration of the irradiation.
Sulphate concentrations were observed to increase in microcosms during the irradiation period of 30 Gy h\(^{-1}\) and this release of sulphate may have masked actual measurements of levels of sulphate reduction in treated systems compared to control systems. It is possible that this generation of sulphate may be due to release of sulphate from dead biomass, or via radiation induced mineralization processes, or due to oxidation of sulphide minerals as a result of oxidizing radiolysis products.\(^{71,103-105}\)

In all control and 0.5 Gy h\(^{-1}\) treated systems containing added carbon, lactate and acetate were removed from solution after 100 days, consistent with their use as an electron donors for microbial respiration (Figure 10). However, in 30 Gy h\(^{-1}\) treated systems, acetate concentrations were observed to increase until 150 days, followed by its subsequent removal from solution. Similar processes occurred in control and 0.5 Gy h\(^{-1}\) treated systems, with the generation and subsequent removal of propionate. Whilst it is possible that this production may be related to radiolysis of complex organics, it is more likely that such processes were a result of fermentation. The differential rates in production and removal of various organic acids from solution between control and 30 Gy h\(^{-1}\) treated systems is, again, likely related to radiation toxicity at the higher dose rate.

Amplification and sequencing of 16S rRNA genes revealed a diverse phylogeny of bacteria in the starting sediment. Members of the Acidobacteria and Proteobacteria dominated initially, however, after 147 days in both control and 0.5 Gy h\(^{-1}\) treatments (56 days irradiation plus 91 days recovery), when organic acids had been consumed and no further Fe(III) reduction was observed, there was a marked increase in members of the Bacteroidetes and Firmicutes. Many species of bacteria affiliated with these phyla are capable of metabolising the organic acids added to these experiments, e.g. via the fermentation of lactate.

Analysis of phylogenetic diversity was also performed on samples from 30 Gy h\(^{-1}\) treatments, taken after 147 days when Fe(II) concentrations were less than half that of controls and acetate concentrations were approximately 12 mM (0 mM in controls and 0.5 Gy h\(^{-1}\) treatments). There was a loss of diversity, with two species of the Firmicutes phylum representing 91% of the total microbial community: an uncultured Clostridiaceae bacterium and a close relative of a Clostridium bowmanii species. Members of the Clostridial family catalysed a mixed acid fermentation, and, as before, the emergence of these species is likely a result of ongoing fermentative processes in these systems. In addition, many such species are also spore-formers, allowing them to survive a range of environmental stresses. As such, it is likely that these species are radiation resistant members of the sediment community and may represent species capable of dominating microbial communities in environments with high radiation fluxes and with fermentable substrates, such as lactate.
In contrast to systems containing added carbon, irradiation of unamended systems with 0.5 Gy h⁻¹ gamma radiation resulted in an enhanced rate and extent of Fe(III)-reduction (Figure 11). Ion chromatography indicated that this effect may be related to radiolysis of nitrate, favouring the early onset of Fe(III) reduction in this treatment in the absence of this competing electron acceptor. Indeed, nitrate concentrations after irradiation were even lower in 30 Gy h⁻¹ treatments, however, a decrease in viability of Fe(III)-reducing species may have precluded enhanced Fe(III)-reduction at these higher doses.

Figure 11. Concentrations of nitrate, 0.5 N HCl extractable Fe(II), and sulfate in microcosms containing no added electron donor. The gray shaded area indicates the duration of the irradiation.

This enhanced Fe(III)-reduction in the 0.5 Gy h⁻¹ treated systems was reflected by the significant enrichment of known Fe(III)-reducing species in this system after 147 days (after the onset of Fe(III) reduction in all treatments, albeit when Fe(II) generation was significantly higher in the 0.5 Gy h⁻¹ treatment). *Geothrix* and *Geobacter* species represented 22% and 3%, respectively, of the total microbial community.

Surprisingly, known Fe(III)-reducing species were also enriched in the 30 Gy h⁻¹ treatment after 147 days, despite Fe(II) generation being similar to that of control samples. *Geobacter* species represented 18% of the total microbial community, demonstrating that Fe(III)-reduction was still possible in sediments that had received a total absorbed dose of nearly 40 kGy. Indeed, the enrichment of Fe(III)-reducing bacteria suggest that these sediments...
may also be poised for enhanced Fe(III)-reduction, though this was likely precluded by a decrease in metabolic activity associated with radiation stress.

Collectively, these observations suggest that a Sellafield-type sediment microbial 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 even 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. Further work would be required to assess the likely impact of the dual stress of radiation and high pH expected in the cementitious near field for ILW disposal of a GDF.


The results summarised by this report and documented in the aforementioned thesis present a range of evidence for the impact of ionizing radiation on microorganisms pertinent to the geological disposal of radioactive waste. Collectively, the experiments of this thesis provide evidence for a range of impacts of ionizing radiation on microorganisms and the substrates they respire. 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 involving abiotic alteration of alternative electron acceptors. Radiation could also potentially enhance microbial activities, via promoting an increase in the bioavailable fraction of organic electron donors. Potentially, such processes may promote the development of redox potentials which favour the reduction of some 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 such radionuclides, this could play an additional role in limiting the migration of some radionuclides from a geological disposal facility.

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, alongside different species of microbes which display a range of radiation sensitivities, gives rise to complex interactions between microbial communities and their environment. In order to directly incorporate our findings into site
selection criteria and post-closure performance assessments, a comprehensive assessment of absorbed doses and dose rates may be considered. However, the observations documented here suggest that biogeochemical processes may not be restricted by dose rates expected in radioactive waste disposal scenarios. Indeed, ionizing radiation fluxes may even provide the basis of novel ecosystems capable of exerting important controls on the biogeochemical evolution of such a facility.

Further work to explore the impact of radiation upon a range of sediment/host rock microbial communities may be of benefit, along with an assessment of radiation damage in specific microbial species e.g. species capable of catalysing metal corrosion, such as sulphate-reducing bacteria.

Much of the experimental work of this study has focussed on the impact of ionizing radiation on electron-accepting processes. However, some intermediate-level waste packages may contain high amounts of organic material, which may (as noted above) also be subject to radiation damage, potentially yielding organic substrates available for microbial respiration. An investigation of the radiolytic degradation products from organic waste materials and from organic polymeric waste encapsulants that may be used and their availability as electron donors, would provide further information on radiation-driven processes which may stimulate the microbial community in and around a GDF.

In addition, the tandem effect of ionizing radiation with multiple other environmental stressors expected in the vicinity of a GDF, namely, high pH (for ILW), temperature (for spent fuel), pressure, and limited availability of nutrients/respirable substrates could also be explored.

7. Acknowledgements

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