The Biodegradation of Isosaccharinic Acid

A thesis submitted to the University of Manchester for the degree of

Doctor of Philosophy

in the Faculty of Engineering and Physical Sciences

2017

Gina Kuippers

School of Earth and Environmental Sciences
[This page is left intentionally blank]
Contents

Abstract 19
Declaration 21
Copyright statement 22
Acknowledgements 23
1. Thesis content and layout 25
   1.1. Project relevance 25
   1.2. Research objectives 27
   1.3. Thesis structure and publication status 27
2. Background and literature review 31
   2.1. The Nuclear Fuel Cycle 31
   2.2. Nuclear Waste Disposal 33
   2.3. Radioactive inventory 37
   2.4. Post-closure GDF evolution 40
   2.5. Cellulose chemistry & degradation 45
      2.5.1. Microbial degradation of cellulose 46
      2.5.2. Radiolytic degradation of cellulose 51
      2.5.3. Chemical degradation of cellulose 51
   2.6. Isosaccharinic acid 56
      2.6.1. Structure and speciation of ISA 57
      2.6.2. Solubility and sorption 60
      2.6.3. Complexation of ISA with radionuclides 62
      Nickel 65
      Americium, Europium & Lanthanum 65
      Thorium 66
      Neptunium 67
      Uranium 67
      Technetium 68
   2.7. Microbial-radionuclide interactions 69
   2.8. Thesis outline and objectives 75
   2.9. References 78
3. Methodology 91
   3.1. Materials 91
3.1.1. Freshwater medium after Lovley et al. (1984) 91
3.1.2. Sulfate-reducing bacterial growth medium 92
3.1.3. α-isosaccharinic acid preparation 92
3.1.4. Ferrihydrite preparation 94
3.2. Culturing techniques 95
3.2.1. Microcosm experiments 95
3.2.2. Isolation of bacteria 95
3.3. Aqueous geochemical analyses 96
3.3.1. Turbidity measurements 96
3.3.2. pH and Eh 97
3.3.3. Light Microscopy 97
3.3.4. Ferrozine assay 98
3.3.5. Bomo PADAP assay 98
3.3.6. Ion Exchange Chromatography 99
3.3.7. Inductively Coupled Plasma Mass Spectrometry & Atomic Emission Spectrometry 100
3.4. Microbial community analysis 101
3.4.1. DNA extraction 102
3.4.2. Agarose gel electrophoresis 102
3.4.3. Polymerase chain reaction & pyrosequencing 103
3.4.4. Sequencing & data analysis 104
3.5. Mineralogical analyses 106
3.5.1. X-ray Diffraction analysis 106
3.5.2. X-ray Absorption Near Edge Spectroscopy & Extended X-ray Fine Absorption Spectroscopy 107
3.5.3. Environmental Scanning Electron Microscopy 110
3.5.4. Transmission electron microscopy 112
3.5.5. PHREEQC modelling 113
3.6. References 113
4. Microbial degradation of isosaccharinic acid under conditions representative for the far field of radioactive waste disposal facilities (research article) 117
4.1. Abstract 118
4.2. Introduction 118
4.3. Material & Methods 121
4.4. Results & Discussion 124
| 4.5. | Conclusion | 131 |
| 4.6. | Acknowledgements | 132 |
| 4.7. | References | 132 |
| 5. | Microbial reduction of Fe(III) coupled to the biodegradation of isosaccharinic acid (ISA) (research article) | 135 |
| 5.1. | Abstract | 136 |
| 5.2. | Importance | 136 |
| 5.3. | Introduction | 137 |
| 5.4. | Materials and methods | 139 |
| 5.5. | Results | 142 |
| 5.6. | Discussion | 150 |
| 5.7. | Acknowledgements | 152 |
| 5.8. | Associated Content | 153 |
| 5.9. | Author information | 153 |
| 5.10. | References | 153 |
| 5.11. | Supplementary information | 160 |
| 6. | The biogeochemical fate of nickel during microbial ISA degradation; implications for nuclear waste disposal (research article) | 165 |
| 6.1. | Abstract | 166 |
| 6.2. | Introduction | 167 |
| 6.3. | Results | 169 |
| 6.4. | Discussion | 175 |
| 6.5. | Methods | 180 |
| 6.6. | Supplemental material | 183 |
| 6.7. | Acknowledgements | 183 |
| 6.8. | Author contributions | 183 |
| 6.9. | Competing financial interests | 184 |
| 6.10. | References | 184 |
| 6.11. | Supplementary information | 191 |
| 7. | Biomineralization of uranium-phosphates fuelled by the microbial degradation of isosaccharinic acid (ISA) (research article) | 197 |
| 7.1. | Abstract | 198 |
| 7.2. | Introduction | 199 |
| 7.3. | Materials and methods | 201 |
| 7.4. | Results | 205 |
I. Figures

Figure 2.1. The closure of the nuclear fuel cycle. After usage of the uranium there are two pathways for the spent fuel: It can be immediately disposed of, or reprocessed to recover usable elements and fuel wastes can be subject to a geological disposal facility (GDF) (adapted from Argonne National Laboratory, 2016).

Figure 2.2. Schematic illustration of a geological disposal facility indicating separate disposal areas for ILW/LLW and HLW/spent fuel (adapted from RWM, 2010b).

Figure 2.3. The radioactive waste inventory of the UK, showing the total radioactivity of the different waste categories as a function of time after GDF operations start at 2040. SF stands for spent fuel (adapted from RWM and NDA, 2015).

Figure 2.4. Total radioactivity in ILW as a function of time (adapted from DEFRA and NDA, 2008).

Figure 2.5. Evolution of the pH in cementitious porewaters in a GDF at 25°C with time as a result of cement dissolution and degradation (adapted from NDA, 2010b).

Figure 2.6. Redox cascade for the degradation of organic matter, describing a potential sequence of electron acceptors changing with time (but also with distance to the GDF), which correlate with a higher energy yield obtained by electron acceptor utilisation. Eh values from Millero, 1996.

Figure 2.7. Cellulose chain linked via β-1,4-glycosidic bonds (adapted from Gurunathan et al., 2015).

Figure 2.8. Simplified schematic of the enzymatic hydrolysis of amorphous and crystalline regions of cellulose by non-aggregating and aggregating cellulase systems (adapted from Ratanakhanokchai et al., 2013).

Figure 2.9. Microbial degradation of cellulose under anaerobic conditions in soils and freshwater sediments. Typically formed fermentation products include lactate, succinate and ethanol, which are further degraded by heterotrophic bacteria. In environments where nitrate, Mn(IV), Fe(III) or sulfate are present, the final products may differ (adapted from Humphreys et al., 2010).

Figure 2.10. Degradation mechanism under alkaline conditions to form either metasaccharinic acid (chemical stopping) or isosaccharinic acid (peeling-off) (adapted from Pavalars et al., 2003).

Figure 2.11. Structural formula of open-chain structures of α-ISA and β-ISA in Fischer’s projection and the corresponding lactones. In the literature α-ISA is also denominated as the erythro– and β-ISA as the threo– form (adapted from Glaus et al., 1999b).

Figure 2.12. Speciation of isosaccharinic acid as a function of –log[H⁺] in 0.1 mol dm⁻³ NaClO₄ at 23°C given as relative concentrations of ISA. The proton concentration is in mol dm⁻³ and
c° is equal to 1 mol dm⁻³. The ISAₜₒₜ is the concentration of HISA and ISA' (adapted from Brown et al., 2010).

Figure 2.13. Possible microbe-metal interactions with extracellular polymeric substances (EPS) that provide binding sites for metals (Me) and radionuclides (adapted from Wang et al., 2010).

Figure 2.14. Dominant oxidation state of key radionuclides at neutral pH, relevant for the geosphere surrounding a GDF (adapted from Brookshaw et al., 2012).

Figure 3.1. Steps of Ca(ISA)₂ synthesis. A - D show degradation reaction, forming Ca(ISA)₂ and other organic degradation products, which is via E boiling and F filtration steps, until G a final Ca(ISA)₂ product is obtained (brown marginal areas are other organics).

Figure 3.2. Principle of PCR: Left showing individual components and right showing one heating cycle (Bioninja, 2017).

Figure 3.3. Set up of a synchrotron beamline showing detection of fluorescence energy (Iₚ) and transmission energy (I) (Ravel and Newville, 2005).

Figure 3.4. XAFS spectrum of FeO (blue line) showing the XANES and EXAFS regions and the smooth background function (red line) with the edge-step indicated as Δµₒ(Eₒ) (after Ravel and Newville, 2005).

Figure 3.5. ESEM principle (http://www.technoorg.hu/news-and-events/articles/high-resolution-scanning-electron-microscopy-1/).

Figure 4.1: Biodegradation of ISA by aerobic microbial cultures at a pH of 7. ISA concentration: (a) test condition with active microbial inoculum; (●) sterile control (autoclaved).

Figure 4.2: Biodegradation of ISA by nitrate-reducing (A-C) and Fe(III)-reducing cultures (D-F) at a pH of 7. A) ISA concentration: (a) test condition with active microbial inoculum; (●) sterile control (autoclaved). B) Test sample: (●) nitrate; (□) nitrite. C) Ions in test condition: (□) ISA; (●) acetate. D) ISA concentration: (a) test condition with active microbial inoculum; (●) sterile control (autoclaved). E) Fe(II) ingrowth: (●) sterile; (□) test condition. F) Ions in test condition: (□) ISA; (●) acetate; (●) n-butyrate; (●) propionate.

Figure 4.3: Biodegradation of ISA by sulfate-reducing cultures (A-C) and in the absence of an added electron acceptor (D-E) at a pH of 7. A) ISA concentration: (a) test condition with active microbial inoculum; (●) sterile control (autoclaved). B) Sulfate concentration: (●) sterile; (□) test. C) Ions in test condition: (□) ISA; (●) acetate; (●) n-butyrate; (●) propionate. D) ISA concentration: (a) test condition with active microbial inoculum; (●) sterile control (autoclaved). E) Ions in test condition: (□) ISA; (●) acetate; (●) n-butyrate; (●) propionate; (●) formate.
Figure 4.4: Gas evolution during the biodegradation of ISA in sediment enrichment slurries containing 2 mM Ca(ISA)\(_2\) under different biogeochemical conditions as follows: (◼) sterile control sterile (autoclaved); (●) 24 mM NaNO\(_3\); (▲) 20 mmol L\(^{-1}\) Fe(III) oxyhydroxide; (▲) 12 mM Na\(_2\)SO\(_4\); (●) no added electron acceptor.

Figure 4.5: Molecular ecology (16S rRNA gene) analysis of microbial ecology of sediment inoculum and microbial enrichments. A) Alpha rarefaction plot showing number or observed species (distinct DNA sequences) in the sediment at day 0 (◼), and after aerobic incubation at day 21 (●), nitrate reduction at day 21 (●), Fe(III) reduction at day 28 (●) and 50 (●), sulfate reduction at day 21 (●) and day 58 (●), and with no added electron acceptor at day 21 (●) and day 58 (●). B) Microbial Community composition by phylogenetic classes in the corresponding samples.

Figure 5.1. Primary sediment enrichments for ISA-degrading, Fe(III)-reducing bacteria at pH values between 7 to 10. A) ISA consumption, B) acetate production and C) Fe(II) production. Symbols are: sterile control (autoclaved cells) dashed line (●); and active microbial cultures with solid line at (●) pH 7 (▲) pH 8; (●) pH 9; and (●) pH 10.

Figure 5.2. Geochemical results obtained from the stable enrichment for ISA-degrading, Fe(III)-reducing bacteria at pH 7. Results are shown for an enrichment set up with ISA, Fe(III) and an active inoculum: A) Ion concentrations [mM] for (◼) ISA, (●) acetate, (●) n-butyrate, (●) propionate and (●) ISA in sterile control added as comparison; B) Fe(II) ingrowth [mM] C) pH and D) Eh [mV] with symbols in B)-D) (◼) test condition; (●) sterile control; (▲) no ISA.

Figure 5.3. TEM (A-J) and ESEM (K) images with corresponding EDS analyses (E, F, G, H, L, M) after incubation. Sterile control shows unaltered ferrihydrite (A, E). In the microbially active enrichments, the mineral phases included crystal aggregates rich in C, O, Ca, Fe with d-spacings matching siderite (F, I), radial ordered crystals rich in Fe-Ca-P (C, G) with d-spacings matching vivianite and an amorphous Fe-S phase (D, H), and bacterially associated iron (arrows in J pointing at cells). ESEM imaging (K) revealed two crystalline structures in an amorphous matrix: (L) comprising Fe, Ca, O and a spherulitic shape identified as siderite and (M) comprising Fe, P, O and a prismatic shape identified as vivianite. X-axis in EDS images is in keV.

Figure 5.4. Molecular community (16S rRNA gene) analysis of stable enrichment cultures at pH 7, showing a sterile control; the ISA degrading, Fe(III)-reducing experiment after ISA degradation at 35 days and an ISA-fermenting control experiment after ISA depletion at 35 days.

Figure 5.5. Butyrate concentration in primary sediment enrichments for ISA-degrading, Fe(III)-reducing bacteria at pH values between 7 to 10. Symbols are: sterile control (autoclaved cells) dashed line (●); and active microbial cultures with solid line at (◼) pH 7 (▲) pH 8; (●)
pH 9; and (●) pH 10. Note that high error bars are as a result that butyrate was not detected in all triplicate bottles.

Figure 5.6. XRD patterns showing iron mineral biotransformations at pH 7-10. Graphs from top to bottom: Sterile control, pH 7, pH 8, pH 9 and pH 10.

Figure 5.7. Relative percentages of vivianite and siderite in the sediment incubations from pH 7 to 10, containing active microbial inocula. Sediment-derived minerals (quartz, anhydrite and albite) are not shown as they were expected to remain the same.

Figure 5.8. Solution chemistry of a stable (7 consecutive transfers) ISA-fermenting enrichment control sample at pH 7 without [no Fe(III)], grey dashed line is ISA concentration of sterile control.

Figure 5.9. Sulfate concentrations in a stable ISA-degrading enrichment culture at pH 7, supplemented with 25 mM Fe(III) (red line). Grey dotted line shows the sterile control.

Figure 5.10. XRD pattern of a precipitate collected from the seventh subculture of an ISA-degrading, Fe(III)-reducing culture at pH 7. Peaks are matched to those of vivianite (red) and siderite (blue) OCDD standards.

Figure 5.11. ESEM image of amorphous Fe(III) oxyhydroxide before the addition of an Fe(III)-reducing inoculum.

Figure 5.12. Light microscopic images of the stable enrichment culture of ISA-degrading, Fe(III)-reducing bacteria at pH 7 after 35 days. Cells were stained using Hoechst stain (DNA shown in blue or cyan). Images are showing: A) partially reduced fine grained Fe(III) oxyhydroxide; B) mainly hexagonal siderite crystals with vivianite crystal in top right corner; C) radial large vivianite crystal; D) two siderite crystals.

Figure 5.13. α-Rarefaction curves showing the species diversity from stable enrichment cultures at pH 7. Blue line: sterile sample; red line: ISA degrading, Fe(III)-reducing experiment; yellow line: ISA fermenting control experiment.

Figure 6.1. Geochemical changes in Ni-ISA biodegradation, Fe(III)-reducing experiment, showing A) pH; B) $E_h$; C) ISA concentration; D) Fe(II) ingrowth; E) acetate and F) butyrate. Symbols are: (●) sterile (autoclaved) control, (●) no inoculum control, and microbially active incubations with (●) 0 mM Ni, (▲) 0.1 mM Ni, or (●) 1.0 mM Ni.

Figure 6.2. ICP-AES analysis of Ni in solution [mM] in the Ni-ISA biostimulation, Fe(III)-reducing experiment. All bottles contained 4 mM ISA and 25 mM Fe(III) oxyhydroxide plus (●) 1 mM Ni only; (●) a sterile (autoclaved) microbial inoculum and 1 mM Ni; a microbially active inoculum with (●) 0.1 mM Ni or (▲) 1 mM Ni.

Figure 6.3. 16S rRNA gene sequencing data from the Fe(III)-reducing Ni-ISA degrading enrichment, sowing phylogenetic classes at selected time points. Shannon H biodiversity index on top of columns is a measure of microbial community diversity.
Figure 6.4. ESEM (A, B) and TEM (C) images of biominerals from the Fe(III)-reducing Ni-ISA degrading enrichment with 0.1 mM Ni with representative EDS profiles. A and B: Biominerals identified were (1) siderite, (2) vivianite and (3) an amorphous phase, comprising S, Fe, and Ni. C: (4) a crystalline area without Ni (4) and (5, 6) weakly crystalline areas with Fe, S and Ni.

Figure 6.5. Pre-test for Ni solubility in freshwater minimal medium (FWM). Tests at 0.1 mM and 1 mM concentrations include (i) FWM only; (ii) Fe(III), (iii) ISA, (iv) Fe(III) and ISA. 191

Figure 6.6. Sulfate concentration in Ni-ISA biostimulation, Fe(III)-reducing experiment. Symbols are: (●) sterile control, (▲) 0 mM Ni, and (■) 0.1 mM Ni. 191

Figure 6.7. α-rarefaction curves 16S rRNA gene sequencing data from Fe(III)-reducing Ni-ISA degrading enrichments showing maximum of distinct species per sample. 191

Figure 6.8. XRD patterns of Fe(III)-reducing Ni-ISA degrading enrichments showing: A) Sterile control, 1.0 mM Ni; microbially active with B) 0 mM Ni, C) 0.1 mM Ni, or D) 1.0 mM Ni; E) Abiotic control without microbial inoculum and with 1.0 mM Ni. 192

Figure 6.9. TEM mapping of a precipitate at the end of the Fe(III)-reducing Ni-ISA degrading enrichment at 0.1 mM Ni. Detected elements are displayed in maps showing O, Fe, P, Ca, Ni and S. 193

Figure 6.10. PHREEQC modelling output file showing Ni-ISA biostimulation, Fe(III)-reducing experiment with 0.1 mM Ni at incubation start (before bioreduction). The system is in equilibrium with ferrihydrite. Saturation indices show that vivianite, siderite and apatite will precipitate (positive value), but no Ni-containing form is expected to precipitate. 194

Figure 6.11. PHREEQC modelling output file showing Ni-ISA biostimulation, Fe(III)-reducing experiment with 0.1 mM Ni when bioreduced (negative pe). The system is in equilibrium with vivianite and millerite. Saturation indices show supersaturation with mackinawite. Thus the simulation indicates millerite and mackinawite will form, which will under natural conditions be a mixed form. 195

Figure 7.1. Solution chemistry of U(VI)-ISA incubation experiment analyzed by IC and ICP-MS showing ISA degradation with Fe(III) at A) 0.1 mM U and at B) 1 mM U and ISA fermentation at C) 0.1 mM U and at D) 1 mM U. Symbols are showing (■) ISA, (●) acetate, (▲) butyrate and (◆) U concentrations in microbially active experiments and (○) ISA in sterile (autoclaved) controls. 206

Figure 7.2. Microbial community fingerprinting of bioreduced U(VI) ISA incubation experiment with Fe(III) and with no Fe(IIII) at day 14 performed by using 16S rRNA gene sequencing. A 16S rRNA gene profile was also analyzed for the U(VI)-ISA incubation experiment with Fe(III) prior to incubation (start). 208
Figure 7.3. Uranium M_{IV}-edge high resolution (HR) XANES data showing in green: U(IV) standard, yellow: U(VI) standard, blue: ISA degradation, Fe(III)-reducing experiment at 1 mM U and red: ISA-fermenting experiment at 1 mM U.

Figure 7.4. Mineralogical analysis of ISA fermentation experiment at 1 mM U(VI) concentration: (A) ESEM image; B), C) TEM images; D) TEM EDS; E) non-phase shift corrected U L_{III}-edge EXAFS data and F) corresponding $k^3$ weighted Fourier transform of EXAFS data. ESEM and TEM images show spherulitic U-containing bioprecipitates which were fitted (dashed line) as uranyl-phosphate from the autunite group.

Figure 7.5. Mineralogical analysis of ISA biodegradation, Fe(III) reduction experiment at 1 mM U(VI) concentration showing A), B) TEM images; C) TEM electron diffraction pattern with rings identified at 2.4 Å, 2.2 Å and 1.62 Å; D) ESEM image; E) ESEM EDS analysis F) non-phase shift corrected Fourier transform of U L_{III}-edge EXAFS data and G) corresponding $k^3$ weighted EXAFS data. ESEM and TEM images show sheeted precipitates, partially folded up and containing U, which were fitted (dashed line) as a ningyoite.

Figure 7.6. ISA biodegradation control experiment with Fe(III).

Figure 7.7. Redox potential evolution in U(VI)-ISA enrichment experiments.

Figure 7.8. pH evolution in U(VI)-ISA enrichment experiments with and without added Fe(III).

Figure 7.9. Abiotic ISA control experiments in freshwater minimal medium (FWM) and with 1 mM U testing ISA solubility in experiments with microbial inocula and without the inoculum.

Figure 7.10. Relative Fe(II) ingrowth compared to total HCl-extractable iron measured with the ferrozine assay.

Figure 7.11. Total Fe in microcosms analyzed by the ferrozine assay after acidification in 0.5 N HCl or 0.25 N hydroxylamine-HCl (Lovley and Phillips, 1986a). Sterile control contains heat-killed microbial inoculum, 1 mM U and Fe.

Figure 7.12. Abiotic U control experiments in freshwater minimal medium (FWM) testing U solubility under experimental conditions.

Figure 7.13. $\alpha$-arefaction curves obtained from 16S rRNA gene sequencing after biodegradation of ISA showing maximum of distinct species per sample.

Figure 7.14. XRD patterns for abiotic experiments. First pattern showing U(VI)-ISA control with Fe(III) oxyhydroxide and a sterile inoculum, second pattern showing U(VI)-ISA control.
Figure 7.15. XRD patterns for experiments after U(VI)-ISA biodegradation. Standards are whitlockite, metaankoleite, siderite, vivianite and ranciete.

Figure 7.16. Uranium L_{III}-edge XANES spectra for U(VI)-ISA experiments with and without Fe(III) after bioreduction collected at beamline B18, Diamond Lightsource, Harwell.

Figure 7.17. Mineralogical analysis of U(VI)-ISA fermentation experiment at 0.1 mM U(VI) concentration: (A) TEM image; B) corresponding EDS; C) ESEM image; D) non-phase shift corrected U L_{III}-edge EXAFS data and E) corresponding k^3 weighted Fourier transform of EXAFS data. ESEM and TEM images show spherulitic bioprecipitates containing Ca-P-Na-U which were fitted (dashed line) as liebigite [Ca_2UO_2(CO_3)_3·11H_2O], a uranyl-carbonate.

Figure 7.18. TEM images showing microbial association with biominerals observed in the U(VI)-ISA fermenting experiment, while in the ISA-U(VI) degradation experiment with Fe(III) such observations were not made, probably due to the high Fe concentrations. A) Clusters of fibrous structure comprising high amounts of U (1) compared to the cell envelope where U was absent (2); C) U-containing precipitates around cell which could be internal or external; D), E) and F) show clusters of round shape containing mainly P and U which seem to be externally.

Figure 7.19. Mineralogical analysis of U(VI)-ISA Fe(III) reduction experiment at 0.1 mM U(VI) concentration showing A) non-phase shift corrected Fourier transform of U L_{III}-edge EXAFS data and B) corresponding k^3 weighted EXAFS data. ESEM and TEM images show sheeted precipitates, partially folded up and containing U, which were fitted (red line) with one oxygen shell containing 8 atoms and a phosphorus shell containing 1 atom.

Figure 7.20. TEM images of ISA-U(VI)-Fe(III) experiment showing cell envelopes. A) cell coated in Fe (TEM EDS below) and B) cells surrounded by partially reduced Fe(II)-precipitates.

Figure 9.1. Geochemical data for sediment incubation with ISA and sulfate at pH 7 to 10, showing A) ISA, B) redox potential [mV], C) sulfate and D) acetate. Symbols are: dotted line (●) sterile control (autoclaved); and solid lines are microbially active experiments at (▲) pH 7 (△) pH 8; (●) pH 9; and (◆) pH 10.

Figure 9.2. ESEM images of late log phase cells from ISA biodegradation experiment poised under sulfate-reducing conditions. Left image shows cells among fine-grained particles, containing Fe and S, right image shows cells surrounding a plated vivianite [(Fe_3PO_4)_2·8H_2O] crystal identified in EDS spectra (not shown).
Figure 9.3. 16S rRNA gene sequencing data from the initial sediment inoculum (T0) and the isolated ISA-degrading, sulfate-reducing consortium (Isolate) showing the microbial community diversity.

Figure 9.4. The class of Deltaproteobacteria in the ISA-degrading, sulfate-reducing consortium (Isolate) analysed by 16S rRNA gene sequencing.

Figure 9.5. Geochemical changes in ISA biodegradation experiment under sulfate-reducing conditions with Ni or U, showing A) ISA [mM]; B) acetate [mM]; C) sulfate [mM] and D) Ni/U [%] concentrations in solution. Symbols are (♀) Sterile control (autoclaved) at 1 mM U/Ni concentration; (♂) no cell control at 1 mM U/Ni; and microbially active experiments: (■) ISA and sulfate without U/Ni; (♀) ISA and sulfate at 0.1 mM U/Ni (▲) ISA and sulfate at 1 mM U/Ni concentration.

Figure 9.6. ESEM images of biominerals from Ni-ISA biodegradation experiment at 0.1 mM Ni concentration showing vivianite [(Fe$_3$PO$_4$)$_2$·8H$_2$O] crystal (top image) and sulfide aggregates containing Fe and Ni (middle and bottom image).

Figure 9.7. ESEM image of biovivianite from U-ISA biodegradation experiment at 1 mM U concentration.

Figure 9.8. TEM and ESEM images of bioprecipitates formed during the U-ISA biodegradation experiment at 1 mM U concentration showing two different mineral phases. A) ESEM and B) TEM images with C) corresponding EDS of U-containing phosphate mineral. D) lose aggregates with B) close up showing nanocrystalline structure in corresponding selected electron diffraction pattern with identified rings at 3.3 Å, 2.5 Å, 2.1 Å, 1.99 Å and 1.78 Å and F) EDS comprising mainly Fe, S and U.

Figure 9.9. Uranium L$_\text{III}$-edge XANES spectra for U(VI)-ISA experiment at 1 mM U concentration after biodegradation collected at beamline B18, Diamond Lightsource, Harwell.

Figure 9.10. A) A label, likely $^{13}$C, is introduced into a microcosm with an environmental culture and then incubated until analyses. B) After the incubation time some of the $^{13}$C label was incorporated and samples will be subjected to analysis, such as PCR and gene cloning, to reveal phylogenetic information about the microorganisms (modified after Dumont and Murrell, 2005).
II. Tables

Table 2.1. Key radionuclides in the nuclear fuel cycle with oxidation states and ways of retardation. From the decay of these radionuclides, future isotopes will arise that are not shown here. Bold italics indicate most stable oxidation states under oxidising conditions, while italics the most stable under reducing conditions (adapted from NDA, 2010f; Brookshaw et al., 2012).

Table 2.2. Best estimates for the radionuclide solubility enhancement factors (SEF) relative to Tc at thermodynamic equilibrium in the presence of varying ISA concentrations (modified after NDA, 2010f). Even though ISA is known to enhance the mobility of Pu, Pu is rarely important in the context of ISA complexation in ILW related to its availability.

Table 3.1. Composition of modified freshwater minimal medium; all compounds are given for dilution in 1 L deionised water (after Lovley et al., 1984).

Table 3.2. Composition of modified freshwater medium after 3.1 for enrichment of sulfate-reducing bacteria; all components are dissolved in 1 L deionised water.

Table 7.1. EXAFS fit parameters for U(VI)-ISA degradation experiments with Fe(III) as terminal electron acceptor and without Fe(III) added. The amplitude factor \( S_0^2 \) was fixed as 1.0 for each sample. Indices are ax for axial atoms and eq for equatorial atoms.

Table 7.2. F-test for U(VI)-ISA incubation experiment with no Fe(III) as an electron acceptor (fermentation) at 1 mM U. Fit No. 127 was chosen as best fit.

Table 7.3. F-test for U(VI)-ISA incubation experiment with Fe(III) as an electron acceptor at 1 mM U. Fit No. 362 was chosen as best fit.

Table 7.4 F-test for U(VI)-ISA incubation experiment with Fe(III) as an electron acceptor at 0.1 mM U. Fit No. 26 was chosen as best fit.
### III. List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AES</td>
<td>Atomic emission spectrometry</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSE</td>
<td>Backscattered electron</td>
</tr>
<tr>
<td>BWR</td>
<td>Boiling water reactor</td>
</tr>
<tr>
<td>Bq</td>
<td>Becquerel</td>
</tr>
<tr>
<td>CDZ</td>
<td>Chemically Disturbed Zone</td>
</tr>
<tr>
<td>CL</td>
<td>Core lipid</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
</tr>
<tr>
<td>DNA</td>
<td>Desoxyribonucleic acid</td>
</tr>
<tr>
<td>DP</td>
<td>Degree of polymerisation</td>
</tr>
<tr>
<td>EDS/EDX</td>
<td>Energy-dispersive X-ray spectroscopy</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESEM</td>
<td>Environmental Scanning Electron Microscopy</td>
</tr>
<tr>
<td>EXAFS</td>
<td>Extended X-ray absorption fine structure</td>
</tr>
<tr>
<td>FEG</td>
<td>Field emission gun</td>
</tr>
<tr>
<td>GBq</td>
<td>Gigabecquerels</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GDF</td>
<td>Geological Disposal Facility</td>
</tr>
<tr>
<td>HAW</td>
<td>Higher Active Wastes</td>
</tr>
<tr>
<td>HAH</td>
<td>Hydroxylamine hydrochloride</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HLW</td>
<td>High Level Waste</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IC</td>
<td>Ion Exchange Chromatography</td>
</tr>
<tr>
<td>ICDD</td>
<td>International Centre for Diffraction Data</td>
</tr>
<tr>
<td>ICP</td>
<td>Inductively-coupled plasma</td>
</tr>
<tr>
<td>ILW</td>
<td>Intermediate Level Waste</td>
</tr>
<tr>
<td>ISA</td>
<td>Isosaccharinic acid</td>
</tr>
<tr>
<td>α-ISA</td>
<td>3-deoxy-2-C-(hydroxymethyl)-D-erythro-pentonic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>β-ISA</td>
<td>3-deoxy-2-C-(hydroxymethyl)-D-threo-pentonic acid</td>
</tr>
<tr>
<td>LCF</td>
<td>Linear combination fitting</td>
</tr>
<tr>
<td>LLW</td>
<td>Low Level Waste</td>
</tr>
<tr>
<td>LLWR</td>
<td>Low Level Waste Repository</td>
</tr>
<tr>
<td>LWR</td>
<td>Light Water Reactor</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>NDA</td>
<td>Nuclear Decommissioning Authority</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>NRVB</td>
<td>Nirex Reference Vault Backfill</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitriloacetic acid</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
</tr>
<tr>
<td>PADAP</td>
<td>2-(5-bromo-2-pyridylazo)-5-diethylaminophenol</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PWR</td>
<td>Pressurised water reactor</td>
</tr>
<tr>
<td>RDP</td>
<td>Ribosomal Database Project</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>RWM</td>
<td>Radioactive Waste Management</td>
</tr>
<tr>
<td>SAED</td>
<td>Selected area diffraction pattern</td>
</tr>
<tr>
<td>SE</td>
<td>Secondary electron</td>
</tr>
<tr>
<td>SF</td>
<td>Spent fuel</td>
</tr>
<tr>
<td>TEA</td>
<td>Terminal electron acceptor</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet visible</td>
</tr>
<tr>
<td>VLLW</td>
<td>Very Low Level Waste</td>
</tr>
<tr>
<td>XAS</td>
<td>X-ray absorption spectroscopy</td>
</tr>
<tr>
<td>XANES</td>
<td>X-ray absorption near edge structure</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
</tr>
</tbody>
</table>
The nuclear waste inventory of the UK comprises large quantities of intermediate level wastes (ILW), which will be immobilised by encapsulation within a cementitious grout in stainless steel containers, followed by disposal in a deep engineered geological disposal facility (GDF) within a suitable geological formation. These wastes contain, in addition to radioactive elements, a heterogeneous mix of organic materials, including plastics, cellulose and rubber. Cellulosic items, such as cloth, tissue, filters, paper and wood, are considered particularly problematic, because they are known to be susceptible to degradation under alkaline conditions, forming small chain organic acids with the ability to complex metals and radionuclides. It is predicted that under alkaline conditions isosaccharinic acid (ISA) will form particularly strong complexes with Ni(II), Am(III), Eu(III), Np(IV), Th(IV), and U(IV). As a result, the presence of ISA could affect the migration behaviour of these elements, by increasing their solubility and reducing sorption, thus enhancing their mobility into the near and far field surrounding a GDF.

During site operation and then after closure of a GDF, microbial communities have the potential to colonise the steep biogeochemical gradients, running from highly alkaline in the GDF “near field” to circumneutral pH conditions in the surrounding geosphere. Within these steep pH gradients microbial processes can control the fate of organic compounds, such as ISA, and have therefore been considered as an effective self-attenuating mechanism to remove ISA from the groundwater.

This thesis aims to deliver a greater understanding of the microbial processes that can potentially use ISA as a carbon source and electron donor, removing it from solution, and thus having a positive impact on radionuclide mobility under GDF-relevant conditions. A microbial enrichment approach was chosen that approaches GDF-relevant conditions to explore the biodegradation of ISA. Cross-disciplinary analyses of water chemistry (pH, Eh, photospectroscopy, IC, ICP), mineralogy (ESEM, XRD, TEM, XAS) and microbiology (light microscopy, next generation sequencing) have demonstrated the ability of bacteria to degrade ISA over a wide range of biogeochemical conditions. Furthermore, key radionuclides (and their
non-active analogues), including Ni(II) and U(VI), were precipitated from the groundwater system during ISA biodegradation. Moreover, in the case of uranium, microbial metabolism led to the reduction of U(VI) to U(IV), which is also less soluble. This study highlights the potential for microbial activity to help remove chelating agents from groundwaters surrounding an ILW GDF, and suggests that safety cases that do not include microbial processes may be overly conservative, over-estimating the impact of ISA on radionuclide transport.
Declaration

No portion of this work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
Copyright statement

The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property University IP Policy (see http://documents.manchester.ac.uk/display.aspx?DocID=24420), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.library.manchester.ac.uk/about/regulations/) and in The University’s policy on Presentation of Theses
Acknowledgements

The work presented in this thesis was made possible thanks to a number of organisations and individuals. First and foremost, I would like to acknowledge Radioactive Waste Management (RWM), without their funding my research would not have been possible. I am grateful for the opportunity that I could travel for my research to some fantastic places, including Mexico, Japan, the Czech Republic and Italy, which gave me the opportunity to meet scientists from a wide background.

I am hugely indebted to my principal supervisor Professor Jon Lloyd. Without his guidance, which included scientific input, critical questions and patience with my ‘Germanic writing’ style, I wouldn’t have been able to accomplish this thesis as it is. I am also grateful for the excellent training I received. A special thank goes to my second supervisor Nick Bryan for his excellent support regarding any chemical question, even though he left Academia. Furthermore, I would like to thank Rebecca Beard, my industrial supervisor, and during her maternity leave Amy Shelton, both of them read through every single piece of work I produced during my PhD and always pointed out helpful literature.

Many other people around me have contributed to the completion of my thesis, which I would like to acknowledge. I would like to give a big thank you to Alistair Bewsher and Paul Lythgoe for measuring endless amounts of samples over the past three years. There is not enough homemade cake or jam that could possibly make up for their efforts. I am also grateful for the fantastic technical support from Chris Boothman, Heath Bagshaw, John Waters and Jon Fellowes; with their support I would not have been able to generate this data. I thank Naji Bassil for teaching me how to set up microcosms and to synthesise ISA, and also for his great support towards my first publication. I give thanks to Pieter, for his endless effort in fixing my EXAFS fits, especially when he was not part of our group anymore. I thank Katherine Morris for her input and involvement during the time she was my internal assessor and also for her patience and help with my radionuclide work.

I owe Sophie a big ‘thank you’ for help with any sort of question in the lab and endless patience with proof-reading of my at stages very chaotic drafts - one day I will stop building English sentences with German grammar! She is not only a colleague for me, but also a running buddy and friend with endless encouraging words.
I would like to further acknowledge my closest colleagues (past and present), who I know mainly in white lab coat and grass-green gloves and people who put up with me in the office, when I was shouting at one of my computers (on a side note: It took 3 computers to get me through this PhD): Adrian, Lynn, Laura, Thanos, Luke, Kurt, Naji and Gianni. Thank you to all of you for helping me to solve scientific problems, for feeding me with sweets or simply as friends.

A big thank you goes home to Germany to my closest friends and family: Mama, Papa, Nora, Sina, Cori und Iain, ich danke euch allen von tiefstem Herzen für eure stetige Unterstützung, die auch wenn manchmal die Verbindung noch so schlecht war, mich durch alle Höhen und Tiefen gebracht hat und mich weitermachen lassen hat - auf euch ist stets Verlass!

Lastly, I am grateful to my closest friends and allies. With the daily late lunches and the numerous amazing trips we made, I was able to keep up my enthusiasm and ambition to complete this thesis. You are the one and only late lunchers, all of them fantastic friends, rock lovers, adventurers and crazy people, which I will miss the most. Soon we will be all scattered across the globe, nevertheless I hope our paths will cross many times in the future in new exciting places.
1. Thesis content and layout

This thesis has been compiled in alternative format, with each chapter presented as an independent body of work. The thesis consists of a general introduction and a full explanation of all methods employed, followed by four research papers that have been prepared for publication and finally a summary outlining the conclusions that can be drawn from the research and future directions. In addition, there is an appendix that contains two sections of unpublished research which is currently in preparation for publication and one section that contains insight into future work. The chapters of this thesis are presented in the following format, as they have already been or will soon be published in a scientific journal.

1.1. Project relevance

With the diminishing reserves of fossil fuels and concerns over global warming caused by their widespread use (Houghton et al., 1996), nuclear power has historically been used as a low carbon alternative source of power to help answer global energy demands. Following the discovery of nuclear fission in 1938, the first nuclear power station at Calder Hall in the UK was launched in 1956 and produced nuclear power until it closed in 2003. Major research advances in the ensuing years have led to the expansion of nuclear power generation globally, while currently holding a share of around 19% of the UK’s energy mix (since 1990).

The history of nearly 70 years of industrial nuclear power activities has contributed to a legacy of significant quantities of nuclear waste that needs to be managed and disposed of safely. The Nuclear Decommissioning Authority (NDA) is responsible for decommissioning and cleaning up existing, publically owned civil nuclear sites and for implementing Government policy on the long-term management of radioactive waste. Radioactive Waste Management (RWM) is a wholly owned subsidiary of the NDA and is responsible for implementing Government policy on geological disposal of higher activity wastes (HAW). UK Government Policy for disposing of the UK’s HAW is through geological disposal that is considered as the safest available long-term solution for the disposal of HAW (DECC, 2014). Geological disposal involves isolating radioactive waste deep inside a suitable geological environment and engineered system which will appropriately contain the waste to ensure that no harmful quantities of radioactivity ever
reach the surface environment or groundwater. Prior to the availability of a geological disposal facility (GDF) in the UK, the existing waste is safely and securely stored on an interim basis by the waste owners on above ground nuclear licensed sites across the UK.

The largest volume of wastes will be Intermediate Level Waste (ILW) and Low Level Waste (LLW), which are typically immobilised by encapsulating the waste within stainless steel containers, filled with a cementitious grout. The containers will then be placed in excavated vaults within a suitable host rock, and backfilled with cement, in order to create an anoxic and alkaline environment. This approach is referred to as a so-called ‘multi-barrier system’ including several natural and engineered barriers. It is important to understand the physical and chemical evolution of the GDF system and the surrounding geosphere following the saturation with groundwater. Specifically with regards to the heterogeneous nature of ILW that contain a high proportion of cellulosic waste components co-disposed with the wastes. Cellulosic materials, such as paper, filters or cotton, may influence radionuclide migration behaviour, as these materials are known to degrade abiotically under the anaerobic and highly alkaline conditions of a cementitious ILW disposal concept to form cellulose degradation products (van Loon and Glaus, 1997). The main degradation product, isosaccharinic acid (ISA), has been shown to be a strong complexant for radionuclides and heavy metals and the formed complexes of ISA and metals/radionuclides are likely to have enhanced solubility and therefore have the potential to travel in the groundwater.

To this end, RWM manages a research portfolio that helps develop concepts to support GDF development and provide the scientific evidence and understanding to underpin the long-term performance of the disposal system. Biogeochemical processes in a GDF or in the surrounding subsurface or ‘far field’, encompass the microbial and mineral constituents of the geosphere, and have a major influence on the speciation, mobility, bioavailability and ecotoxicity of radionuclides in the environment (van Hullebusch et al., 2005). Understanding interactions between the waste itself, the engineered GDF components and the natural barrier provided by the geological environment, is a complex area of research, but significant in terms of underpinning the long-term performance of the disposal system. To this day, there remain knowledge gaps relating especially to biogeochemical processes that may occur within the barrier system of a GDF, and how they will impact on the transport of radionuclides from the
GDF to the surrounding biosphere, over the long timescales required to allow the decay of key radionuclides (up to one million years). This study was designed to investigate the biogeochemical fate of ISA and associated ISA-radionuclide complexes under GDF-relevant conditions.

1.2. Research objectives
The primary aims and objectives of the work described in this thesis were to investigate the potential for microbial degradation of ISA in the geosphere surrounding a GDF. This was followed by investigations of the fate of metals and radionuclides that may be complexed to ISA.

From past research, it was inferred that ISA may be microbially degraded under alkaline conditions \((pH = 10)\) and over a wide range of biogeochemical conditions, representative of the ‘near field’ surrounding a GDF. Based on this research, it cannot be excluded that ISA may travel in groundwater into the ‘far field’ environment. This research focuses on the role that microorganisms may have in controlling the biogeochemical fate of ISA and the impact on the fate of key radionuclides under such “far field” conditions.

A comprehensive experimental study was designed that used microbial enrichment cultures to study the biodegradation of ISA by microorganisms retrieved from an alkaline legacy lime workings site in Buxton, U.K., a model system that approaches aspects of the far field geosphere of a GDF. Various geochemical conditions were established, including a range of pH values and reducing conditions, to explore the biodegradation of ISA under different scenarios that may develop in the geosphere surrounding a GDF. Metals and radionuclides, relevant to nuclear waste disposal, were introduced into these systems, to determine the impact of microbial ISA degradation on the fate of these elements.

1.3. Thesis structure and publication status

**CHAPTER 1**

Thesis content and layout

**CHAPTER 2**

Background and literature review

**CHAPTER 3**
Methodology

**CHAPTER 4**

*Microbial degradation of isosaccharinic acid under conditions representative for the far field of radioactive waste disposal facilities*

(This Chapter is published in Mineralogical Magazine, November 2015, Vol. 79(6), pp. 1443–1454, DOI: 10.1180/minmag.2015.079.6.19)

Gina Kuipers, Naji M. Bassil, Christopher Boothman, Nicholas Bryan, Jonathan R. Lloyd

Author contributions: G. Kuipers – primary investigator, preparation and monitoring of experiments, data acquisition and analysis; N.M. Bassil - help with preliminary experimental set up; C. Boothman - sample analysis for microbial ecology (sequencing); N.M. Bassil and N. Bryan– manuscript review; J.R. Lloyd - planning of experiments with G. Kuippers, data interpretation and manuscript review.

**CHAPTER 5**

*Isosaccharinic acid (ISA) biodegradation and the impact on iron mineralogy under conditions relevant to nuclear waste disposal (research article)*

Gina Kuipers, Heath Bagshaw, Christopher Boothman, Rebecca Beard, Nicholas Bryan, Jonathan R. Lloyd

Author contributions: G. Kuipers – primary investigator, preparation and monitoring of experiments, data acquisition and analysis; H. Bagshaw - support with TEM and ESEM imaging; C. Boothman - sample analysis for microbial ecology (sequencing); R. Beard and N. Bryan– manuscript review; J.R. Lloyd – planning of experiments with G. Kuippers, data interpretation and manuscript review.

Status: Submitted to Applied and Environmental Microbiology (Date: 16/06/2017)

**CHAPTER 6**

*The biogeochemical fate of nickel during microbial ISA degradation; implications for nuclear waste disposal (research article)*

Gina Kuipers, Christopher Boothman, Heath Bagshaw, Michael Ward, Rebecca Beard, Nicholas Bryan, Jonathan R. Lloyd
Author contributions: G. Kuipers – primary investigator, preparation and monitoring of experiments, data acquisition and analysis; C. Boothman – sample analysis for microbial ecology (sequencing); H. Bagshaw – support with ESEM imaging; M. Ward – support with TEM imaging; N. Bryan – support with PHREEQC modelling; J.R. Lloyd – planning of experiments with G. Kuippers, data interpretation; J. Lloyd, R. Beard and N. Bryan – manuscript review.

Status: Submitted to Scientific Reports (Date: 28/08/2017)

Chapter 7

Biomineralization of uranium-phosphates fuelled by the microbial degradation of isosaccharinic acid (ISA)

Gina Kuipers, Kurt Smith, Pieter Bots, Katherine Morris, Christopher Boothman, Samuel Shaw, Nicholas Bryan, Jonathan R. Lloyd

Author contributions: G. Kuipers – primary investigator, preparation and monitoring of experiment and data acquisition and analysis; K. Smith, K. Morris, S. Shaw and P. Bots – support with XANES & EXAFS data analysis and interpretation; C. Boothman - sample analysis for microbial ecology (sequencing); J.R. Lloyd – planning of experiments with G. Kuippers, data interpretation, manuscript review; K. Morris, R. Beard and N. Bryan – manuscript review.

Status: In preparation for submission to Environmental Science & Technology

Chapter 8

Conclusions and future directions

Chapter 9

Appendices
2. Background and literature review

2.1. The Nuclear Fuel Cycle

The nuclear fuel cycle involves the production of energy by the utilisation of fissile materials obtained from the mining of uranium. Uranium is a relatively widespread element that occurs in low percentages naturally in the Earth’s crust and is mined in a number of countries. Uranium deposits, or so-called ores, such as uraninite, contain two different isotopes of $^{238}\text{U}$ (99.28%) and $^{235}\text{U}$ (0.71%). $^{235}\text{U}$ is fissile because its atomic nucleus is susceptible to fission when struck by a neutron and is the isotope that is used for energy generation. In contrast, the nucleus of $^{238}\text{U}$ will nearly always absorb a neutron, resulting in radioactive $^{239}\text{U}$, which then naturally decays to $^{239}\text{Pu}$, also a fissile isotope.

The extraction of the uranium ore occurs either via excavation and then processing to enrich for $^{235}\text{U}$, which is left to precipitate and pressed into pellets. The pellets are encased in zirconium alloy tubes to form fuel rods, which are arranged into a fuel assembly to be introduced into a reactor vessel (Figure 2.1). Fuel cycles are differentiated by the sort of fuels used, the conditioning and the manner of removing fuel from the reactor at the end of a fuel’s useful lifetime, when it is burned off (‘spent fuel’). Nuclear power generation can be divided into an open cycle (or a once-through cycle) when fuels are not reprocessed, and a closed cycle when spent fuels are reprocessed (Figure 2.1). In this cycle, some of the fissile material that originated from a reactor during nuclear power generation may be removed after it has reached the end of its useful life and may be reprocessed such that it may be recycled for new fuel.

Nuclear reactors create power by fission, a process in which neutrons are fired at the fuel, e.g. $^{235}\text{U}$. At collision, the uranium nucleus consumes neutrons and releases new neutrons. The emerging neutrons can be used to cause fission of more $^{235}\text{U}$ atoms, leading to the release of additional neutrons, which triggers a nuclear chain reaction. On average, for each neutron consumed $\sim 2.5$ (=\(\nu\)) new neutrons are released (Choppin et al., 2013). The neutron ratio can be controlled so that for each fission reaction exactly one of the new neutrons undergoes further fission, resulting in a steady state. The high energy of the fission fragments is promptly converted to heat through collision. To process the resulting heat there are several designs of
nuclear power plant, each having its advantages and draw-backs. About 79% of the currently operational reactors worldwide are of the light water reactor type (LWR), i.e. pressurised water reactors (PWR) or boiling water reactors (BWR) (Choppin et al., 2013). Principally, each reactor contains a core, which encloses the fuel material surrounded by cooling water. The cooling water circulates within the vessel, ensuring a constant operating temperature. In most of the reactors (63% worldwide), the heated water leaves the core as hot water at high pressure (PWR), which then passes through a steam generator; the steam drives one or more turbines, which in turn operate electric generators.

The fuel can be used for approximately 18-36 months until an excess of fission products and heavy metals have accumulated, competing for the neutrons and thus rendering the fuel unviable. Spent fuel contains nearly all of the elements of the periodic table, although the long-term risk arises from only 1% of the fuel, primarily the transuranic elements plutonium, neptunium, americium, and curium and the long-lived isotopes of iodine and technetium. Short-term radioactivity emerges in high doses from mainly cesium and strontium. Once removed from

Figure 2.1. The closure of the nuclear fuel cycle. After usage of the uranium there are two pathways for the spent fuel: It can be immediately disposed of, or reprocessed to recover usable elements and fuel wastes can be subject to a geological disposal facility (GDF) (adapted from Argonne National Laboratory, 2016).
a reactor, used fuel is transferred to a storage pond, and submerged in water to shield from emitted radiation and absorb heat released by the decaying waste. After the initial very high levels of radioactivity have decayed, a process taking several months or years depending on the material, the spent fuel can be passed to long-term management. Currently, there are different approaches to spent fuel management, including 1) direct disposal without reprocessing, 2) reprocessing to recover and recycle usable elements and 3) storing the fuel for a later decision.

2.2. Nuclear Waste Disposal

The development of a disposal facility for the long-term management of radioactive waste is among one of the largest engineering projects in the UK and other countries that produce radioactive wastes. To this day, most of the LLW from the UK is disposed of in near-surface facilities, for example at the already existing Low Level Waste Repository (LLW Repository Ltd) near Drigg in Cumbria (Nirex, 2007b). The small proportion of LLW that cannot be disposed of in a near-surface facility, as well as any ILW and HLW, are currently stored in surface facilities until a site for a long-term underground disposal has been found. RWM has developed a set of illustrative geological disposal facility (GDF) concepts for the UK, which are planned to be implemented in depths between 200 and 1,000 m (NDA, 2010c; d; f, RWM, 2010b; c). For the construction of a GDF in the UK a large range of potentially suitable geological settings have been suggested, which belong under the categories of higher strength rocks, lower strength sedimentary rocks or evaporites (NDA, 2010g).

A GDF makes use of a combination of engineered and natural barriers to isolate and contain the waste from the surface environment and biosphere (Figure 2.2; NDA, 2010a; b). The proposed disposal concept is designed as a multi-barrier containment system that provides physical containment, chemical conditioning and geological isolation and containment (NDA, 2010d). Physical containment of ILW is achieved by immobilisation and packaging of wastes in a cementitious grout within a steel or concrete container. The encapsulated waste content is backfilled into the waste containers and moved via a waste access shaft into the vaults of the GDF, which are sealed with cement (Figure 2.2). Whilst the physical containment is sufficient to contain the great majority of radionuclides, it will not persist long enough to contain the radionuclides with half-lives greater than a few hundred years (Crossland and Vines, 2001).
Over these extended time periods, in the order of a million years, chemical containment will be provided through the conditions established when re-saturating groundwater interacts with the cementitious components of the engineered barrier system (Harris, 1997; Harris et al., 1997). Here, after several decades of operation, closure of the GDF by backfilling with cement will create hyperalkaline conditions upon groundwater intrusion (NDA, 2010d; e). The cement barrier materials will provide abundant surfaces for radionuclide sorption and the high pH environment helps to immobilise most radionuclides. In addition, chemical buffering at high pH is considered to potentially minimise most microbial activities (NDA, 2010d; e). Simultaneously, initial corrosion of the wastes and containers will rapidly consume any remaining oxygen. Thus, chemical conditioning will be achieved by a combination of low oxygen, high pH and increased sorption capacity (Crossland and Vines, 2001). Emplacement of the waste packages in vaults excavated deep underground within suitable geological sediment layers or host rock will provide geological isolation, and will retard contaminants released from the GDF via small pore spaces with low water flow. The geological barrier will be characterised by slightly elevated pH
conditions compared to undisturbed rock. Its task is to isolate the waste from the terrestrial biosphere and to retain the radionuclides until they have decayed to levels at which radiological impacts are acceptably low (NDA, 2010d).

Planning for geo-disposal in the UK requires the definition of the types and quantities of wastes. The ‘inventory for disposal’ is therefore defined in the 2014 Implementing Geological Disposal White Paper (DECC, 2014). The inventory for disposal includes: High Level Waste (HLW) arising from reprocessing spent fuel, ILW from existing nuclear sites and defense, medical, industrial, research and educational activities. The small proportion of Low Level Waste (LLW) that is not suitable for disposal in the existing Low Level Waste Repository (LLWR), includes spent fuel from commercial reactors and research reactors that is not reprocessed, spent fuel and ILW from a new nuclear build program, plutonium stocks not re-used in new fuel manufacture, uranium stocks arising from enrichment and fuel fabrication activity and irradiated fuel and nuclear materials from the UK defense program. Some of these radioactive materials are not currently classified as waste and include spent fuel, irradiated fuel and some nuclear materials for which reprocessing is uncertain and may change in the future (Morris et al., 2011). The volumes of these wastes are regularly reviewed and made publically available, as they are subject to change due to improved operation and waste treatment (Figure 2.3). Currently the total volume of wastes destined for geological disposal is estimated to be approximately 656,000 m³ with an activity of ca. 232,000,000 TBq at 2040 (RWM and NDA, 2015).

LLW will comprise 11,600 m³ at 2040, with an overall low radioactive content of 0.9 TBq (RWM and NDA, 2015). LLW is mainly generated from hospitals and industry, as well as the nuclear fuel cycle. Major components include building rubble, soil, steel and some cellulosic items, e.g. clothes.

ILW is estimated to comprise 456,000 m³ (1,930,000 TBq at 2040). These wastes are classified based on exceeding upper limits of radioactive content for LLW, without need for heat to be taken into account, which is required for storage or disposal of HLW (DECC, 2014). Most of the ILW content arises during spent fuel reprocessing, nuclear decommissioning and from medical, industrial, research and educational activities, but will also comprise the small proportion of LLW that is not suitable for disposal in the existing Low Level Waste Repository. ILW consists of a heterogeneous mixture of materials, including graphite, steel, aqueous waste, sludge, flocs and

![Figure 2.3](image_url). The radioactive waste inventory of the UK, showing the total radioactivity of the different waste categories as a function of time after GDF operations start at 2040. SF stands for spent fuel (adapted from RWM and NDA, 2015).

The amount of HLW is relatively small, approximately 9.290 m$^3$ (around 1% of the total packaged waste up to year 2040), but contains around 15% of the radioactivity (35 Mio TBq; RWM and NDA, 2015). HLW in the UK is the residue produced during spent fuel reprocessing. The nature of reprocessing means that HLW is produced as an acidic liquid, mainly comprising 96% unreacted uranium, 1% plutonium and 3% waste products (BEIS and NDA, 2017b). Since it is also physically hot, it needs cooling as well as shielding. Spent fuel that is not reprocessed is not currently declared as waste in the UK and is stored to be either be disposed of directly or reprocessed at some point in the future.

Before disposing of nuclear wastes in a GDF, some waste types need to be conditioned in order to minimise the hazards and to reduce their volumes (DECC, 2014). Conditioning processes, such as vitrification and cementation, are used to transform the waste into a stable or solid waste form, and to immobilise radionuclides. HLW is conditioned by vitrification, which means the immobilisation by encapsulation in glass, and packaging in stainless steel canisters that are packaged into a disposal container. In contrast, spent fuel assemblies are assumed to be
disposed of, intact, in a disposal container (RWM and NDA, 2015). ILW and LLW are routinely compacted and cemented in stainless steel canisters (Nirex, 2007b). LLW that is combustible is also incinerated as a form of pre-treatment, before the ashes are compacted to further reduce the volume. The packed canisters are then placed into large metal containers, which are backfilled with cement and sealed, before they are stored in concrete-lined vaults. The waste containers are designed to maintain integrity for 500 years (RWM, 2010a), which secures containment of short-lived soluble radionuclides. Following their corrosion, the multi-barrier system will be the most important mechanism in the retardation of radionuclides.

### 2.3. Radioactive inventory

Key radionuclides from the inventory can be distinguished into naturally occurring radionuclides, originating from the natural decay series with $^{235}$U, $^{238}$U and $^{232}$Th, and man-made radionuclides, produced as a result of either nuclear fission or activation (NDA, 2010f) (Table 2.1). Nuclear fission products are produced during energy generation from either spontaneous reaction or bombardment with neutrons or charged particles (see Chapter 2.1), whereas activation products arise as a result of irradiation of structural components within a nuclear reactor (e.g. steel, cement, and graphite). It should be noted that the table highlights the most important radionuclides during disposal, but further products will arise from decay. However, reprocessing of fuel will separate enriched uranium and plutonium, so they can be further used for fabricating new fuel to be used in a new fuel cycle (see Chapter 2.1; RWM and NDA, 2015).
Table 2.1. Key radionuclides in the nuclear fuel cycle with oxidation states and ways of retardation. From the decay of these radionuclides, future isotopes will arise that are not shown here. Bold italics indicate most stable oxidation states under oxidising conditions, while italics the most stable under reducing conditions (adapted from NDA, 2010f; Brookshaw et al., 2012).

<table>
<thead>
<tr>
<th>Fission products</th>
<th>Isotope</th>
<th>Type of radiation</th>
<th>Half-life [y]</th>
<th>Oxidation states</th>
<th>Mobility retarding mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenium</td>
<td>79Se</td>
<td>β</td>
<td>1.2 x 10^6</td>
<td>VI, IV, II, -II</td>
<td>Reduction, sorption</td>
</tr>
<tr>
<td>Strontium</td>
<td>90Sr</td>
<td>β</td>
<td>29.1</td>
<td>II</td>
<td>Sorption (cation exchange), incorporation/co-precipitation</td>
</tr>
<tr>
<td>Zirconium</td>
<td>93Zr</td>
<td>β, γ</td>
<td>1.5 x 10^6</td>
<td>IV, III, II, I, -II</td>
<td>Precipitation, sorption, incorporation, hydrolysis</td>
</tr>
<tr>
<td>Technetium</td>
<td>99Tc</td>
<td>β</td>
<td>2.15 x 10^5</td>
<td>IV, VII</td>
<td>Reduction, sorption</td>
</tr>
<tr>
<td>Tin</td>
<td>126Sn</td>
<td>β, γ</td>
<td>1.0 x 10^5</td>
<td>IV, III, II, I, -I, -II, -III, -IV</td>
<td>Sorption, incorporation/(co-)precipitation</td>
</tr>
<tr>
<td>Iodine</td>
<td>129I</td>
<td>β, γ</td>
<td>1.57 x 10^7</td>
<td>VII, VI, IV, III, I, 0, -I</td>
<td>Anion exchange; interactions with organic matter</td>
</tr>
<tr>
<td>Caesium</td>
<td>137Cs</td>
<td>β</td>
<td>30.17</td>
<td>I</td>
<td>Sorption (cation exchange)</td>
</tr>
</tbody>
</table>

**Actinides**

| Thorium         | 230Th   | α                 | 7.5 x 10^4    | IV               | Sparingly soluble            |
| Radium          | 226Ra   | α                 | 1.6 x 10^3    | II               | Co-precipitation             |
| Uranium         | 238U    | α                 | 4.47 x 10^9   | III, IV, V, VI  | Reduction, sorption          |
| Neptunium       | 237Np   | α                 | 2.14 x 10^6   | III, IV, V, VI, VII | Reduction, sorption |
| Plutonium       | 238Pu   | α                 | 87.7          | III, IV, V, VI, VII | Reduction, sorption |
|                 | 239Pu   | α                 | 2.41 x 10^4   |                   |                              |
|                 | 240Pu   | α                 | 6.55 x 10^5   |                   |                              |
|                 | 241Pu   | β                 | 14.4          |                   |                              |

**Activation products**

| Chlorine        | 36Cl    | β                 | 3.0 x 10^5    | VII, VI, V, IV, III, II, I, -I | Isotopic exchange, anion exchange |
| Carbon          | 14C     | β                 | 5.7 x 10^3    | IV, III, II, I, 0, -I, -II, -III, -IV | Incorporation/(co-)precipitation, reduction |
| Nickel          | 63Ni    | β                 | 9.9 x 10^1    | IV, III, II, I, -I, -II | Incorporation/(co-)precipitation |
| Niobium         | 94Nb    | β                 | 4.47 x 10^9   | V, IV, III, II, I, -I, -III | Hydrolysis, precipitation, incorporation |
Chemical properties of the key radionuclides are relevant with regard to behaviour in aqueous solutions and interactions with solid surfaces. The most important processes that control radionuclide concentrations in solution include sorption, precipitation, co-precipitation and reduction (Table 2.1). Most radionuclides typically have both, a stable and a radioactive nuclide, with identical chemical properties, in which cases studies can be performed on the stable nuclide that allows drawing conclusions for the radionuclide. The redox state of aqueous solutions is also of importance for the solubility of several key elements, as their solubility is greater at a higher oxidation state. Other factors determining radionuclide speciation or interaction with minerals includes their size, their ability to form or associate with colloids (Bots et al., 2014) and microbially-mediated processes (see Chapter 2.7).

Figure 2.4. Total radioactivity in ILW as a function of time (adapted from DEFRA and NDA, 2008).
The most important radionuclides in ILW and their total radioactivity as a function of time is shown in Figure 2.4. A major factor in support of the safety of geological disposal of HAW is the radioactive decay of the wastes over time. A radionuclide either decays directly to a stable, non-radioactive isotope or in other cases, particularly for actinides, decay occurs via a radioactive ‘daughter’ isotope before a stable isotope is formed. Therefore, the total activity of the waste, here for ILW, needs to be considered for an inventory as a function of time (Figure 2.4; NDA, 2010a). In the first thousand years post-closure of an ILW-GDF, the hazard from the wastes will be the highest. In this time period, the shorter lived radionuclides will have decayed significantly, which include Fe-55, Ni-63, Sr-90 and Cs-137. In the next thousands to 100,000 years, the components of the engineered barrier system are expected to break down and the geosphere surrounding a GDF in conjunction with the buffer is expected to further retard the migration of radionuclides. In this time period, Ni-59, and Am-241 will decay significantly. Until about a million years, some of the barrier components, in particular clay-based barriers, may retain their original properties and the geosphere will continue to provide isolation. Most of the radioactivity in a GDF for ILW will have decayed at this stage, but the longest lived radionuclides, including U-238, U-234, Th-230 and Ra-226, and betagamma daughter isotopes will continue to persist. In the very long term of several millions of years the GDF will become part of the natural geological system (NDA, 2010f).

2.4. Post-closure GDF evolution
The imposed environmental conditions in a GDF are largely different from natural soil environments. Major influences are pH, temperature, oxygen availability and the presence of ions. As introduced in Chapter 2.2, following the backfilling of ILW vaults with cementitious material, groundwater re-saturation of the disposal system establishes favourable conditions in the GDF near-field for the chemical containment of radionuclides. The GDF near-field comprises the immediate surrounding vaults sealed with a cementitious backfill that generate a chemical barrier of highly alkaline pH and reducing conditions upon groundwater re-saturation surrounding the repository contents (Greenfield et al., 1992). The groundwater plume evolving in the GDF and moving away from the near-field will be conditioned by the cement backfill to a high pH. This alkaline plume will interact with the surrounding layers of host rock, the far field, and will create a chemical gradient termed the chemically disturbed zone (CDZ). The interplay
of these barriers will be important during GDF evolution to retard the transport of radionuclides into the environment.

A crucial part of the multi-barrier system will be the encapsulation of radioactive waste within a cementitious grout. This is intended to create a very high pH inside a GDF of around pH ≥13.4 (van Loon and Glaus, 1997, 1998b; Pavasars et al., 2003). In detail, after closure of the GDF, oxygen, ions and organic substances can get imported into the GDF with groundwater ingression, but leaching from the cement phase will take place simultaneously. Over long timescales, the groundwater flow undergoes chemical changes when moving through the GDF (van Loon and Glaus, 1997, 1998b; Pavasars et al., 2003). The weathering can be divided into four stages (Figure 2.5) that begin with stage I, the dissolution of NaOH and KOH and saturation with respect to calcium-silicate-hydrates (CSH) and Ca(OH)$_2$ (portlandite). This initial dissolution will rise the pH to a hyper-alkaline level of 13.4, with a predicted Ca$^{2+}$-concentration of 2 mM and OH$^{-}$ concentration 0.3 M and be stable for hundreds to thousands of years (van Loon and Glaus, 1997, 1998b; Askarieh et al., 2000). When NaOH and KOH have been leached out completely, stage II begins with a decrease of the pH to around 12.5. This stage is governed by Ca(OH)$_2$ dissolution (around 20 mM) and is predicted to be stable for periods of
the order of $10^5$ years (Berner, 1992). In stage III, hydrated CSH starts dissolving after Ca(OH)$_2$ has been dissolved completely, creating a pH range between 12.5 and 10. Phase IV marks the time after closure, when the pH drops to the same value measured in percolating groundwaters in the GDF, which will lead to CaCO$_3$ dissolution. Thus, the most abundant ion in solution is Ca$^{2+}$, but significant amounts of Na$^+$ and K$^+$ will be present at the beginning of the backfilling (Berner, 1992; Glaus et al., 1999a). In addition to the hyperalkaline conditions created from the leaching of cement, reducing horizons will be maintained by corrosion of iron from steel containers and steel used in repository construction (Greenfield et al., 1992).

Gases will also be produced in the near-field following closure of the GDF, with the most important gas generating processes being the corrosion of metals and degradation of organic materials. Radiolysis will present a minor contribution to total gas generation (Agg et al., 1996; Williams, 2012). The gaseous products include flammable, radioactive and chemotoxic species, such as hydrogen gas (H$_2$), methane (CH$_4$) or radioactive C-14 gases. Even though a GDF will be constructed in a way that enables gas release, understanding the gas generating processes and monitoring them carefully, especially in post-closure times, is essential in order to meet environmental standards.

H$_2$ will comprise the predominant gas by volume and will be mainly generated from anaerobic corrosion of steel and reactive metals in post-closure times (Williams, 2012). Smaller amounts of molecular H$_2$ are also produced enzymatically by hydrogenase which happens characteristically in the presence of iron or sulfur (Bachofen, 1991). If hydrogen is not consumed during methanogenesis or other respiratory processes, it will accumulate and inhibit the degradation of organics (Bachofen, 1991). However, normally H$_2$ is removed by hydrogen-consuming microorganisms.

Cellulose items are the most significant gas-generating components of organic waste and therefore in ILW (Agg et al., 1996). After closure, oxygen will be consumed first as the most favourable terminal electron acceptor (TEA) for microbial processes and during corrosion of metal items and is thus depleted rapidly. Once anoxic conditions are established, some microorganisms are able to use alternative electron acceptors, such as nitrate or sulfate to metabolise organic materials (Figure 2.6). It should be noted however, that microbial activity will be constrained to the individual tolerability towards alkalinity. This leads to the formation of
gases including CO\(_2\), N\(_2\)O, NO\(_x\), or H\(_2\)S and also to organic acids as intermediate products (Bachofen, 1991). CO\(_2\) is produced along the whole redox cascade during complete degradation of organic matter under aerobic conditions, and in the absence of oxygen with nitrate or sulfate as electron acceptors or in fermentative metabolism (Bachofen, 1991). This gas can also be consumed by methanogens and acetogens, which can act as a sink for CO\(_2\) (Small \textit{et al.}, 2008). During the operational phase of a repository and shortly after closure, CO\(_2\) production from mainly aerobic respiration will diffuse rapidly (Askarieh \textit{et al.}, 2000), whereas in the post-operational time CO\(_2\) can accumulate. However, altogether no net production of this gas will occur because it reacts with cementitious materials to precipitate as CaCO\(_3\). When the redox potential falls to a value of -300 mV or less, CH\(_4\) can be produced from methanogenesis of short chain fatty acids or CO\(_2\) and H\(_2\), thus representing a sink for both climate-relevant gases (Askarieh \textit{et al.}, 2000). This will maintain the cycling of electrons, even though at decreased rates. Fermentation of many organic substrates, especially cellulose, may also result in the biological formation of variable amounts of hydrogen and CO\(_2\).

![Redox cascade for the degradation of organic matter](image)

Figure 2.6. Redox cascade for the degradation of organic matter, describing a potential sequence of electron acceptors changing with time (but also with distance to the GDF), which correlate with a higher energy yield obtained by electron acceptor utilisation. Eh values from Millero, 1996.
The engineered components of the GDF multi-barrier system are designed to allow gas migration. Controlled ventilation is implemented to meet environmental standards that do not exceed agreed limitations. This, in combination with the properties of the host rock, provides passive protection against gas over-pressurisation that could otherwise cause disruption of the barriers (NDA, 2010b).

Another influencing factor in ILW GDF evolution is temperature, which can have important implications for the chemical degradation of cellulose (section 2.5.3). Following backfilling of the GDF vaults intended for disposal of ILW, the first few months will be dominated by a short-lived temperature increase associated with the curing exotherm from the cement backfill. During this time, it is expected that temperatures could reach up to 80°C, dominated in the center of the disposal vaults (Chambers et al., 1995; Knill and Kennedy, 2003). Within approximately one year after closure the maximum vault temperatures will drop to 50°C and will stabilise at around 30°C for the next 100 years (Knill and Kennedy, 2003).

Despite the high pH and anoxia residing in a GDF, chemical transformations in repositories still occur at low rates, and these are relevant to studies representing radionuclide migration behaviour. Especially when the pH values decrease, as described above, the degradation of organic polymers present in LLW or ILW by radiolysis or via chemical or microbial processes may be facilitated (Greenfield et al., 1990). Chemical and radiolytic processes will be dominant at high pH values, whilst some microbial activity is expected to take place in microhabitats of lower pH that may influence the whole repository (Greenfield et al., 1990; Colasanti et al., 1991; Rosevear, 1991). The impact of microbial processes will become more important with increasing distance from the GDF, which will be discussed in greater detail in section 2.5.1.

The degraded EBS barriers continue to retard the release of contaminants, although at this time the majority of the radioactive inventory would have decayed (NDA, 2010f). Thus, in the very long term (many hundreds of thousands to millions of years) the safety of a GDF will depend upon the effective retention and retardation of radionuclides by the geosphere and the most appropriate provisions have to be made in order to minimise migration or to immobilise radionuclide movement.
2.5. Cellulose chemistry & degradation

Cellulosic wastes, including a range of polymeric organic materials, such as cloth, tissue, filters, paper and wood, will comprise a significant proportion of the organic materials in ILW. Cotton is composed to a large extent of cellulose, whereas wood and paper contain a range of polymers. The cellulose in wood is usually associated with other plant substances for which a precise chemical composition cannot be given (Pettersen, 1984). However, by approximation, cellulose comprises the major constituent (40-50%) with varying portions of hemicellulose (20-35%) kept together by lignin (18-35%), which gives the molecule its strength (Timell and Syracuse, 1967). The content of hemicellulose in paper is much lower (<10%; Humphreys et al., 2010), and it does not contain lignin because it is removed during the pulpung process. The manufacturing of paper uses heat and strong alkali that influence the degree of polymerisation and the crystallinity of cellulose (Humphreys et al., 2010).

![Cellulose chain](image_url)

Figure 2.7. Cellulose chain linked via β-1,4-glycosidic bonds (adapted from Gurunathan et al., 2015).

Cellulose is a polysaccharide, produced photosynthetically by CO₂ fixation in plants and is the main constituent of the plant cell wall. Cellulose is a crystalline homopolymer made up of repeating β-1-glucose units linked together by (1→4)-glycosidic bonds to linear macromolecules (Humphreys et al., 2010), giving the chemical formula (C₆H₁₀O₅)n as shown in Figure 2.7. Cellulose chains are grouped by means of hydrogen bonding and van der Waals’ forces to generate microfibrils, of which bundles form fibers that are aggregated laterally in parallel sheets, which are orientated in different ways to increase the molecule’s stability. The cellulose microfibrils are usually embedded in a matrix of other polymers, including hemicellulose, pectins and lignin. The role of cellulose in plant tissue is to increase mechanical strength and resistance of plants to osmotic pressure. The combination of these structural properties gives cellulose its high tensile strength.
It has to be considered that the process of assembly of the glucose chains remains poorly understood. However, it is believed that the stereochemistry of the β (1→4) glycosidic bonds induces a rotation by 180° of each glucose molecule from its neighbour, resulting in a twisted backbone which facilitates the chair (C₆) conformation with intramolecular dense hydrogen bonding (Humphreys et al., 2010). By inter- and intramolecular hydrogen-bonding and further strengthening of the cellulose molecule by Van der Waals forces, the typical microfibril structure is facilitated, which in turn forms fibers. The biosynthesis of cellulose microfibrils proceeds by chain elongation occurring from the non-reducing end that result in a size of approximately 2 to 3 nm in cross-sectional dimension (in wood). As the chain emerges, it polymerises to form both, crystalline and amorphous regions.

As stated earlier, under repository conditions cellulose can be degraded by microbial, chemical or radiolytic processes (Greenfield et al., 1990). During cellulose degradation, the crystallinity of the molecule will change, together with its chemical and physical properties. Although it is not known how the different chemical and physical properties influence alkaline degradation, it is certain that crystalline regions of cellulose are less susceptible to degradation (Askarieh et al., 2000).

Research has demonstrated that chemical degradation of cellulose under alkaline, anaerobic conditions and expected temperatures of <170°C is of particular relevance in the context of geological disposal (Bradshaw et al., 1986, 1987). Microbial degradation of cellulosic wastes in a GDF is feasible in the operational phase under aerobic conditions, but post closure will most likely be initiated chemically under anaerobic, alkaline conditions. The different forms of cellulose degradation that might take place in a GDF are explained in the following sections.

2.5.1. Microbial degradation of cellulose

The insoluble, crystalline microfibril nature of cellulose makes it highly resistant to enzymatic hydrolysis. Nevertheless, bacteria and fungi, usually occurring in mixed populations, are known to produce enzymes capable of extracellular degradation, either in association with the cell surface or in the external environment away from the cell (Leschine, 1995; Lynd et al., 2002). These enzymes encompass a complex set of cellulases, whose primary function is to hydrolyse β-1,4-glycosidic linkages of cellulose that generates oligosaccharides of various lengths (Gilbert
and Hazlewood, 1993). The reaction to break β-1,4-glycosidic bonds proceeds through an acid hydrolysis mechanism using proton donors and bases.

Three major types of enzymes are known for microorganisms to depolymerise insoluble cellulose (Figure 2.8). Described in a simplified way, they comprise a synergetic complex working closely together, comprising a) endoglucanases, which randomly hydrolyse β-1,4-glycosydic bonds within cellulose molecules, producing reducing and non-reducing ends; b) exoglucanases or cellobiohydrolase, which cleave disaccharide units (cellobiose) from the non-reducing ends; and c) β-glucosidases, which hydrolyse cellobiose and low-molecular-weight cellodextrins, thereby yielding glucose (Gilbert and Hazlewood, 1993; Leschine, 1995). The synergy of these enzymes can be explained by their concerted actions, starting with the endoglucanase attacking random amorphous regions of the cellulose, cutting oligosaccharides of various lengths, which consequently creates new reducing and non-reducing ends. Exoglucanases act on the ends, initiating hydrolysis of more crystalline regions, forming cellobiose. Finally, closing the cycle β-glucosidases hydrolyse the cellobiose to glucose in order to prevent accumulation of this disaccharide, which would inhibit the activity of cellobiohydrolase. The products are typically low-molecular weight compounds.

Figure 2.8. Simplified schematic of the enzymatic hydrolysis of amorphous and crystalline regions of cellulose by non-aggregating and aggregating cellulase systems (adapted from Ratanakhanokchai et al., 2013).
There are two known different types of cellulose hydrolysis, aggregating and non-aggregating ones (Figure 2.8). The non-aggregating systems are found in aerobic environments and are better studied. In many aerobic bacteria and fungi, these enzymes are secreted individually (non-aggregating) and can act synergistically, whilst many anaerobic bacteria (e.g. Clostridia) are known to produce cell-associated, large complexes tightly bound to a scaffolding protein (aggregating; Figure 2.8; Ratanakhanokchai et al., 2013). The (anaerobic) aggregating complex occurs mainly in bacterial consortia formed with other microorganisms, and is known as the cellulosome. The cellulosome comprises at least 14 distinct polypeptides including numerous endoglucanases and xylanases and at least one β-glucosidase that are acting synergistically. There is little activity in this system against the crystalline regions of cellulose. Such cellulosomes are stable enzyme complexes that are bound to the bacterial cell wall and at the same time flexible enough to bind to microcrystalline cellulose (Lynd et al., 2002).

Microbial cellulose utilisation can either proceed via aerobic or anaerobic organisms, but not both at the same time. Most of the cellulose on Earth is degraded aerobically according to the reaction to CO₂ and water, while approximately 5-10% is fermented to methane and CO₂ and another approximately 5-10% is degraded anaerobically (Ehhalt, 1974; Haichar et al., 2007).

Aerobic degradation

Well-aerated topsoil layers are the most important habitats for aerobic biota involved in cellulose degradation, whereas in an underground GDF aerobic microbial degradation is expected to occur only during the operational time and shortly after repository closure. In the presence of sufficient amounts of oxygen, complete degradation of cellulose to CO₂ and water is probable. Most known cellulose degraders today were found in aerobic environments (Haichar et al., 2007). In order to access the cellulosic substrate, lignin degraders, in particular Actinomycetes, disintegrate the lignin matrix. Among the most intensively studied aerobic cellulolytic bacteria are Cellulomonas, Pseudomonas, Thermomonospora and Microbiosa (Béguin and Aubert, 1994). Species include Myxobacteria that are specialised in cellulose degradation (Yan et al., 2003). Bacteria related to the Dyella show β-glucosidase activity and can utilise cellulose as sole carbon source (An et al., 2005). Mesorhizobium belongs to the Rhizobia group of bacteria and has cellulolytic and pectinolytic enzymes that can break glycosydic bonds in plant cells.
(Hubbell et al., 1978, Mateos et al., 1992). Another important group are the *Sphingomonas*, ubiquitously distributed soil bacteria that exhibit cellulase activity (Männistö and Häggblom, 2006). *Streptomyces sp.* are also distributed widely and well known for their ability to degrade cellulose (Wachinger et al., 1989; Semedo et al., 2000; Wirth and Ulrich, 2002). Fungi, capable of aerobic cellulose degradation include the well-studied *Trichoderma reesi* (Béguin and Aubert, 1994).

### Anaerobic degradation

Anaerobic microbial degradation of cellulose occurs in a broad range of habitats with many different microorganisms involved, resulting in the production of carbon dioxide and methane or other reduced compounds, such as $\text{H}_2\text{S}$, in the presence of sulfate as a terminal electron acceptor (Béguin and Aubert, 1994). In a GDF, anaerobic microbial degradation is especially expected to take place in post-closure times, when oxygen becomes depleted.

When an aerobic environment, such as soils or fresh water, changes to anaerobic conditions or cellulose is transported to an anoxic site, the availability of terminal electron acceptors (e.g. nitrate, Mn(IV), Fe(III), sulfate) is crucial to maintain cellulose degradation (Leschine, 1995). If both, oxygen and alternative electron acceptors are absent, fermentation takes over (Figure 2.9). Cellulolytic microbes that are adapted to these low-energetic conditions produce enzymes to depolymerise cellulose, thereby producing cellobiose, celloextrins, and some glucose (Leschine, 1995). These intermediates can be further degraded by the cellulase system producing alcohols, organic acids, $\text{H}_2$, $\text{CO}_2$ and eventually methane. Methanogens and acetogens use the emerging $\text{H}_2$ to produce methane or acetate, respectively. Acetate can be used by methanogens to produce methane and $\text{CO}_2$. Thus, through the combined activity of syntrophic bacteria and archaea, the cellulose molecule is completely converted to $\text{CO}_2$ and $\text{CH}_4$. In environments with high sulfate levels, sulfate-reducing bacteria may compete with the methanogens for acetate, resulting in $\text{H}_2\text{S}$ production. Known cellulose fermenting organisms are typically Gram-positive *Clostridia*, *Ruminicocci* and *Caldicellulosirupta*, but contain a few Gram-negative species, such as *Butyrivibrio* and *Acetivibrio* (Lynd et al., 2002).
When the conditions in a GDF near-field become anaerobic, microorganisms will be additionally exposed to extremely harsh alkaline conditions, elevated temperatures, radiation, low water contents and a lack of key nutrients (Colasanti et al., 1991; Stevens et al., 1993; Chicote et al., 2004; Rizoulis et al., 2012; Williamson et al., 2013; Brown et al., 2015). Microbial activity in such environments was seen to be mainly limited to pH values of 10-11 (Rizoulis et al., 2012; Williamson et al., 2013; Bassil et al., 2015b), however activity has been observed up to a maximum pH of 12.25 (Colasanti et al., 1991). Consequently microbial activities are expected to be greatly reduced for the first tens of thousands of years (Askarieh et al., 2000). Therefore, the environmental conditions within a GDF may not completely prevent microbial activities and microbial communities might develop in micro niches in and around the wasteforms. In the nutrient depleted deep geosphere, the cellulose content of the GDF might also present a substantial energy source for alkaliphilic microbes that survive in such niches within the wastes. Despite the possibility for small amounts of microbial cellulose degradation to occur shortly after

Figure 2.9. Microbial degradation of cellulose under anaerobic conditions in soils and freshwater sediments. Typically formed fermentation products include lactate, succinate and ethanol, which are further degraded by heterotrophic bacteria. In environments where nitrate, Mn(IV), Fe(III) or sulfate are present, the final products may differ (adapted from Humphreys et al., 2010)
GDF closure, most cellulose is assumed to be degraded via radiolytic (section 2.5.2) and chemical degradation (section 2.5.3), under evolving repository conditions.

### 2.5.2. Radiolytic degradation of cellulose

Radiolysis of cellulose is known to occur in a GDF, but compared to chemical degradation (2.5.3) it is not the predominant degradation pathway (Arthur and Hinojosa, 1971; David and Bergh, 1982; Bradshaw *et al.*, 1986, 1987).

When cellulosic materials are exposed to ionising radiation, energy will be absorbed resulting in the dissociation of covalent bonds and the generation of free radicals. The degree of structural change or damage depends on the amount of radiation energy absorbed, whereby the radiation resistance varies with the polymer structure (Humphreys *et al.*, 2010). The free radicals generated can cause mid-chain scission or cross-linking. The manner in which free radicals react in a polymer depends on several factors, such as the chemical and physical structure of the polymer, the environment, the temperature and finally the irradiation dose rate (Humphreys *et al.*, 2010). During pre- and post-closure times, cellulose will be exposed to alpha, beta and gamma radiation, with the gamma dose rate declining rapidly over the first few hundred years post-closure since its emissions are dominated by $^{60}\text{Co}$ and $^{137}\text{Cs}$. Alpha doses, on the other hand, will be dominated by a significant inventory of long-lived radionuclides (Heath and Williams, 2005). The effects of gamma radiation on cotton result in increased amounts of carbonyl groups, carboxyl groups and chain cleavage in a ratio of 20:1:1, a decrease in tensile strength and increased water and alkali solubility (Blouin and Arthur, 1958).

### 2.5.3. Chemical degradation of cellulose

The chemical degradation of cellulose is mainly influenced by the pH. Under the alkaline conditions expected in the evolved GDF near-field, chemical processes are likely to dominate, degrading cellulose to water-soluble, low molecular weight compounds (Whistler and BeMiller, 1958).

The chemical degradation of 4-$O$-substituted glucose derivatives, such as 4-$O$-methyl-$\beta$-glucose, maltose, amylase and cellulose is particularly important under these conditions. The type of degradation products formed depends strongly on the solution chemistry in contact with cellulose: In the Ca$^{2+}$-rich cementitious porewaters though, the formation of 3-deoxy-2C-
(hydroxymethyl)-erythro and threo-pentonic acids (β-glucosiosaccharinic acids) is favoured (Nef, 1907, 1910; Nef et al., 1917; Whistler and BeMiller, 1958). Mechanisms involved include a ‘peeling-off’ reaction, which initiates the reaction, and a ‘stopping’ reaction, which competes simultaneously with the former reaction at different endings of cellulose molecule (Nef, 1907, 1910; Nef et al., 1917).

The alkaline degradation of cellulose is initiated at relatively rapid rates in terms of geological timescales, and is governed by the peeling-off mechanism that depends on the concentration of reducing end groups. During degradation, glucose units are eliminated from the cellulose chain in a stepwise process, starting at the reducing end group, while the β-1,4 glycosidic linkages are alkali-stable and cannot be reduced.

The peeling-off reaction takes place from the beginning of cellulose degradation and attacks the reducing end groups of the cellulose molecule (Knill and Kennedy, 2003). The first step in the peeling-off reaction involves the production of an enediol, via keto–enol tautomerism (Figure 2.10). This is followed by the production of an enediol anion via deprotonation by hydroxide ions. Anion isomerisation then takes place, resulting in a mixture of equilibrium intermediate anions with either aldose or ketose end groups (Pavasars et al., 2003). The anions then undergo β-elimination, forming diketodeoxyglycitol which is again transformed via keto-enol tautomerism to a hexose. The reaction progresses depending on the C-atom at which the β-elimination takes place, if the β-elimination occurs at the C-4 atom, one hexose monomer is split off and peeling off continues at the next glucose molecule, while if it occurs at any other position, the hexose remains associated to the cellulose molecule and induces chemical stopping (Pavasars et al., 2003). Finally, after either type of elimination the diketodeoxyglycitol intermediate can undergo benzilic acid rearrangement to produce metasaccharinic acid or isosaccharinic acid (ISA), the most common products (Nef, 1907, 1910; Nef et al., 1917; Pavasars et al., 2003). Around >50 components were observed among the cellulose degradation products, of which glucosiosaccharinic comprised around ~70 to 85% (Pavasars et al., 2003).
Benzilic acid rearrangement transforms an α-diketone into the salt of an α-hydroxyacid via a base-catalysed reaction. For this to take place, a hydroxide ion is added to a carbonyl group, producing an intermediate that undergoes an intramolecular rearrangement followed by a proton-transfer yielding to an α-hydroxy acid. This base-catalysed process requires only one molecule of base to bring one molecule of benzil and water is not a necessity (Vercammen et al., 1999a). In this way, the yield of rearrangement products increases with a more basic pH as it is base-catalysed, however, surprisingly the yield is also enhanced under acidic conditions, suggesting that not exclusively OH\(^-\) groups, but also H\(^+\) catalyse rearrangement (Comisar et al., 2005). The presence of Ca\(^{2+}\) catalyses benzilic acid rearrangement, which generates ISA, consequently Ca\(^{2+}\) in solution increases the yield of ISA. Moreover, ISA preferentially forms complexes with Ca\(^{2+}\) in solution at a postulated ratio of 1:1 (Vercammen et al., 1999a).

![Figure 2.10. Degradation mechanism under alkaline conditions to form either metasaccharinic acid (chemical stopping) or isosaccharinic acid (peeling-off) (adapted from Pavasars et al., 2003).](image)

The aforementioned stopping reaction distinguishes between a chemical and a physical stopping mechanism and will be explained in the following section. If no other reactions would compete with the peeling-off reaction, cellulose would eventually be degraded completely. However, studies of the kinetics of the peeling process indicate that as the reaction proceeds,
more reducing end groups are transformed by a pseudo-first order mechanism, and this limits the number of reducing end units compared to non-reducing end groups (metasaccharinic acids), resulting in an alkali-stable structure (van Loon et al., 1999a). There are two processes that can terminate cellulose degradation. First, chain termination arises when the degradation directly undergoes β-elimination, forming metasaccharinic acids which undergo keto-enol tautomerism and then benzilic acid rearrangement (Figure 2.10; Haas et al., 1967). The alkali structures produced are no longer available for degradation, therefore this reaction is referred to as chemical stopping, and inhibits complete degradation (Van Loon and Glaus, 1997).

Additionally, cellulose degradation can be terminated at certain points of the molecule due to physical properties, referred to as ‘physical-stopping’. This stopping is induced by a change in crystallinity, whereby amorphous regions are readily degraded, while highly ordered crystalline regions are less susceptible to degradation processes (Humphreys et al., 2010). The reason is the lower solubility of crystalline regions, which makes them more resistant to alkali (Van Loon and Glaus, 1997). The reaction is not stopped abruptly, but rather via a transitional change in more crystalline regions (Haas et al., 1967). However, based on X-ray studies looking at different reagents, there seems to be no strong correlation between crystallinity and susceptibility (Rowland, 1985).

In contrast, alkaline hydrolysis of glycosidic bonds produces new reducing end groups, which will reinitiate the peeling-off reaction, referred to as ‘mid-chain scission’ (van Loon et al., 1999a; Pavasars et al., 2003). Mid-chain scission is the hydrolysis of cellulose by alkali attack of the cellulose chain at random positions (van Loon et al., 1999a; Pavasars et al., 2003). It is not a major reaction pathway for the alkaline degradation of cellulose until a temperature of about 170°C is reached (Askarieh et al., 2000). At temperatures of more than 170°C, independently from the availability of oxygen, mid-chain scission leads to great weight losses of cellulose. Although these high temperatures are not credible in an ILW disposal vault (Knill and Kennedy, 2003), mid-chain scission may also be initiated by radiolytic processes. When cellulosic wastes are exposed to radiation, non-volatile degradation products transform into a homologous series of glucose units from cellobiose to celloheptaose (Askarieh et al., 2000). These products are indicators for mid-chain scission that will regenerate reducing end-groups and allow the peeling reaction to start anew (Knill and Kennedy, 2003). Basically, the glycosidic linkages will be
cleaved either between the oxygen and the glucosyl group or the oxygen and the aglycone (Knill and Kennedy, 2003).

A model for the mechanisms and kinetics of cellulose degradation that assumed the peeling-off reaction to be a pseudo-first-order reaction, dependent on the number of reducing end groups and on the degree of polymerisation, found that the production of ISA depends on the same parameters (van Loon and Glaus, 1997). However, the degradation rate and the arising products will also depend on other factors such as the cellulose/water ratio, pH, temperature, available cations, and electron acceptors (Pavasars et al., 2003). Chemical properties of the repository, such as the OH$^-$ concentration (or pH), which lies around 0.3 M and the Ca$^{2+}$ concentration, around 20 mM in the initial cementitious porewater, can have a governing effect on the peeling-off reaction (van Loon and Glaus, 1997; van Loon et al., 1999a). A study showed an increase in the rate constant for the peeling-off reaction with the OH$^-$ concentration up to 0.3 M and remained constant at higher concentrations (Van Loon and Glaus, 1997). Later research showed that the kinetics changed after a peeling-dominated period for about three years, with degradation rates becoming slower and mainly limited by alkaline cleavage of glycosidic bonds to non-reducing ends (Pavasars et al., 2003). The effect of Ca$^{2+}$ in solution catalyses benzilic acid rearrangement, as well as chemical stopping, thereby the overall effect is an increased rate of ISA production (Machell and Richards, 1960; Van Loon and Glaus, 1997).

Modelling and long-term studies have been carried out, to make predictions on the time scales and the course of cellulose degradation (Van Loon and Glaus, 1997; Glaus et al., 1999a; Pavasars et al., 2003). These studies showed a change of degradation mechanism from the first three years that will be dominated by the peeling-off reaction, producing mainly ISA, to alkaline hydrolysis in later stages, increasing the ratio of other organic acids compared to ISA (Van Loon and Glaus, 1997). A model by van Loon & Glaus (1997) predicted initial degradation of up to 10% of the cellulose in 1,000 years with degradation being complete between 100,000 and 1,000,000 years after closure. A follow-up model refined these results, concluding a rapid initial degradation, removing around 15 to 25% of the cellulose in the first three years, during which time ISA dominated; after seven years this dominance decreased in favour of other degradation products (Pavasars et al., 2003). From their results, these authors presumed a much faster degradation rate and estimated the cellulose content of a GDF to be degraded...
completely after approximately 150 to 550 years. Glaus and van Loon reworked their results (2008), and proposed an edited model based on data of a 12 year incubation experiment which includes another hidden reaction. The suggested reaction takes place at the reducing end of glucose that is hidden from alkali in the crystalline part of the molecule, but will become temporarily available to alkali attack, followed by the peeling-off reaction. In this regard, with the high numbers of reducing end groups that were hidden, the rate of cellulose degradation will be accelerated and whole decomposition could occur in 1,000 to 5,000 years (Glaus and van Loon, 2008). The different time scales estimated in studies by van Loon and Glaus (1997; 2008) and Pavasars and colleagues (2003), result from different assumptions, such as rate constants for alkaline hydrolysis or temperatures. In conclusion, evaluations about cellulose degradation rates under GDF conditions might be updated over coming years with new experiments, however a core statement derived from these studies implies that, for geological time-scales, fast and complete cellulose degradation occurs with ISA arising as the main stable degradation product.

2.6. Isosaccharinic acid
At moderate pH values (pH <9) cellulose is degraded mainly by biological processes to carbon dioxide and methane, while non-biological reactions, such as the peeling reaction, are expected to take over at high pH conditions (Svensson et al., 2007). As explained in section 2.5.3, under the highly alkaline environment of a GDF, cellulose is unstable and can degrade abiotically, producing water-soluble, low molecular weight compounds (e.g. ISA) with metal-complexing properties. Because of their complexing ability, the presence of ISA could potentially lead to higher solubilities and mobilities of radionuclides (Whistler and BeMiller, 1958). The specific composition of the products of cellulose degradation depends on the chemical conditions, such as the cations present and the pH (Machell and Richards, 1960). However, at highly alkaline conditions and in contact with Ca$^{2+}$, which is abundant in cementitious repositories, ISA has been reported to be the main product from the chemical degradation of cellulosic material (Machell and Richards, 1960; Van Loon and Glaus, 1997; Glaus et al., 1999a), remaining stable in long-term experiments (Pavasars et al., 2003).

Chromatographic analyses of leachates from anaerobic alkaline cellulose degradation experiments, revealed additional byproducts, contributing a small percentage (5%) of the total organic carbon (van Loon et al., 1999a). An extensive review combining results from studies
under a wide range of temperatures (20-200°), lists the commonly identified degradation products (Knill and Kennedy, 2003). The major differences noted were thought to be due to changes in the chemical conditions including the relative concentrations of substrates. In total, more than 50 products were identified, while the main byproducts were lactic acid, glycolic acids, pentonic acids, α-glucosiosaccharino-1,4-lactone, and β-glucometasaccharinic acid in a complex mixture of products.

Another industry that generates significant amounts of ISA is the alkaline pulping of wood to produce paper and card based products (Niemelä and Sjöström, 1986). In this process lignin is dissolved creating a so-called ‘black liquor’ that undergoes partial degradation by which a complex mix of organic compounds is being produced. The incineration of municipal solid waste incineration (MSWI) dry scrubber residues is another industrial sector handling vast amounts of cellulose. However, not all cellulosic material is incinerated due to insufficient capacity of the incineration plant which will lead to co-disposal of ash together with cellulose which facilitates ISA formation (Svensson et al., 2007). Moreover, MSWI dry scrubber residue is known to contain high amounts of metals (Chandler et al., 1997), which can be chelated by the presence of ISA from unburnt co-disposed cellulosic material (Svensson et al., 2007). For this reason, there exists interest from other industrial sectors to study the fate of ISA in the environment.

### 2.6.1. Structure and speciation of ISA

Isosaccharinic acid has two diastereomers, α-ISA (3-deoxy-2-C-(hydroxymethyl)-erythro-pentanoic acid) and β-ISA (3-deoxy-2-C (hydroxymethyl)-threo-pentonic acid), as displayed in Figure 2.11. Both stereomers together comprise 70 to >80% of cellulose degradation products and are produced in approximately equal amounts (van Loon et al., 1999a; Pavasars et al., 2003; Ekberg et al., 2004). Because α-ISA is known to form strong complexes with actinides and other metals present in ILW (explored in detail in section 2.6.3), knowledge of the behavior of the acid is crucial for the potential mobility of radionuclides in the environment of a nuclear waste repository.
ISA is a weak acid that is present either as a protonated (HISA) or deprotonated (ISA-) molecule. As a result of different methodological approaches, there exists a great deal of disagreement in the literature about the deprotonation and lactonisation of ISA (Rai and Kitamura, 2016), which will be discussed in the following.

Both ISA diastereomers have two enantiomeric lactone confirmations (ISA_L) (Figure 2.11), which form from the open-chain molecule by losing a water molecule and condensation into ring-molecules (van Loon et al., 1999a). The lactone is a propionic ring of 4-C atoms containing an alcohol group (-OH) and a carboxylic acid group (-COOH). The open-chain- or ring-structures are known to inter-convert depending on the pH (Ekberg et al., 2004). Several studies have shown that under acidic conditions, with a pH < 5, the lactone form is prevalent (Rai et al., 1998; Ekberg et al., 2004; Brown et al., 2010). In more detail, the equilibrium reaction for the deprotonation of the base form of HISA can be written as:

$$HISA \leftrightarrow ISA^- + H^+$$

(eq. 2.1)

for which the logK can vary between -3.27 to -4.04 (Cho et al., 2003; Pavrasars et al., 2003; Rai et al., 2003; Shaw et al., 2012; Almond et al., 2016). The acid-catalysed lactonisation reaction that transforms HISA to ISA_L can be written as:

$$HISA_{(aq)} \leftrightarrow ISA_L + H_2O$$

(eq. 2.2)
The transformation constant into the lactone was estimated to vary from \( \log K = 0.37 \) to 0.84 (Cho et al., 2003; Rai et al., 2003; Rai and Kitamura, 2016).

\(^{13}\)C NMR studies of the HISA molecule demonstrated that the carboxylic acid is the most acidic site of the molecule with the respective acid dissociation constant \((K_a)\) being:

\[
K_a = \frac{[ISA^-][H^+]}{[HISA]} \quad \text{(eq. 2.3)}
\]

An initial acid dissociation constant was estimated to be \( \log K = -4.46 \) (Rai et al., 1998). However, this constant is questionable because they did not record if the transformation reaction had been taken into account. Nevertheless, they re-interpreted their work, only using the dissolution constant of Ca\((ISA)_2\) (into \(Ca^{2+}\) and \(ISA^-\)), giving a \(\log K\) value of \(-3.27 \pm 0.01\) (Cho et al., 2003; Rai et al., 2003). This group also found by \(^{13}\)C and \(^1\)H NMR spectroscopy studies that the most acidic site of the neutral HISA molecule is the carboxylic acid, which is largely deprotonated at \(pH = 5.3\) (Cho et al., 2003). Another research group did further work on the equilibrium constant, correcting the dissociation constant to \(\log K = 3.86 \pm 0.05\), while the transformation into ISA\(_L\) gave \(\log K = 6.88 \pm 0.64\) (Ekberg et al., 2004). They also determined the forward reaction of the transformation rate from HISA to ISA\(_L\), which was relatively slow and had a half-life of \(t_{1/2} = 122.2 \pm 0.6\) min, while the backward reaction was faster.

![Figure 2.12. Speciation of isosaccharinic acid as a function of \(-\log [H^+]\) in 0.1 mol dm\(^{-3}\) NaClO\(_4\) at 23°C given as relative concentrations of ISA. The proton concentration is in mol dm\(^{-3}\) and \(c^0\) is equal to 1 mol dm\(^{-3}\). The ISA\(_{tot}\) is the concentration of HISA and ISA\(^-\) (adapted from Brown et al., 2010).](image-url)
More recently, the equilibrium dissociation constant has been re-examined kinetically and also substituted by two kinetic rate constants, giving higher accuracy for the two constants (Brown et al., 2010). The first constant only describes the dissociation of HISA into ISA− and the sum of HISA and ISA_L to give ISA− (Brown et al., 2010). Figure 2.12 presents the constants of relative concentrations of [ISA] + [HISA] which are in accordance with the thick line representing the total ISA concentration (ISA_tot), equilibrated by Brown et al. (2010). Data points in the same Figure indicate measurements from measurements of the speciation of ISA in 0.1 mol dm⁻³ NaClO₄ that are in agreement with the constants (Ekberg et al., 2004). From these constants the transformation constant from ISA to ISA_L was derived as logK = 0.79 ± 0.04. Finally, these results enabled the estimation of the acid dissociation constant at an ionic strength of zero for ISA_L as logK = -4.83 ± 0.07, while for HISA alone the logK = -4.04 ± 0.06, and for both HISA and ISA_L the logK was -4.9 ± 0.07 (Brown et al., 2010).

### 2.6.2. Solubility and sorption

In the presence of ISA, radionuclides can form strong aqueous complexes (Greenfield et al., 1992; Rai et al., 1998; van Loon et al., 1999b; Ekberg et al., 2004). Thus, it may be expected that ISA can increase the mobility of radionuclides in cementitious porewaters, unless its concentration is lowered by the formation of sparingly soluble complexes and/or the sorption of ISA on cement. In solution ISA competes with radionuclides for precipitation sites. In cementitious porewaters it has been observed that ISA was taken up by the cements, as a result of the formation of stable Ca(ISA)₂ salts in the presence of the high Ca²⁺ concentrations (van Loon et al., 1997; Vercammen et al., 1999a):

\[
Ca(ISA)_2(c) + 2H^+ \leftrightarrow Ca^{2+} + 2HISA_{(aq)} \quad (eq. 2.4)
\]

Whereby an indexed \(c\) stands for a crystalline and \(aq\) for an aqueous phase. The general dissolution equilibrium of Ca(ISA)₂(c) from (eq. 2.4), can involve a number of reactions, depending on the pH (Rai et al., 1998):

\[
Ca(ISA)_2(c) + 2H^+ \leftrightarrow Ca^{2+} + 2 HISA \quad (eq. 2.5)
\]

\[
Ca(ISA)_2(c) \leftrightarrow Ca(ISA)_{2(aq)} \quad (eq. 2.6)
\]

\[
Ca(ISA)_2(c) \leftrightarrow Ca^{2+} + 2ISA^- \quad (eq. 2.7)
\]
The reaction is dissociated completely into Ca\(^{2+}\) and the protonated form of ISA (HISA) at pH <4.5, increasing the concentration of both ions (eq. 2.5). The thermodynamic equilibrium coefficient was estimated at pH <4.5 with Ca\(^{2+}\) and ISA\(^{-}\) in equilibrium as \(\log K = 1.3\). In contrast, the concentrations of Ca\(^{2+}\) and ISA remain stable in the pH range from 4 to 12, indicating dominance of (eq. 2.6) or (eq. 2.7) (Rai et al., 1998), with a thermodynamic equilibrium coefficient found to be \(\log K = -2.22\) in this pH range. When the Ca\(^{2+}\) concentration is elevated as a function of pH from 4 to 12, reaction (eq. 2.8) is likely to proceed, wherefore ISA\(^{-}\) decreases from solution. For this equation \(\log K\) was estimated to be in the range between -6.22 to -6.53 (Rai and Kitamura, 2016). At pH values beyond 12, Ca\(^{2+}\) precipitates as Ca(OH)\(_2\), liberating ISA into solution (Rai et al., 1998; Vercammen et al., 1999a). The precipitation of portlandite, results in the release of ISA\(^{-}\) into solution, which can bind with small amounts of Ca\(^{2+}\) remaining in solution to form a weak complex of Ca(ISA)\(^{+}\), with an approximate \(\log K\) of 1.7 (Vercammen et al., 1999a; Rai et al., 2003; Hummel et al., 2005; Rai and Kitamura, 2016):

\[
Ca^{2+} + ISA^- \leftrightarrow Ca(ISA)^+ \tag{eq. 2.8}
\]

At very high pH (>12), another proton may be removed from a hydroxyl group of the ISA\(^{-}\), leading to the formation of Ca(ISA\(^{-}\)H\(_0\))\(^0\):

\[
Ca^{2+} + ISA^- \leftrightarrow Ca(ISA^-H)^0 + H^+ \tag{eq. 2.9}
\]

with an equilibrium coefficient of \(\log K = -10.4\) (Vercammen et al., 1999a). An acid dissociation constant for the hydroxyl groups of ISA has also been found (Cho et al., 2003).

Finally, Rai and his colleagues (1998) calculated the logarithm of the solubility product (\(\log K_{sp}\)) for the ISA dissociation into Ca\(^{2+}\) and ISA\(^{-}\) to be \(\log K_{sp} = -7.62\) for pH <12 with the reaction:

\[
K_{sp} = a_{Ca} \cdot (a_{ISA})^2 \tag{eq. 2.10}
\]

whereby \(a_{Ca}\) is the activity of Ca and \(a_{ISA}\) is the activity of ISA\(^{-}\) in solution. This value was later corrected to be \(\log K_{sp} = -6.36\) for the pH 10.8 to 12 (Vercammen et al., 1999a) and was in the range of \(\log K_{sp} = -6.22 \pm 0.03\) at neutral pH (van Loon et al., 1999b). The complexation constant for the formation of a complex between Ca and ISA was reported as \(\log K = 5.40\) (Rai et al., 1998). Conservative estimates of the maximum concentration of ISA generated in solution were also calculated at \(~5 \times 10^{-2}\) M (Bradbury and van Loon, 1997; van Loon and Glaus, 1998b).
For considerations of ISA sorption onto cement, a study using artificial cement pore water (pH = 13.3, 2 mM Ca) found with an increasing degree of cellulose degradation, more ISA sorbed to cement, that led to an increase of Ca in solution (van Loon and Glaus, 1998a). Even though there will be electrostatic repulsion between the negatively charged surfaces of cement and ISA, strong interactions will result in quick sorption of ISA on cement, almost reaching equilibrium after one day (van Loon et al., 1997; Heath and Williams, 2005). The water content in the cement also influences ISA sorption, whereby lower water content results in stronger sorption of ISA (Heath and Williams, 2005). The total sorption capacity of cement for ISA is high and has been estimated to be around 0.3 mol kg$^{-1}$ (Bradbury and Sarott, 1995; van Loon and Glaus, 1997).

**2.6.3. Complexation of ISA with radionuclides**

Studies of the behaviour of radionuclides under repository conditions have practical importance for performance assessment and the development of safety cases for radioactive waste management. In particular the influences of complexing agents with regards to the radionuclide solubility and sorption characteristics are important to consider during the evolution of a GDF. Complexing agents (ligands) are compounds that most commonly contain elements such as oxygen or nitrogen, which have high electron-donating abilities to metal ions or radionuclides. Chelation takes place when a ligand binds to a radionuclide through more than one donor atom. The stability of the interaction is very dependent on the chemistry of the participating radionuclides and ligands. Organic ligands are already present in ILW, including oxalic acid, citric acid, ethylenediaminetetraacetic acid (EDTA) or nitriloacetic acid (NTA; Francis, 1998; Hummel et al., 2005; Suzuki and Suko, 2006), which have been used in decontamination operations. A second group comprises ligands that form in situ from the degradation of wastes, of which ISA is the most important organic complexant. ISA has been shown to affect the sorption and solubility of many cations of the transition metal, lanthanide and actinide series (e.g. Greenfield et al., 1992; Baston et al., 1994a; Bourbon and Toulhoat, 1996; Vercammen et al., 1999a; Warwick et al., 2003). Since ISA forms under alkaline conditions, most research has focused on complexation behaviour of ISA and resulting transport behaviour of radionuclide-ISA complexes under these conditions (Greenfield et al., 1992, 1997; van Loon and Glaus, 1998a;
Almost equal amounts of α-ISA and β-ISA arise from the alkaline degradation of cellulosic wastes, however since the strength of complexation of metals or radionuclides for the β-form is ~2 orders of magnitude lower (van Loon and Glaus, 1998a) most research has focused on experiments using α-ISA. Thermodynamic modelling has shown that deprotonated hydroxyl groups play a major role in the complexation with radionuclides (Greenfield et al., 1990, 1992, 1994; Bourbon and Toulhoat, 1996). Two different processes have been considered during complex formation under alkaline conditions, either the addition of OH⁻ groups, or the removal of an equal number of H⁺ groups from the ISA molecule (Cho et al. 2003; Gaona et al. 2008), and will be discussed in more detail for key radionuclides below. ISA is a polyhydroxy ligand with polyhydroxy behaviour. ISA (L⁻) has two potential donor atoms for multivalent metal ions which allows complexation with one or more metals or radionuclides. For a 1:1 complex following equation can be applied:

$$M^{z+} + L^- \leftrightarrow ML^{z-nH_-^{-1}} + nH^+$$  \hspace{1cm} (eq. 2.11)

A generalised coordination-ionisation scheme postulated for polyhydroxy carboxylic acids predicts \( n \) to be 0 for the binding of Ca²⁺ at pH values 4-10, whereas at higher pH >10, \( n \) is expected to be 1 (Vercammen et al., 1999a).

A key factor in radionuclide complex generation is the concentration of ISA, which depends on the cellulose loading in a GDF, its degradation rate and the cement porosity (van Loon et al., 1997; van Loon and Glaus, 1998a). In UK waste, the amount of cellulose will likely average around 1% (NDA and DECC, 2014), from which up to 0.1 M ISA can be generated under conservative assumptions in the cement porewater (van Loon et al., 1997). This amount was judged to be sufficient to complex the present radionuclides, which could significantly alter radionuclide migration by enhanced solubility (Baston et al., 1994b; van Loon et al., 1997). Solubility enhancement factors for different radionuclides at different concentrations of ISA are shown in Table 2.2 (NDA, 2010f). The strongest solubility enhancement was measured for Pu(IV) (Greenfield et al., 1992; Bradbury and Sarott, 1995). In addition, the effect of ISA on radionuclide sorption ranges around one or two orders of magnitude (van Loon and Glaus,
1998b; Heath and Williams, 2005 and references therein). Thus the apparent influence of ISA on solubility limits appears to be far greater than on sorption (Bradbury and Sarott, 1995).

It should be noted that in laboratory tests the solubility enhancement of ISA on plutonium was studied, however ISA-Pu complex formation will not be relevant to geological disposal, because reprocessing of the wastes will separate plutonium which can be resued in the fuel cycle (RWM and NDA, 2015), whilst ISA will be produced in wastes containing cellulose (mainly ILW and some LLW). For this reason, complexation of ISA with plutonium will not be further discussed in this thesis.

<table>
<thead>
<tr>
<th>Element</th>
<th>Best estimate for solubility enhancement factors (SEF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cellulose loading 10 wt%</td>
</tr>
<tr>
<td>Pu</td>
<td>4.6 x 10^5</td>
</tr>
<tr>
<td>Am</td>
<td>1.6 x 10^5</td>
</tr>
<tr>
<td>Th</td>
<td>2.0 x 10^4</td>
</tr>
<tr>
<td>U</td>
<td>2.2 x 10^2</td>
</tr>
<tr>
<td>Np</td>
<td>1.7 x 10^4</td>
</tr>
<tr>
<td>Tc</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Cellulose loading 1 wt%</td>
</tr>
<tr>
<td>Pu</td>
<td>4.6 x 10^3</td>
</tr>
<tr>
<td>Am</td>
<td>2.4 x 10^3</td>
</tr>
<tr>
<td>Th</td>
<td>5.4 x 10^0</td>
</tr>
<tr>
<td>U</td>
<td>3.4 x 10^1</td>
</tr>
<tr>
<td>Np</td>
<td>2.5 x 10^1</td>
</tr>
<tr>
<td>Tc</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Cellulose loading 0.1 wt%</td>
</tr>
<tr>
<td>Pu</td>
<td>5.3 x 10^0</td>
</tr>
<tr>
<td>Am</td>
<td>3.7 x 10^1</td>
</tr>
<tr>
<td>Th</td>
<td>1.8 x 10^0</td>
</tr>
<tr>
<td>U</td>
<td>3.1 x 10^1</td>
</tr>
<tr>
<td>Np</td>
<td>1.3 x 10^0</td>
</tr>
<tr>
<td>Tc</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.2. Best estimates for the radionuclide solubility enhancement factors (SEF) relative to Tc at thermodynamic equilibrium in the presence of varying ISA concentrations (modified after NDA, 2010f). Even though ISA is known to enhance the mobility of Pu, Pu is rarely important in the context of ISA complexation in ILW related to its availability.
Previous results relating to the impact of ISA on the radionuclides/analogues of relevance to this work are outlined in the following sections:

**Nickel**

Nickel (Ni) based alloys are valuable in minimising the corrosion of steel (Platt et al., 1997). Ni-63 (half-life 9.9 x 10^1 years) and Ni-59 (half-life 7.6 x 10^4 years) are activation products formed from the irradiation of steel nuclear reactor components (NDA, 2010f, 2014). Upon release following corrosion of these metal alloys, nickel shows low solubility in the alkaline porewaters (pH 11.5 to 13.5) and high sorption onto cement (Bradbury and Sarott, 1995; Scheidegger et al., 2000). However, in presence of ISA, Ni solubility is shown to be enhanced (Bradbury and Sarott, 1995; Baker et al., 2003). The formation of different aqueous complexes that are responsible for enhanced solubility are dependent on the pH (Warwick et al., 2003; Evans et al., 2013; Almond et al., 2016). At pH values <7 a 1:1 Ni:ISA stoichiometry is expected, resulting in Ni(ISA)^+, whereas between pH 7 and 10 a 2:1 ratio produces Ni_2ISA(OH)_3, and at pH >10 a Ni_2ISA(OH)_4 complex will be dominant. At neutral pH the stability of a Ni-ISA complex is much weaker with log_{10}β = 2.2 to 2.58, whereas at pH >10 the stability constant was estimated as log_{10}β = 29.9 (Warwick et al., 2003; Almond et al., 2016).

**Americium, Europium & Lanthanum**

Americium (Am), europium (Eu) and lanthanum (La) are trivalent elements of the lanthanide and actinide series with stable and synthetic radioactive isotopes. La is often used experimentally as analogue for Am, as it provides a stable, non-radioactive metal (Tolley et al., 1995; Yong and Macaskie, 1998), however little research is currently available on its complexation with ISA. Most work has focused on Am and Eu for which complexation reactions are presented.

It is assumed that under alkaline conditions, polyhydroxy carboxylic acids form complexes with trivalent cations associated with deprotonation of one or more OH groups of the ligand (Tits et al., 2005), which can be written as:

\[ \text{Am}^{3+} + \text{ISA}^- \leftrightarrow \text{Am(OH)}_4\text{ISA}^- + 4H^+ \]  

(eq. 2.12)

Studies on the sorption behaviour of europium (Eu) onto cement phases showed a high affinity for Eu sorption. The presence of ISA showed a concentration-dependent decrease in sorption at 10^{-4} M to 10^{-3} M equilibrium ISA concentration at pH = 11 (van Loon and Glaus, 1998b) or
above 10^{-5} M at pH = 13.3 (Tits et al., 2005) due to complex formation. The complexation of europium with ISA was studied at high pH (10.7 to 13.3) and in the presence and absence of Ca (van Loon and Glaus, 1998a). Based on sorption data, these authors suggested, a 1:1 complex, Eu:ISA (eq. 2.13). However, re-interpretations of their work, based on the assumption that three protons were released lead to Eu(OH)$_3^{0}$ (aq) formation as most dominant species in solution and the following Eu(III)-ISA complexes (eq. 2.14; Vercammen et al., 2001; Tits et al., 2005).

\[ \text{Eu}^{3+} + \text{ISA}^- + 4H_2O \leftrightarrow \text{Eu(OH)}_4^-\text{(ISA)}^- + 4H^+ \]  
\[ \text{Eu}^{3+} + \text{ISA}^- + 4H_2O \leftrightarrow \text{Eu(ISA)}^- + 3H^+ \]  

(eq. 2.13)  
(eq. 2.14)

**Thorium**

Thorium-230 (Th) is a daughter in the uranium-238 natural decay series and has a half-life of 7.5 x 10^4 years. Th is mainly found in the redox state +IV, with sorption K_d values found to be $\geq1$ m$^3$ kg$^{-1}$ in the absence of ligands (Wieland et al., 1998). ISA was found to decrease Th sorption significantly at concentrations higher than 10^{-4} M (van Loon and Glaus, 1998a; Wieland et al., 2002), whilst lower concentrations of 10^{-5} M ISA were subsequently reported to also have to reduce Th(IV) sorption (Tits et al., 2005).

The stoichiometric complex that forms between Th and ISA in the absence of Ca and at high pH (12.8 and 13.3) was proposed to have a ratio of 1:1 and to be associated with the release of four protons (Vercammen et al., 1999b):

\[ \text{Th}^{4+} + \text{ISA} \leftrightarrow \text{Th(OH)}_4\text{(ISA)}^- + 4H^+ \]  

(eq. 2.15)

Parallel research however proposed a ratio of 1:2 Th:ISA from batch sorption experiments at pH = 13.3 and in the presence of Ca, which seemed to affect the stoichiometry (Wieland et al., 1998, 2002):

\[ \text{Th}^{4+} + 2H_4\text{ISA}^- + \text{Ca}^{2+} \leftrightarrow \text{CaTh(OH)}_4\text{(ISA)}_2 + 4H^+ \]  

(eq. 2.16)

Vercammen and co-workers (1999a) also found a ratio in the presence of Ca of 1:2 Th:ISA with one or two Ca ions. However, the Ca stoichiometry could not be resolved satisfactorily, because when using high performance anion exchange chromatography experiments, a 1:2:2 Th:ISA:Ca ratio was found, while from batch experiments a 1:2:1 Th:ISA:Ca complex was derived (eq. 2.16; Vercammen et al., 2001). In a subsequent study adopting the 1:2:1 stoichiometry,
equilibrium constants were calculated as \( \log K = -10.1 \pm 0.2 \) for the 1:2:1 ratio and \( \log K = -3.6 \pm 0.2 \) for the 1:1 ratio when Ca was absent (Vercammen et al., 2001). Later on, the 1:2:1 Th:ISA:Ca ratio estimated by Vercammen and colleagues was also adopted by Tits et al. (2005) in their model as the most likely formation. They estimated \( \log K = -5 \) (Tits et al., 2005).

**Neptunium**

Neptunium-237 (Np) is produced from uranium-235 by neutron capture in reactor cores. Np occurs as Np(V) in its most stable state, but Np(IV) is less soluble. Solubility experiments performed with amorphous NpO₂ in the presence of different ISA concentrations and under varying pH values have led to the postulation of four species that could form, as described below (Rai et al., 2003). At pH values <7 a ratio of 1:1 Np:ISA with the addition of three OH⁻ groups was proposed (eq. 2.17) or a ratio of 1:2 with three OH⁻ groups (eq. 2.18). For pH >7, there are 1:1 Np:ISA (eq. 2.19) and 1:2 (eq. 2.20) forms, both with four attached OH⁻ groups.

\[
\text{Np}^{4+} + 1ISA^- \leftrightarrow \text{Np}(OH)_3(ISA)_{aq} + 3H^+ \quad \text{(eq. 2.17)}
\]
\[
\text{Np}^{4+} + 2ISA^- \leftrightarrow \text{Np}(OH)_2(ISA)_{aq}^- + 4H^+ \quad \text{(eq. 2.18)}
\]
\[
\text{Np}^{4+} + ISA^- \leftrightarrow \text{Np}(OH)(ISA)_{aq}^- + 4H^+ \quad \text{(eq. 2.19)}
\]
\[
\text{Np}^{4+} + 2ISA^- \leftrightarrow \text{Np}(OH)_{aq}^2(ISA)_{aq}^2^- + 4H^+ \quad \text{(eq. 2.20)}
\]

**Uranium**

Uranium (U) is the most dominant radionuclide by mass in ILW, and has three naturally occurring isotopes, including \(^{238}\text{U}\), which is the most dominant (99.27%) and long-lived \(^{235}\text{U}\) and some \(^{234}\text{U}\), which are all alpha-particle emitters. Under environmental conditions, U has two stable oxidation states, U(VI) is found under oxic conditions as the uranyl moiety formally containing U-O bonds. It is mobile at neutral pH, usually occurring as \(\text{UO}_2^{2+}\)or its hydroxyl complexes above a pH of ~6.5, or as uranyl carbonate complexes at higher pH (Choppin et al., 2002). In the absence of carbonate, the uranyl ion sorbs strongly to mineral surfaces, such as iron oxyhydroxides, or organics (e.g. Andersson et al., 2001). Under reducing conditions, U usually occurs as nanoparticulate uraninite with the oxidation state U(IV), which is relatively insoluble and immobile. Sorption of U(VI) is pH dependent and is greatest between pH 5 to 8 (Barnett et al., 2000). Above this pH soluble U(VI)-carbonate complexes dominate. Complexing
agents, such as humic and fulvic acids, and competing cations, such as Ca\(^{2+}\) and Mg\(^{2+}\), decrease its sorption (Siegel and Bryan, 2003). In nuclear wastes, the solubility of U has been shown to be influenced by the presence of organic acids, such as ISA (Baston et al., 1994b).

The solubility of the reduced form of uranium U(IV) was experimentally derived using UO\(_2\)·2H\(_2\)O in the presence of ISA (0.04 and 0.06 M) and over pH in the range 3 to 14, resulting in U(OH)\(_4\)(ISA)\(^-\) as the major complex (Warwick et al., 2004):

\[
U^{4+} + ISA^- \leftrightarrow U(OH)_4(ISA)^- + 4H^+
\]  
(eq. 2.21)

However, this study neglected ISA protonation and proposed a notional tetrahydroxide complex. Therefore, the complex with a log\(K = 49\) seems likely at high pH (tested at pH 13.5; Warwick et al., 2004).

**Technetium**

Technetium (Tc) is a long-lived radionuclide with a half-life of 2.13 \(\times\) 10\(^5\) years that arises as fission product from \(^{235}\)U fission during the production of nuclear power (Lloyd, 2003). \(^{99}\)Tc is a \(\beta\)-particle emitter that can exist in oxidation states +7 to -1 (Kotegov et al., 1968). Under oxic conditions Tc occurs as negatively charged pertechnetate (Tc(VII)O\(_4\)^-) and does not sorb to most clay surfaces, whereas in its reduced form, Tc(IV), it associates with minerals very strongly (Kienzler et al., 2009). The soluble pertechnetate ion has a high bioavailability as an analogue of sulfate (Lloyd, 2003). Since the reduced Tc(IV) form is insoluble as Tc(IV)O\(_2\), bacterially-mediated reduction seems to be a promising strategy to remove it from groundwater (e.g. Lloyd et al., 1998). Reduced iron-bearing sediments have a strong influence on Tc, probably because they can reduce Tc(VII) abiotically and immobilise the radionuclide as Tc(IV) (Lloyd & Macaskie, 2000; Morris et al., 2008). Data on the complexation of Tc by ISA are scarce (Bradbury and Sarott, 1995; Lloyd, 2003; Maset et al., 2006).

In summary, ISA may have the potential to form stable complexes with a wide range of radionuclides present in ILW. Complexation can therefore influence radionuclide speciation, solubility limits and sorption behaviour, enhancing the release of radionuclides into the geo- and biosphere. In addition, chelate complexes can be very stable because they increase entropy during complex formation and therefore are particularly important in actinide chemistry.
Research on radionuclide sorption has demonstrated that over timeframes within which ligands have a significant stability, they would not migrate out of the engineered barrier in the first 100 years post-closure (Heath and Williams, 2005). Regarding the geodisposal of radioisotopes with long half-lives, the containment of radioactive wastes has to be ensured for their respective half-lives, which, in some cases, will require the isolation and containment over time scales of a million of years. The construction of a GDF is designed to fulfill this function over the required timeframes (NDA, 2010c; d).

2.7. Microbial-radionuclide interactions

Microbiology was first considered in the planning for a nuclear waste disposal programme for HLW in Sweden (Pedersen, 1999). This coincided with the recognition that a subterranean biosphere exists in the nutrient-poor, anoxic groundwaters of the deep subsurface (Ekendahl et al., 1994). The interactions of microbes with metals and radionuclides has received much attention and is well documented in both natural and engineered environments, including systems such as bioremediation, biomining, wastewater treatment and microbial corrosion (Ike et al., 2011). The UK-based Nirex programme proposed cementitious grout for the disposal of ILW, to facilitate hyperalkaline conditions, which were aimed at immobilising radionuclides and minimising microbial activity (Nirex, 2003). Nevertheless, the resulting waste form will be complex, releasing CO₂, CH₄ and a range of electron donors, such as chemical decontamination agents or ISA and substantial amounts of H₂ from the corrosion of the high amount of steel within a GDF. This will present microorganisms with a complex mix of growth substrates, together with local areas of reduced alkalinity that could potentially allow microbial communities to develop in “micro niches”. Previously it was demonstrated that such high pH conditions (pH 10 to 12) are not sufficiently harsh to prevent microbial metabolism supported by a range of electron accepting processes (Rizoulis et al., 2012; Williamson et al., 2013; Bassil et al., 2015b). In addition, radiation effects associated with the waste content indicated they may have a minimal effect on microorganisms, or in some cases can stimulate microbial metabolism (Brown et al., 2014). Thus, microbial communities may influence the biogeochemical evolution of a GDF during its operational phase and after closure, including the fate of radionuclides. Therefore the role of microbial processes needs to be considered for the implementation of a GDF. Microbes may decrease the bioavailability and mobility of toxic metal and radionuclides.
via a range of processes. Such microbial interactions are underpinned by a range of mechanisms, including biosorption, bioaccumulation, biotransformation and biomineralisation which have been reviewed extensively (Figure 2.13; Lloyd and Macaskie, 2002; Newsome et al., 2014).

**Biosorption**

The term biosorption is used to describe the metabolism-independent sorption of metals and radionuclides to biomass (Gadd and White, 1989), which includes bacteria, fungi and algae (Volesky and Holan, 1995). Ligands in the cell wall involved in metal sorption include carboxyl, amine, hydroxyl, phosphate and sulfhydryl groups (Beveridge and Murray, 1980; Lloyd and Macaskie, 2002). For example, the metal lanthanum, a surrogate for americium was demonstrated to readily bind to the biomass as LaPO$_4$ (Kazy et al., 2006). Biosorption can occur with living or dead material, although several studies have shown that dead biomass often sorbs higher amounts of metal than live biomass (Voilesky and Holan, 1995).

![Figure 2.13. Possible microbe-metal interactions with extracellular polymeric substances (EPS) that provide binding sites for metals (Me) and radionuclides (adapted from Wang et al., 2010).](image)

**Bioaccumulation**

Uptake of metals is an energy-dependent process that has been demonstrated for most physiologically important metal ions which are vital for many microorganisms, including Fe, Zn, Cu, Ni and Co. These metals are often required for enzyme cofactors that are important for metalloproteins. As these processes are, in contrast to biosorption, metabolism dependent, they
can be affected by the concentration and toxicity of the metallic ions (Volesky, 1990). Microorganisms have developed mechanisms to restrict the amount of toxic metals taken up intracellularly and/or convert them to more innocuous forms (Gadd, 1990). Especially important for this study to mention is Ni, a transition metal that represents an essential trace element for several microbial enzymes (Nielsen and Ollerich, 1974; Babich and Stotzky, 1983; Pümpel et al., 2003; Madigan et al., 2015). Accumulation of this metal in Pseudomonas species was localised in the periplasm and membrane, in the form of Ni phosphide and Ni carbide crystals (Sar et al., 2001). However, Ni, as well as other metals, poses a toxic threat to microorganisms at higher concentrations, although the lethal dose can differ between microorganisms (Poulson et al., 1997; Ruggiero et al., 2005). Bioaccumulation of U has been recognised as well, with intracellular uptake of U into Pseudomonas cells in U(VI) bioreduction studies reported (e.g. Marqués et al., 1991; VanEngelen et al., 2010; Choudhary and Sar, 2011). However, it is presumed that U could accumulate intracellularly as a result of increased membrane permeability which is caused by the high toxicity of the radionuclide (Suzuki and Banfield, 1999).

**Bioreduction**

Microbial processes may also affect the speciation of metals and radionuclides via reductive biotransformation, a process that can decrease the mobility of the transformed metals. The speciation can be altered by microbes in different ways, for example, by changing the oxidation state directly during the degradation processes (coupled to oxidation/reduction) or indirectly by influencing ambient redox/pH conditions that affect mineral-surface reactivities, metal speciation and soil-fluid properties (e.g. Macaskie, 1991; Macaskie et al., 1995; Tolley et al., 1995; Lloyd et al., 2005). During reduction, a bacterial cell acts as a redox catalyst which oxidises an electron donor (e.g. glucose, acetate or here, ISA) to gain energy for its metabolism, while transferring excessive electrons to a chemical (electron acceptor) that gets reduced. In this process, oxygen is the preferred electron acceptor, which is reduced first, but when oxygen becomes depleted, the use of alternative electron acceptors takes over in a sequence starting with NO$_3^-$, then Mn(IV) and Fe(III), followed by SO$_4^{2-}$, and eventually carbon dioxide (Figure 2.14), progressively providing less energy. High valence metals and radionuclides, such as U(VI) (Lovley, 1993) and Tc(VII) (Lloyd and Macaskie, 1996; Lloyd et al., 1998), have also been shown to be utilised by
specialist microorganisms. At neutral pH, U(VI)/U(IV) has a similar redox couple to Fe(III)/Fe(II) and acts as a surrogate for Fe(III)-reducing bacteria that can also reduce U(VI) (Lovley et al., 1991a). Other groups of microorganisms capable of U(VI) reduction include sulfate-reducing bacteria (Lovley and Phillips, 1992) and fermentative bacteria (Francis et al., 1994). The enzymatic reduction of U(VI) proceeds via U(V), which is highly unstable and disproportionates to U(IV) and U(VI) (Renshaw et al., 2005). Even though most studies suggest that U(VI)

Figure 2.14. Dominant oxidation state of key radionuclides at neutral pH, relevant for the geosphere surrounding a GDF (adapted from Brookshaw et al., 2012).

reduction occurs enzymatically (Williams et al., 2012), there are examples of abiotic reduction attributed to Fe(II) minerals (e.g. Latta et al., 2012) and biominerals (O’Loughlin et al., 2010; Veeramani et al., 2011, 2013). Bioreduced U(IV) is insoluble and often precipitates as insoluble nanoparticulate uraninite [UO$_2$] (Lovley et al., 1991a; Suzuki et al., 2002), however this form of uranium removal has been shown to be susceptible to reoxidation (Fredrickson et al., 2002; Senko et al., 2007; Newsome et al., 2015b). More stable forms involve precipitation as phosphates (see biomineralisation).
For an electron acceptor to be accessible to microbial reduction, many microbes require dissolved species that can enter the cell prior to reduction. However, Fe(III), one of the most abundant electron acceptors in sedimentary environments (Lovley, 2000), is usually present as extracellular insoluble Fe(III) (hydr)oxides, presenting a difficulty for microbial reduction. One possibility to facilitate dissolution of their Fe(III) moieties is by organic ligands (e.g. ISA or humic acids), which can bind to the metal and keep it in solution. Another way for the cell to access an insoluble electron acceptor, is by direct contact involving a suitable electron transfer protein, such as outer membrane c-type cytochromes. This requires the cell to be in close contact with the respective electron acceptor (Myers and Myers, 1992). Such c-type cytochromes have been localised in the outer cell membrane of for example *Shewanella putrefaciens MR-1* (Myers and Myers, 1992) and *Geobacter sulfurreducens* (Lloyd et al., 2003). In contrast, the reduction of Tc(VII) by species, including *Geobacter sulfurreducens* (Lloyd et al., 2000b) and *Desulfovibrio desulfuricans* (Lloyd et al., 1998), follows a different mechanism involving hydrogenase activity located in the periplasm of the Gram-negative cell.

**Biomineralisation**

Microorganisms can also control the solubility of metals and radionuclides via biomineralisation reactions, commonly divided into two processes: They can include respiratory processes such as enzymatic reduction, liberating for example Fe(II) and Mn(II) with strong complexing abilities for metals and radionuclides, or changes of the local geochemistry by microbial metabolism via enzymatically-generated ligands, including phosphate, sulfide, carbonate or hydroxides or metabolism-driven changes in pH.

Biogenic carbonate precipitation relies on microbially-mediated release of carbonate, which in turn raises the pH and alkalinity required for precipitation of carbonates as a result of supersaturation. Calcite precipitation has been shown to immobilise Pu, Np and $^{90}$Sr (Meece and Benninger, 1993; Thorpe et al., 2012, 2014). Phosphates are another group of ligands with very low solubility (Langmuir, 1978). The advantage of phosphate mineralisation, over that of carbonates, is their high stability with respect to dissolution processes. Natural analogues have proven the longevity of phosphates and resistance to oxidation over geological timescales (Sato et al., 1997; Finch and Murakami, 1999; Jerden and Sinha, 2003). Biogenic phosphates can be produced via hydrolysis of stored polyphosphates (Boswell et al., 1999). An alternative
mechanism, which has been proven using a *Citrobacter* (now reclassified as *Serratia*) species, involves the degradation of organophosphates, such as glycerol phosphate. The glycerol phosphate is cleaved via enhanced cell phosphatase activity, releasing an inorganic phosphate ligand, which precipitates uranium as hydrogen uranyl phosphate mineral [H(UO\(_2\))PO\(_4\)] abbreviated to ‘HUP’) from solution (Macaskie *et al.*, 1992). Advantageous of biogenically precipitated HUP is a high ion-exchange capacity that can capture further metals and radionuclides by co-crystallisation, including radiotoxic \(^{60}\)Co, \(^{90}\)Sr, \(^{137}\)Cs (Paterson-Beedle *et al.*, 2006), and Ni (Bonthrone *et al.*, 1996; Basnakova and Macaskie, 1997). In addition to uranium precipitation as HUP, recent studies have focused on bioreduced U(IV)-phosphates, which have been described as ‘desired end products’ from bioremediation due to their high resistance to reoxidation (Khijniak *et al.*, 2005; Newsome *et al.*, 2015a). Another important group of phosphates include hydroxyapatites [Ca\(_5\)(PO\(_4\))\(_3\)(OH)] that form in the presence of Ca. These minerals immobilise metals or radionuclides, including Sr, Co, Eu and U, via sorption or ion exchange (Shelobolina *et al.*, 2004; Handley-Sidhu *et al.*, 2011, 2014; Gangappa *et al.*, 2017). It was further demonstrated that biogenically produced hydroxyapatite can have higher removal capacities for metals and radionuclides over respective synthetic products (Handley-Sidhu *et al.*, 2011). Other metals known to be captured by phosphates include La (Tolley *et al.*, 1995) and Np (Lloyd *et al.*, 2000a). In the case of Np(V), reduction to Np(IV) allowed sequestration of this actinide as Np(IV)-phosphate (Lloyd *et al.*, 2000a). Similar to HUP, sequestration of Np was significantly enhanced by pre-conditioning of the biogenic phosphate. For example, the addition of La in a flow-through reactor resulted in LaPO\(_4\) precipitation, which was more effective for capturing Np, due to combined bioaccumulative and chemisorptive mechanisms (Basnakova and Macaskie, 1998). Sulfides, produced from sulfate reduction, a process which can reduce acidity from solution, are also important for metal precipitation, including Ni, Zn, Cu, Fe, and Pb (Lewis, 2010).

In conclusion, the effect of microbial activity includes for example biofilm formation, mineral dissolution, secondary phase formation, or adsorption onto and sorption into cells (e.g. Lloyd and Macaskie, 2000; Renshaw *et al.*, 2007). The described interactions of microbes with metals and radionuclides also involves the interaction with minerals present in the
geosphere/biosphere. Microbial activity affecting pH and redox potential and introducing organic compounds, may influence the wider geochemical environment, and thus the longevity of GDF multi-barrier containment. Uncertainties remain about the rates and extent of microbial processes at high pH and within steep biogeochemical gradients characterising the CDZ. These alkaline and reducing horizons are dominated by fermentative and methanogenic activities, but more oxidising layers can facilitate microbial nitrate-, iron-, and sulfate-reduction, which may eventually be dominated by aerobic processes, closer to the surface. Along these redox-gradients, which continuously decrease in alkalinity, the transport of organic growth substrates is expected to stimulate a successively increasing bacterial diversity, presenting a complex biosphere. The inferred microbial colonisation of the surrounding geosphere of a GDF may influence radionuclide dispersion and transport characteristics. This form of biological attenuation may prevent the transport of radionuclides, offering a “bio-barrier” that will complement the physical and chemical barrier system designed into the more conventional multi-barrier concept, employed in nuclear waste management plans worldwide.

2.8. Thesis outline and objectives
Given the uncertainty surrounding the transport of a radionuclide-ISA complex that may eventually leave the GDF after groundwater resaturation, research focused on identifying its fate in the wider environment is warranted. Following considerations presented in the literature review, it became clear that the GDF conditions characterised by high pH, elevated temperatures and low nutrient availability may not be sufficient to fully prevent microbial activity, but rather specially adapted microorganisms will likely survive in micro-niches of the wastes (e.g. Rizoulis et al., 2012; Williamson et al., 2013). These microorganisms can be either present in the sub-surface and wastes or enter with the groundwater, and although exposed initially to a nutrient poor environment, may be stimulated by the presence of organic waste compounds, such as ISA. Therefore, microbial activity has been the focus of research to estimate its impact on the fate of ISA. Not only could microbial metabolism remove ISA from solution, but it could also immobilise radionuclides complexed to it, which are expected to subsequently precipitate. It was also hypothesised that metal-reducing anaerobic microorganisms may either directly or indirectly reduce certain key radionuclides, which would again decrease their solubility.
Previous research has been carried out to study the ability of such specialised microbial communities to colonise the wastes and biodegrade ISA. A preliminary study looked at an analogue for the chemically disturbed zone of a GDF, or the “near field”, dominated by an alkaline pH (=10) and investigated alkali-tolerant bacteria in their ability to degrade ISA (Bassil et al., 2015b). However, under such harsh alkaline conditions, microorganisms may be unable to break down the ISA completely; sulfate reduction in particular (supported by high levels of sulfate in UK groundwaters) was shown to be minimal under the simulated GDF conditions. Thus, it is possible that an alkaline plume that migrates from the GDF may carry ISA in its leachates through the CDZ into the anoxic surrounding “far field”, and eventually delivers radionuclides into the accessible biosphere. For this reason, research of microbial ISA degradation under less alkaline conditions, representative for the “far field” is necessary.

The primary aims and objectives outlined in this thesis aim at exploring microbial ISA degradation under moderate pH conditions in the “far field”, highlighting the impact of ISA on the geochemical surroundings, changes of the indigenous microbial community and the fate and speciation of some key radionuclides and metals. To address the first question, neutrophilic microorganisms, retrieved from sediments contaminated by a lime kiln site at Harpur Hill near Buxton, representing the “far field” surrounding a GDF, were incubated under a range of biogeochemical conditions (Chapter 4). Initial incubations were created exploring the redox cascade, including aerobic respiration, denitrification, iron- and sulfate-reduction, organic acid fermentation and methanogenesis. In particular, the presence of significant amounts of ferric iron was considered as this may be either released from the weathering of subsurface minerals or generated from the corrosion of steel components in a GDF, promoting an Fe(III)-reducing community. Therefore Chapter 6 studied the pH range (7 to 10) under which ISA degradation is feasible during Fe(III) reduction and addressed the impact of ISA biodegradation on the iron mineralogy, as part of the wider geosphere. However, deep aquifer UK groundwaters are commonly rich in sulfate (Metcalf et al., 2007), so sulfate reduction might become the dominant terminal electron accepting process. Thus, following research under Fe(III)-reducing conditions, the impact of sulfate-reducing bacteria using ISA as an electron donor was investigated, including studying the associated microbial community and mineralogy under a range of pH conditions (7 to 10; Chapter 9.1).
After gaining insight of the biogeochemical cycling of ISA under a wide range of conditions, questions remained regarding the fate of radionuclides in the system, which comprise the second part of this thesis. It is noteworthy that a study exploring the biodegradability of a radionuclide-ISA complex had not been carried out previously. Special focus was placed on the ability of microorganisms to degrade a metal/radionuclide-ISA complex with the subsequent formation of mineral phases, such as sulfides and phosphates. Factors that might inhibit microbial degradation of a radionuclide-ISA complex could result from increased radiotoxicity or chemotoxicity, but also the stability of a complex with bonds too strong for bacteria to cleave. Experiments were designed using the priority radionuclides, nickel and uranium, relevant for the disposal of ILW (NDA, 2014). The first model metal/radionuclide explored was nickel, which is present in Fe-Ni alloys used for reactor components, as it minimises their corrosion (Platt et al., 1997). The fate of a nickel-ISA complex was studied under key electron accepting processes, starting with Fe(III)-reducing conditions (Chapter 6), and followed by sulfate-reducing conditions (Chapter 9.2). After exploration of the fate of divalent Ni(II), hexavalent U(VI), which is a key radionuclide in ILW, with a complex geochemistry, was explored. In these experiments, the fate of U(VI) added to Fe(III)-reducing or fermenting enrichment cultures grown on ISA, was explored (Chapter 7). This Chapter presents data from geochemical, mineralogical and microbial characterisation, and includes an analysis of the uranium species. The speciation is important, as immobilised U(IV) is considered to be more recalcitrant than U(VI). In addition, preliminary results are presented for the biodegradation of ISA under sulfate-reducing conditions and exposed to a range of U(VI) concentrations (Chapter 9.2).

A comprehensive experimental study was designed that used microbial enrichment cultures to study the biodegradation of ISA by microorganisms retrieved from an alkaline legacy lime workings site in Buxton, U.K., a model system that approaches aspects of the far field geosphere of a GDF. Various geochemical conditions were established, including a range of pH values and reducing conditions, to explore the biodegradation of ISA under different scenarios that may develop in the geosphere surrounding a GDF. Metals and radionuclides, relevant to nuclear waste disposal, were introduced into these systems, to determine the impact of microbial ISA degradation on the fate of these elements. A cross-disciplinary approach was used to analyse geochemical parameters, including ISA removal from solution and gas
generation (using IEC, pH, Eh, spectrophotometry, GC-TCD), adaptation of the microbial community (using 16S rRNA gene sequencing), and the potential impact of ISA on biomineral formation (using XRD, TEM, SAED, EDX, ESEM and light microscopy) and on the fate of priority radionuclides (Ni, U) (using ICP-AES/MS, TEM, SAED, EDX and ESEM). Finally, end-member radionuclide speciation and their coordination environment was characterised for uranium (using synchrotron techniques; XAS).

The results presented in this thesis extend our knowledge of biogeochemical cycling of ISA under a wide range of geochemical conditions, and for the first time the biodegradability of a radionuclide-ISA complex was demonstrated. This outcome highlights that an in situ sediment microbiome could be able to remove ISA from solution via a self-attenuated process. This form of biological attenuation may prevent the transport of radionuclides, offering an adventitious “bio-barrier” that will complement the physical and chemical barrier system designed into the more conventional multi-barrier concept, currently employed by nuclear waste management organisations worldwide.

2.9. References


3. Methodology

In this chapter, experimental background and analytical methods common to all methods are described, whilst the chapters presenting research manuscripts (Chapters 4, 5, 6 and 7) and unpublished work (Chapters 9.1 and 9.2) describe experimental set ups specific to those studies.

All chemical reagents used were analytical grade and for solutions deionised water of 18 MΩ was used. Experimental preparation, sampling and data analysis were carried by the author. Analyses performed by technical laboratory staff or with their support, are indicated as appropriate.

3.1. Materials

3.1.1. Freshwater medium after Lovley et al. (1984)

For the purpose of enriching a bacterial community representative of the geosphere surrounding a GDF, a freshwater minimal medium was chosen (Lovley et al., 1984) and modified, as listed in Table 3.1. Mineral mix and vitamin mix were filter-sterilised. Prior to incubation, the medium was autoclaved and then degassed with a mixture of N₂:CO₂, thereby adjusting the required pH.

<table>
<thead>
<tr>
<th>Media composition</th>
<th>Mineral mix composition</th>
<th>Vitamin mix composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient</td>
<td>Amount [g]</td>
<td>Ingredient</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.5</td>
<td>Nitrilotriacetic acid</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>25</td>
<td>MgSO₄</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>0.6</td>
<td>MnSO₄·H₂O</td>
</tr>
<tr>
<td>KCl</td>
<td>0.1</td>
<td>NaCl</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.5</td>
<td>FeSO₄·7H₂O</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10 mL</td>
<td>CaCl₂·2H₂O</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>10 mL</td>
<td>CoCl₂·6H₂O</td>
</tr>
<tr>
<td>pH</td>
<td>7 to 7.5</td>
<td>ZnCl₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CuSO₄·5H₂O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AlK(SO₄)₂·12H₂O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H₂BO₃</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na₂MoO₄</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NiCl₂·6H₂O</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na₂WO₄·2H₂O</td>
</tr>
</tbody>
</table>

Table 3.1. Composition of modified freshwater minimal medium; all compounds are given for dilution in 1 L deionised water (after Lovley et al., 1984).
3.1.2. Sulfate-reducing bacterial growth medium

The culture medium from 3.1.1 in combination with Na$_2$SO$_4$ as the sole electron acceptor did not result in the stimulation of sulfate-reducing bacteria after culture transferal. The use of other known SRB growth media (e.g. Postgate medium B; Postgate, 1984) was avoided because they contain poorly defined substrates such as yeast extract, which could stimulate other heterotrophic bacteria not involved in ISA degradation. Therefore, a modified freshwater medium based on that described in section 3.1 was prepared (Table 3.2), and sustained growth over a number of consecutive transfers. This medium was consequently adopted for all experiments involving SRB (Chapters 4, 9.1 and 9.2). The modified medium comprised:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaHCO$_3$</td>
<td>2.5 g</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>1.0 g</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$ · H$_2$O</td>
<td>0.6 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.1 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.5 g</td>
</tr>
<tr>
<td>MgSO$_4$ · 7H$_2$O</td>
<td>2.0 g</td>
</tr>
<tr>
<td>FeSO$_4$ · 7H$_2$O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>1.7 g</td>
</tr>
<tr>
<td>Vitamin mix (same composition as in 3.1)</td>
<td>10 mL</td>
</tr>
<tr>
<td>Mineral mix (same composition as in 3.1)</td>
<td>10 mL</td>
</tr>
<tr>
<td>pH</td>
<td>7 to 7.5</td>
</tr>
</tbody>
</table>

Table 3.2. Composition of modified freshwater medium after 3.1 for enrichment of sulfate-reducing bacteria; all components are dissolved in 1 L deionised water.

3.1.3. α-isosaccharinic acid preparation

This study used pure salts of ISA, including sodium isosaccharinic acid (Na(ISA)) and calcium isosaccharinic acid (Ca(ISA)$_2$), the most important alkaline degradation products from cellulose under GDF conditions to stimulate microbial growth.

Ca(ISA)$_2$ was prepared from α-lactose monohydrate and Ca(OH)$_2$ following a protocol of Vercammen et al. (1999a). 500 mL deionised water were flushed with Argon gas under low pressure in a three neck round bottom flask for 3 hours. 50 g of α-lactose monohydrate and 13.6 g of Ca(OH)$_2$ were added to the sealed flask and the solution was left to stir for three days.
During the reaction time, ISA and other organic acids were produced, accompanied by a noticeable change of colour from cream, over orange and brick-red to brownish red (Figure 3.1 A-D). The solution was then boiled in a reflux system for ~6 hours (Figure 3.1 E). Afterwards the hot solution was filtered through a Whatman filter No. 3, which collected unreacted lactose and Ca(OH)$_2$, whilst ISA and other small organics passed through the filter paper (Figure 3.1 F). The supernatant was reduced to ~100 mL and stored overnight at 4°C. A white precipitate formed which was centrifuged at 4,000 g for 10 min to separate from the supernatant. The precipitate was washed with deionised water and ethanol and left to dry overnight at 50°C. In the next cleaning step, the precipitate was re-dissolved in deionised water in a ratio of 1.2 g to 100 mL and brought to boil. Whilst hot, the solution was filtered through a Whatman filter No. 3 and the residues were rinsed with acetone followed by ethanol. After reduction to 10 mL volume, the solution was poured into a wide and flat tray covered with aluminum foil. A few milliliters of acetone were added to bind the residual organics and carry them to the sides of the tray, whilst the acetone-insoluble Ca(ISA)$_2$ descends to the bottom. The solution, containing Ca(ISA)$_2$, was dried at 50°C overnight. In the last step, Ca(ISA)$_2$ was collected from the bottom of the tray by carefully not collecting any organics (dark brown colour; Figure 3.1 G). The purity of the synthesised product was examined by ICP-MS and compared to Ca(ISA)$_2$ of known quality.

![Figure 3.1](image)

Figure 3.1. Steps of Ca(ISA)$_2$ synthesis. A - D show degradation reaction, forming Ca(ISA)$_2$ and other organic degradation products, which is via E boiling and F filtration steps, until G a final Ca(ISA)$_2$ product is obtained (brown marginal areas are other organics).
Na(ISA) was produced by contacting Ca(ISA)$_2$ salt by alkaline hydrolysis with 3 M NaOH at a known concentration (Vercammen et al., 1999a; Rai et al., 2003; Bontchev and Moore, 2004). The solution was left on a stir plate for about 5 days for exchange of Ca with Na which resulted in precipitation of dissolved Ca as Ca(OH)$_2$ and left Na(ISA) in the aqueous phase. By centrifugation (4,000 g for 10 min), the supernatant, containing Na(ISA), was recovered.

Na(ISA) consists of ISA/H$_2$O layers connected to O$_{ISA}$-Na-O$_{ISA}$ in a 2D network, making the bonds less stable compared to Ca(ISA)$_2$, which in contrast has layers in a 3D network due to the higher coordination of Ca (Bontchev and Moore, 2004). The structural difference of Na(ISA) gives it a greater water solubility than Ca(ISA)$_2$ (Bontchev and Moore, 2004), which consequently enhances the radionuclide mobility. Accordingly Na(ISA) is experimentally more suitable with respect to investigations of radionuclide mobility. However, the addition of alkaline Na(ISA) to experiments, resulted in a pH increase to >10.5, which was impractical for most of the experiments performed at neutral pH. Acid neutralisation (using concentrated HCl) was attempted to bring down the pH, but as a result microbial ISA degradation was prevented or incomplete. Furthermore, the addition of HCl produces an interfering peak with ISA in IC signals and would make a quantitative analysis impossible. It was therefore concluded that Na(ISA) is more suitable for high pH investigations (>10), such as in studies by Bassil (2015), whilst for (near) neutral pH investigations Ca(ISA)$_2$ is more appropriate.

### 3.1.4. Ferrihydrite preparation

Experiments simulating Fe(III)-reducing conditions in the geosphere surrounding a GDF, were performed using ferrihydrite, a Fe(III) oxyhydroxide mineral. The large surface area and poor crystallinity of ferrihydrite make it a suitable electron acceptor for microbial respiration. Ferrihydrite was added in form of iron gel to microcosm experiments.

For the preparation, 108 g of FeCl$_3$ were dissolved in 1 L of deionised water under constant stirring to avoid clumping of the Fe(III). The pH was monitored throughout and adjusted to 7 by adding a few drops of 10 M NaOH to the solution. The crude iron gel was left to settle for 30 min before the pH was re-adjusted. Chloride was washed off in six repeated cycles of centrifuging the crude iron gel (5,000 g for 20 min) and replacing the supernatant with deionised water. After separating the last supernatant, the gel was re-suspended in deionised water to create a gel and the Fe(III) concentration was estimated with the ferrozine assay (section 3.3).
3.2. Culturing techniques

3.2.1. Microcosm experiments

This study used a cultivation enrichment approach, to select for microorganisms capable of ISA degradation. This paragraph describes in brief the general concept of the incubation set up. For full experimental details and specific individual manipulations, the reader is referred to the respective chapters, for example manipulations of pH (Chapters 5 and 9.1), electron acceptors (Chapter 4) or experiments with nickel (Chapters 6 and 9.2) or uranium (Chapters 7 and 9.2).

In brief, incubation experiments were set up in glass serum bottles containing a microbial growth medium as described in section 3.1.1 or 3.1.2. The medium was spiked with ISA as electron donor to a final concentration of 4 mM, and appropriate electron acceptors were added depending on the experiment. Where necessary the pH was adjusted using NaOH. Serum bottles were sealed loosely with a foam sponge to maintain aerobic conditions, whilst for anaerobic conditions serum bottles were sealed with a butyl stopper and de-aerated with a N₂/CO₂ (80:20) gas mixture. After autoclaving, the bottles were inoculated with 1% v/v inoculum. Inocula used were fresh sediments (primary enrichments), or stable enrichments, using a transferred microbial inoculum from previous enrichment cultures. Depending on the focus of the experiment, a filter-sterilised solution containing a metal or radionuclide was added. Cultures were incubated in the dark at 20°C.

3.2.2. Isolation of bacteria

Understanding the individual role of microorganisms in the experiments performed is crucial to help identify the potential of a soil microbiome to remove ISA from solution and to reduce metals or radionuclides to less soluble forms, e.g. U(VI) to U(IV). Thus, the microbial community can have a significant impact on the geochemical behaviour of metals and radionuclides, and with this the waste integrity.

For the isolation of bacteria, a medium as presented in section 3.1.1 or 3.1.2 was prepared and Ca(ISA)_2 added. Before autoclaving 1% of agar was added to allow the medium to solidify in petri dishes. The medium was let to cool down (but it must be sufficiently warm so that no solidification takes place), and the respective amount of vitamin solution was added. In a laminar flow cabinet, the medium was poured in labelled petri dishes whilst still warm and left for
at least 40 min to cool down. Petri dishes were closed and put upside-down in a sterile bag, which was closed in a way that air exchange was maintained. The bag was transferred into an anaerobic cabinet and left for at least 7 days to ensure that all oxygen had been removed from the plates. Plates were inoculated in the anaerobic chamber by dropping 20 µL of sample aliquot from an enrichment experiment on the plate and streaking it with a 1 µL sterile disposable loop. Inoculated plates were sealed with parafilm and stored upside-down in a GasPak, equipped with oxygen indicator paper and a GasPak™ EZ anaerobe container system to remove residual oxygen. The GasPak was incubated at 20°C in the dark and checked regularly for colony formation. When visible colonies had formed, sub-culturing was done by picking isolated colonies with a 1 µL disposable loop and streaking them on a new plate. The plate was incubated under the same conditions as before. After several sub-cultures, liquid media were inoculated with clearly visible isolated colonies, to test if the isolates were still able to degrade ISA. For this purpose, ISA-containing liquid medium of the same composition as used for the plates was made up, but agar was omitted, and divided into glass serum bottles which were purged with a N₂/CO₂ (80:20) gas mixture. The glass serum bottles were inoculated with isolates from the sub-cultured plates. Results from the isolation of bacteria are presented in Chapter 9.1.

3.3. Aqueous geochemical analyses

3.3.1. Turbidity measurements

Microbial growth was monitored at regular time intervals during incubation experiments by spectrophotometry. An increase in absorbance indicated cell multiplication, whilst when the signal levelled off growth had reached the stationary phase, indicating that cells had to be transferred to fresh growth medium. A drawback of the method is that measurements are only possible in clear media, which excluded Fe(III) and sulfate reduction experiments, which contained light scattering mineral phases.

Samples were added to cuvettes, either directly or after dilution in medium if necessary. After blanking the spectrophotometer against a solution of medium and ISA, samples were analysed at 600 nm.
3.3.2. pH and Eh

The pH of samples was measured using a Fisherbrand FB68801 electrode connected to Mettler Toledo FEP20 digital meter, which was calibrated using Fluka pH 4, 7 and 10 buffer solutions. For the redox measurements, a Denver Instrument Accumet digital meter equipped with a Mettler Toledo Inlab Redox Micro ORP electrode was used. Calibration was done with a Mettler Toledo 220 mV redox buffer solution.

3.3.3. Light Microscopy

Spectrophotometric measurements were made to monitor cell growth rates, however, incubations focusing at Fe(III)- and sulfate-reducers contained light scattering mineral phases, which interfered with the measurement. Therefore light microscopic studies were useful in getting a fast but not quantitative estimate of microbial growth in the experiments.

Aliquots were viewed under both translucent light and fluorescent light using Hoechst 33342 nucleic acid stain. Hoechst is a cell-permeant nuclear counterstain that emits blue fluorescence when bound to double stranded DNA. Here the dye was applied to microbial cells from sediment particles and/or minerals.

The stain was dissolved in deionised water (0.1 mg L⁻¹) and applied in aliquots (5 μL stain to 500 μL sample). After mixing, the stained samples were coated on a glass slide (1.2x1.5 mm thickness), which was covered with a cover slip (22x26 mm, #1 Menzel Gläser) and viewed immediately under the microscope. Slides were examined with a Zeiss Axio Imager A.1 microscope equipped with a Zeiss Axiocam 506 mono camera. The lenses used were a 40x/0.65 Ph2 A-Plan (401051), a 100x/1.3 Oil Ph3 EC Plan-NEOFLUAR and a 100x/1.25 Oil Ph3 ACHROPLAN (440081). Samples were imaged under both, translucent light, without using any filters, and under fluorescent light, using an Fs49 filter. Both images were combined in one, using ZEN 2012 software (blue edition by © Carl Zeiss Microscopy GmbH, 2011), to make fluorescent cells visible among precipitates. The same software was used for image processing and analysis.
3.3.4. Ferrozine assay

In order to assess Fe(III)-reduction, the ferrozine assay was applied to monitor Fe(II) production in samples containing Fe(III) oxyhydroxide or natural Fe(III)-containing minerals present in the sediment (Lovley and Phillips, 1987).

For the assay, standards with concentrations of 1, 5, 10, 20 and 50 mM FeSO$_4$$\cdot$H$_2$O were prepared. A ferrozine solution was made by dissolving 1 g ferrozine and 11.9 g HEPES in 1 L H$_2$O. Briefly, samples (20 µL) were taken from the incubations and dissolved in 980 µL of 0.5 M hydrochloric acid (HCl). These were left to digest for one hour. After mixing the samples, an aliquot of 20 µL was taken and added to 980 µL of the ferrozine solution. The absorbance was measured at a wavelength of 562 nm, and compared to measurements made for the samples and standards.

Total iron concentrations (Fe(II)+Fe(III)) were measured only at the beginning and end of the experiment. Here a fresh 6.25 M solution of the strong reductant hydroxylamine hydrochloride (HAH) was prepared. 1.88 mL of 0.5 M HCl, together with 80 µL HAH and 40 µL sample were mixed and shaken. They were left overnight to reduce all the iron and then diluted in HCl in a ratio of 4:1, and the ferrozine assay was performed as described above. Results from this technique are presented in Chapters 4, 5, 6 and 7.

3.3.5. Bomo PADAP assay

The bromo PADAP assay (using the reagent 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol), was used to measure the amount of soluble U(VI) immediately after sampling and mixing with the reagent (Johnson and Florence, 1971). This measurement gives an indication of the U(VI) concentration in solution, whilst ICP-MS measurements were carried out on supernatants for precise quantitative analysis of total uranium in solution. Before measuring the U(VI) concentration, the following reagents were prepared. The complexing agent comprised 2.63 g CY-DTA (trans 1,2 dianimocyclohexane – NN, N', N' tetra-acetic acid), 0.5 g NaF, and 6.5 g sulphosalicyclic acid, which were added to a beaker containing 80 mL deionised water, neutralised to pH 7.85 with 40 % v/v NaOH solution and diluted to 100 mL with deionised water. Similarly, a buffer solution, containing 14.9 g triethanolamine in 100 mL deionised water, was prepared and adjusted to pH 7.85 with 9 M HClO$_4$. The bromo-PADAP indicator solution was
prepared with 0.05 g 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol in 100 mL AnalaR ethanol. For the assay, 260 µL deionised water, 100 µL sample, 80 µL complexant, 80 µL buffer, 400 µL pure ethanol and 80 µL bromo-PADAP solutions were added to a cuvette and sealed with parafilm. Note that samples were diluted accordingly when concentrations were >30 ppm. Calibration standards covering the range of 1 to 25 ppm were prepared following the same protocol. After mixing, the solutions were left for 60 min to react before analysis using a spectrophotometer at 578 nm, and compared to the standard curve. Results from the assay are presented in Chapters 7 and 9.2.

3.3.6. Ion Exchange Chromatography

For the measurement of analytes in solution an ion exclusion high performance liquid chromatography (IE-HPLC) technique was used. IEC is based on the principle of retaining ions or polar molecules on a stationary phase (column) and separating them based on their affinity to a mobile phase and eluting them on a detector.

At the end of the incubation experiments, frozen aliquots of samples taken at selected time points of the incubation experiments were thawed and mixed, then centrifuged at room temperature (14,000 g for 10 min) to remove all biomass and any biominerals present. The supernatant was transferred to clean centrifuge tubes and analysed with IEC. The IE-HPLC used was a Dionex ICS5000 Dual Channel chromatograph, fitted with a Dionex AS-AP autosampler that can hold 120 glass vials of a 2 mL volume. For background reduction a Dionex ACES300 Chemical Suppressor was used. Compounds were detected using a CD20 conductivity detector. The instrument was operated in two channels, depending on the target compound: Channel 1 was used to analyse sulfate and phosphate, while channel 2 analysed nitrate, nitrite, acetate, formate, lactate, propionate, butyrate and ISA. Conditions for both channels were as follows.

Channel 1: The chromatograph was equipped with a Dionex Capillary (50 x 2 mm) AG18 guard column and a Dionex Capillary (250 x 2 mm) AS18 analytical column. The mobile phase used was a concentrated KOH which was mixed with high purity water to produce the mobile phase (eluent) at a flow rate of 0.025 mL/min and a backpressure of 2,100 PSI. Background suppression was set at 23 mA. Samples were injected at a volume of 0.1 µL to a run at 38 mM and for 10 minutes.
Channel 2: The chromatograph was equipped with a Dionex Capillary (50 x 0.4 mm) AG11-HC 4u guard column and a Dionex Capillary (250 x 0.4 mm) AS11-HC 4u analytical column. The mobile phase used was 11.7 M KOH which was mixed with high purity water to produce the mobile phase (eluent) at a flow rate of 0.015 mL/min and a backpressure of 3,200 PSI. Background suppression was set at 13 mA. Samples (0.4 µL) were injected to a run starting at 1 mM KOH for 10 minutes, which was increased to 38 mM up to minute 25 and re-equilibrated until 40 minutes. Results from IC are presented in Chapters 4, 5, 6, 7, 9.1 and 9.2. Sample analysis was performed by Mr A Bewsher.

3.3.7. Inductively Coupled Plasma Mass Spectrometry & Atomic Emission Spectrometry

Inductively-coupled plasma (ICP) was used for the quantification of a range of metals and radionuclides in solution. Detection was achieved by different detectors, depending on the metal analysed: For analysis of U and La, the ICP was connected to mass spectrometry (ICP-MS) and for Ni to atomic emission spectrometry (ICP-AES). ICP-MS was used in preference when low levels of metal were targeted, as it achieves detection limits of 0.01 ppb which are much lower than for ICP-AES with a limit of 10 ppb. However, samples for ICP-MS have to be very dilute (i.e. below 0.1%) in order to avoid damage to the detector, whilst ICP-AES allows 1-5% concentration.

The technique is based on a nebuliser that generates an aerosol mist from the sample, which is then injected into an argon plasma (ICP). The dissociation of molecules emits electrons of specific mass/charge ratios, which enter the quadrupole. Applying a characteristic voltage causes only the ions of interest to reach the detector. When the ICP, containing the ions of interest, reaches the detector, the intensity of ions is recorded and compared to standards of known concentration to deduce the concentration of the measured element.

Samples for ICP-MS and ICP-AES were prepared by centrifugation (14,000 g, 10 min) to remove any particles from solution and aliquots of the supernatant were diluted in 2% HNO₃ at the required concentrations (<100 ppb for ICP-MS and < 10 ppm for ICP-AES). Standards, of the element of interest, were diluted from VWR certified standard dilutions, and were run after every 10 samples. Analyses were done on an Agilent 7500 CX (ICP-MS) or a Perkin-Elmer
Optima 5300 DV (ICP-AES). Results are presented in Chapters 6 and 9.2. Sample analysis was performed by Mr P Lythgoe.

3.4. Microbial community analysis
16S rRNA gene profiling was used to examine the initial bacterial community composition from soils used as inocula and to identify changes in the community due to the imposed (bio)geochemical pressures imposed in the experiments. Microbial community information facilitates correlation of changes in community structure that adapts to ISA degradation, helps to identify the organisms involved in ISA break down and further metabolisation of intermediates. Next generation sequencing (NGS) technologies were used to identify the 16S rRNA genes present in the samples. NGS platforms produce millions of short sequence reads of about ~800 base pairs (bp) in length.

Sequencing started with the extraction of DNA from a sample. The amount of DNA collected is usually not enough to be analysed, a constraint that is overcome by amplification of the target regions of DNA using a polymerase chain reaction (PCR) that involves primers for the amplification of 16S rRNA gene sequences. The 16S rRNA gene sequence comprises around 1,500 bp, responsible for protein synthesis; an essential process for all microorganisms and hence conserved across all prokaryotes.

Figure 3.2. Left-hand side showing individual components needed for PCR and right-hand side showing one PCR cycle (adapted from Bioninja, 2017).
PCR involves three steps: denaturing, annealing and extension (Figure 3.2). In the denaturation process, DNA is heated up (94-96 °C) to separate the double-stranded DNA. In the second step, annealing (55-65 °C) is necessary for the amplification of the target DNA which is defined by PCR primers. PCR primers are short pieces of single-stranded DNA, usually around 20 nucleotides in length, that carry base sequences complementary to segments of the single DNA strands. Two primers are used in each PCR reaction, flanking the target region that will be copied from both sides. In the third step, the temperature is lowered to enable the primers to pair with complementary DNA strands. The next step takes place at 72°C, where a thermostable polymerase enzyme attaches to the annealed primers to create new complementary strands of DNA for each template by incorporating nucleotides (DNA building blocks). After this cycle the amount of DNA in the sample has doubled and a second cycle starts by raising the temperature again. PCR is a sensitive technique that creates millions of copies from a single gene during 30 to 38 PCR cycles, and this allows detection of even uncultivated soil microorganisms in low abundances. To visualise the DNA and identify the size of amplified products, agarose gel electrophoresis is used. Gel electrophoresis is also used to purify the DNA by slicing areas of target, prior to sequencing using an appropriate NGS platform.

### 3.4.1. DNA extraction

DNA was extracted from 200 µL of the original soils (as slurry) and the subcultured enrichment cultures, using a PowerSoil DNA Isolation Kit (MoBio PowerLyzer™ UltraClean Microbial DNA Isolation Kit, Laboratories, Inc., Carlsbad, CA, USA).

### 3.4.2. Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualise DNA and as such it served as a quick test to check if the DNA extraction was successful. Furthermore, it was applied to identify the size of DNA bands generated by PCR. Agarose gel is a polymeric matrix used to separate the DNA according to the length of its fragments. A tray containing wells is submerged in the gel and the samples are loaded into individual wells. An electric current is applied through the gel matrix which makes the samples migrate towards the positive electrode, whereby the lighter DNA bands travel the furthest distance. One well will contain a DNA ladder containing fragments of known base pair lengths, used to calculate the size of the samples applied to the gel.
For the procedure, aliquots of the PCR product were mixed with 12.5 µL of 5x gel-loading dye, and 35 µL of the mixture was loaded on a 2% Tris-Acetate-EDTA/agarose gel. A 2,000 to 100 bp DNA ladder was also loaded on the gel that was run at 80 mV for 2 hours. The DNA bands were viewed on a Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA, USA) and the band corresponding to 400 bp size for each sample was excised from the gel. DNA extraction and cleanup from the excised gel were performed using a QIAquick Gel Extraction Kit (Quiagen, Limburg, the Netherlands), according to the manufacturers protocol.

3.4.3. Polymerase chain reaction & pyrosequencing

Pyrosequencing was initially performed using a Roche 454 platform, results of which are presented in Chapters 4 and 5. This platform was discontinued mid-way through this thesis, necessitating a change to a new platform, the Illumina MiSeq platform, from which results are presented in Chapters 6, 7, 9.1 and 9.2. The new platform has many advantages, such as lower costs and a higher throughput of data, compared with the 454 platform. In addition, 454 pyrosequencing has a higher error rate, which routinely leads to up to 15% artificial in vitro amplification of the original DNA templates (Gomez-Alvarez et al., 2009). In the following the two pyrosequencing techniques will be described.

Roche 454 pyrosequencing:

PCR for amplicon 454 pyrosequencing was performed using Roche’s ‘Fast Start High Fidelity PCR system’ that employs tagged fusion bacterial primers 27F (Lane, 1991) and 338R (Muyzer et al., 1995), targeting the V1-V5 hypervariable region of the bacterial 16S rRNA gene. The primers were synthesised by IDTdna (Integrated DNA Technologies, BVBA, Leuven, Belgium) and designed for sequencing using a Roche 454 Life Sciences GS Junior system. The fusion 27F primer (5’-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG ACG AGT GCG T AG AGT TTG ATC MTG GCT CAG -3’) comprises the 454 Life Sciences “Lib-L Primer A”, a 4 base “key” sequence (TCAG), a unique ten base multiplex identifier “MID” sequence for each sample (NNNNNNNNNNN), and bacterial forward primer 27F. The reverse fusion 338R primer (5’-GCW GCC TCC CGT AGG AGT-3’) contained the 454 Life Sciences “Lib-L Primer B”, a 4 base “key” sequence (TCAG), and bacterial primer 338R.
For the PCR master mix, 40 µL sterile purified H₂O, 5 µL of 10x PCR reaction buffer, 1 µL dNTP mix, 0.8 µL of 25 mM 338R primer and 0.4 µL High Fidelity Enzyme Blend were mixed per sample and added to a 50 µL PCR reaction tube. 0.8 µL of one of each barcoded 27F primer and 2 µL of DNA, taken from the sample corresponding to each specific barcode, were added to separate tubes. A negative control for the PCR reaction, which contained 2 µL sterile H₂O, was also prepared. The PCR conditions included an initial denaturation step at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s and extension at 72°C for 45 s, followed by a final extension step at 72°C for 5 min. In the next step, agarose gel electrophoresis was performed as described above (section 3.4.2). DNA was quantified on a Nanodrop ND-1000 (Thermo Scientific) and all samples were diluted to 10 ng/µL. The DNA product was then stored at 4°C prior to emulsion PCR and then sequencing was performed using a 454 GS Junior pyrosequencing system (Roche), using the facility in the Faculty of Life Sciences, University of Manchester.

**Illumina MiSeq pyrosequencing:**

16S rRNA sequencing was conducted with the Illumina MiSeq platform (Illumina, San Diego, CA, USA) which used the same Roche ‘Fast Start High Fidelity PCR System’ (Roche Diagnostics Ltd, Burgess Hill, UK) as above, however different primers were used, 515F and 806R, targeting the V4 hyper variable regions for 2 × 150-bp paired-end sequencing (Caporaso et al., 2011, 2012). The fusion 515F primer used for PCR amplification had the sequence 5′-GTG YCA GCM GCC GCG GTA A-3′ and the reverse 806R primer was 5′-GGA CTA CHV GGG TWT CTA AT-3′. PCR was performed in 50 µL reactions, starting with the denaturation at 95°C for 2 min, followed by 36 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension step of 5 min at 72°C. The PCR products were cleaned up and normalised to ~20 ng each using the SequalPrep Normalisation Kit (Fisher Scientific, Loughborough, UK). The PCR amplicons from all samples were pooled in equimolar ratios. The run was performed using a 4 pM sample library spiked with 4 pM PhiX, a control library of genomic DNA, to a final concentration of 10% (Kozich et al., 2013).

**3.4.4. Sequencing & data analysis**

The pyrosequencing reads from the 454 platform were processed using Qiime 1.8.0 release (Caporaso et al., 2010), which removed low quality reads and short sequences, and both
forward and reverse primers were discarded. Noise reduction and chimera removal were achieved with USEARCH (Edgar, 2010). Operational taxonomic units (OTU), sequences grouped according to their similarity, were picked at 97% sequence similarity using Qiime. Taxonomic classification of all reads was performed in Qiime using the Ribosomal Database Project (RDP) at 90% confidence threshold (Cole et al., 2009), 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.

Pre-analysis of amplified products for sequencing on the MiSeq platform was performed using an Agilent BioAnalyzer at the Centre for Musculoskeletal Research, Manchester (Kozich et al., 2013). Raw sequences were divided into samples by barcodes (up to one mismatch was permitted) using a sequencing pipeline. Quality control and trimming was performed using Cutadapt (Martin, 2011), FastQC (Andrews, 2010), and Sickle (Joshi and Fass, 2011). MiSeq error correction was performed using SPAdes (Nurk et al., 2013). Forward and reverse reads were assembled to full-length sequences with PANDAseq (Masella et al., 2012). Using ChimeraSlayer the sequences were screened for chimeras which were removed subsequently (Haas et al., 2011). Then similar to the 454 platform, OTU’s were generated with UPARSE (Edgar, 2013) and classified by USEARCH (Edgar, 2010) at the 97% similarity level, and singletons were removed. The taxonomic assignment was performed by the RDP classifier (Wang et al., 2007).

Rarefaction analyses of the sequences were conducted to describe microbial diversity using the original detected OTUs in Qiime (Caporaso et al., 2010). Values accounting for the evenness of the distribution were assigned by the Shannon-Weaver H index (van Elsas et al., 2006). For this purpose, $p_i$, the proportion of individuals belonging to the whole group of species ($i$), was calculated for each species, and multiplied by the natural log (ln). The index is computed from the negative sum of the numbers for each species.

$$H = -\sum_{i=1}^{H} p_i \ln p_i$$  \hspace{1cm} (eq. 3.1)

DNA extractions were performed by Mr C Boothman, 454 pyrosequencing was carried out by Mr G. Fox in the Faculty of Life Sciences sequencing Facility, Manchester and MiSeq
pyrosequencing was done by Mr C Boothman at the Centre for Muscoskeletal Research, Manchester. Data processing was done by the author and Mr C Boothman.

3.5. Mineralogical analyses
The analysis of (bio)minerals formed from biodegradation of ISA is described in the following section. Using Environmental Scanning Electron Microscopy (3.5.3) and Transmission Electron Microscopy (3.5.4), it was also possible to study microorganisms in context of biomineralisation.

3.5.1. X-ray Diffraction analysis
X-ray powder Diffraction (XRD) crystallography was used to identify crystalline precipitates from microbial ISA degradation. Amorphous phases are rarely characterised, whilst crystalline phases are usually detected when the concentration is >5% by mass. It is an elegant technique because of its speed and ease of performance that can be used to conduct semi-quantitative analyses.

The principle is based on constructive interference between a source of X-rays and a crystalline sample. Each crystalline material has its individual atomic molecular structure, which consequently emits a characteristic diffraction pattern. When X-rays hit a crystallographic inter-plane spacing and create a reflection (constructive interference = n is an integer), the Bragg’s law is satisfied following the equation:

\[ 2d \sin \theta = n\lambda. \]  
(eq. 3.2)

This law relates the wavelength of electromagnetic radiation [\(\lambda\)] to the diffraction angle [\(\theta\)] and the lattice \(d\) spacing [\(d\)] in a crystal. Since the powdered material contains randomly ordered crystals, the sample will be scanned through two angles, to attain all possible diffraction directions of the lattice. After signal processing, a characteristic fingerprint for each element is obtained that can be compared to a database of standards with known peak angles and intensities.

For XRD analysis, precipitates from biodegradation experiments were collected at the end of individual incubations. Sample preparation was performed anaerobically by centrifugation (14,000 g, 10 min) and washing in an anoxic bicarbonate buffer (30 mM NaHCO\(_3\)). After discarding the supernatant, the wet pellet was coated onto a glass slide and dried before placing it into an anaerobic sample holder fitted with a knife-edge. The slides were analysed
using a Bruker D8 Advance instrument, fitted with a Gobel Mirror and a Lynxeye detector. Cu Kalpha1 X-rays were used in a scan mode from 5 to 70° with a step size 0.02° and a count time of 0.5 sec per step. Mineral identification was performed with Eva v14 software to match patterns to standards from the ICDD database. A semi-quantitative analysis of relative mineral proportions was carried out using Topas v4.2 software. Results from XRD are presented in Chapters 5, 6, 7, 9.1 and 9.2.

3.5.2. X-ray Absorption Near Edge Spectroscopy & Extended X-ray Fine Absorption Spectroscopy

XRD is a useful spectroscopic technique for the analysis of chemical structures of compounds, such as uranium minerals, when the targeted element is sufficiently concentrated (>1%) and is present in a crystalline phase. However, such requirements are not always met in biological and environmental systems. Thus, X-ray absorption spectroscopy (XAS) is advantageous as it allows for the analysis of speciation and coordination of specifically targeted elements such as uranium at very low concentrations, and it can be used to characterise non-crystalline phases, such as uranium sorbed onto minerals.

![Figure 3.3. Set up of a synchrotron beamline showing incident beam ($I_0$), and following the absorption event the detection of fluorescence energy ($I_f$) and transmission energy ($I_t$) (adapted from Ravel and Newville, 2005).](image)

XAS relies on high intensity monochromatic X-rays produced by synchrotrons. In the synchrotron, a continuous beam of high-energy electrons circulate in a ring and then either a bending magnet or wigglers are used to generate a magnetic field, where the electrons are deflected and generate high energy X-rays in the process. An X-ray beam is emitted towards the monochromator that is tuned to select the absorption edge of the desired element (Figure 3.3). Incident X-rays of energy that equal to the binding energy of the core-level atom interact with the atom when hitting the sample thereby generating absorption. Absorption occurs when an electron is promoted to an unoccupied energy level or when an electron is ejected from the
atom. Absorption edges are broadly characteristic for each element, e.g. the uranium L_{III} edge is at approximately 17166 eV depending on the oxidation state (National Physical Laboratory, 2017). Following an absorption event, the atom is said to be in an excited state and left with an empty electronic level from the core-level (K, L, or M shell), referred to as core hole, and a photo-electron. Two mechanisms can be responsible for the core hole to relax, either a higher energy electron drops to fill the core hole, known as fluorescence, or an electron drops from a higher electron level and a second electron is emitted, described as the Auger effect. Fluorescence is more likely to occur in the hard energy regime (> 2 keV), whilst the Auger effect usually occurs in the soft X-ray regime (Ravel and Newville, 2005).

Spectra are mainly obtained by measuring either in transmission or in fluorescence mode (Figure 3.4). The transmission mode describes absorption, which is measured directly from the transmitted energy through the sample:

\[ I = I_0 e^{-\mu(E)t} \]  

(eq. 3.3)

\[ \mu(E)t = -\ln \left( \frac{I}{I_0} \right) \]  

(eq. 3.4)

where the intensity of an X-ray beam passing through a material is given with the absorption coefficient \( \mu \), \( I_0 \) is the intensity of the X-ray before hitting the sample and \( I \) is the transmitted signal through the sample. The fluorescence mode detects the re-filling of a deep core hole:

\[ \mu(E)t \propto \frac{I}{I_0} \]  

(eq. 3.5)

where \( I \) is the emitted energy.

![Figure 3.4. XAFS spectrum of FeO (blue line) showing the XANES and EXAFS regions and the smooth background function (red line) with the edge-step indicated as \( \Delta \mu_0(E_0) \) (adapted from Ravel and Newville, 2005).](image-url)
Photo-electrons are absorbed by all matter (here the absorber atom is uranium) through the photo-electric effect and spectra can be collected for the X-ray absorption fine structure (XAFS). These spectra include a ‘near edge’ region, or XANES (X-ray absorption near edge spectroscopy), and an ‘extended region’, or EXAFS (X-ray absorption fine structure) (Figure 3.4). XANES spectra are appropriate for determining an atom’s oxidation state, as there are shifts of 1 to 3 eV in edge position for each valence unit change. By contrast, oscillations in the EXAFS region are created by constructive interference of electrons that have been ejected from the central absorber and are scattered by the surrounding atoms, thus providing information about the local coordination environment of atoms around the absorbing atom.

XAS techniques were used to determine the oxidation state of uranium as well as study the local coordination environment of uranium species after incubation. XAS data were collected for the M_{IV}-edge and L_{III}-edge of U at different beamlines.

For studies of the U M_{IV}-edge High-Energy Resolution Fluorescence Detection X-Ray absorption near edge structure (HERFD XANES), aliquots from incubations containing 240 ppm U were spun down (5,000 g, 20 min) and a wet paste was layered in a sample holder. Samples were prepared strictly anaerobically and kept in a Kilner jar for double containment, which was purged with Ar to maintain an anoxic atmosphere and stored at -80 °C until analysis. These spectra were collected on the ID26 beamline at the European Synchrotron Radiation Facility (ESRF) in Grenoble (Gauthier et al., 1999) using a Si(111) monochromator and a X-ray emission spectrometer (Glatzel et al., 2013). The intensity of the Mβ emission line (3336.0 eV) was recorded using five spherically bent Si crystal analysers aligned at 75° Bragg angle within a helium atmosphere (Kvashnina et al., 2013, 2014).

For U L_{III}-edge data analysis, two methods of sample preparation were used dependent on the uranium concentration. Experiments containing around 24 ppm uranium, were centrifuged (5,000 g, 20 min) and pellets were mounted as sludge in an airtight cryovial containing wax to maintain the sample position within the vial. Samples with higher uranium concentration were collected by centrifugation (5,000 g, 20 min) and dried in an anaerobic chamber. The dry material was diluted to approximately 1% U w/w by adding cellulose to a final weight of 1.5 g. The sample was powdered and mixed with a pestle and mortar and then transferred to a pellet press that produced a 2 mm thick pellet. The pellet was mounted in the same airtight XAS
holder as above. All samples were prepared strictly anaerobically and kept in a Kilner jar for double containment, which was purged with Ar to maintain an anoxic atmosphere and stored at -80 °C until analysis at the Diamond Light Source, on B18 XAS beamline. U L_{III}-edge spectra were collected at room temperature, in fluorescence mode using a 9-element Ge detector (Dent et al., 2009). Standard spectra were also collected in transmission mode for U(VI) (as schoepite, UO_2·2H_2O) and U(IV) (as uraninite, UO_2). Spectra were merged and the background subtracted using Athena software (Ravel and Newville, 2005). Energy calibration was completed by parallel measurement of a Y-foil calibrated to 17038 eV. Resulting EXAFS spectra were k^3-weighted in the wavevector space (x(k)) and spectra were Fourier transformed (FT) over the k-range of approximately 3 to 12 Å\(^{-1}\). Typically, EXAFS spectra include single and multiple scattering paths which were fitted using Athena and Artemis software (Ravel and Newville, 2005) and compared with spectra from the relevant literature. Theoretical phases and amplitudes were calculated with FEFF6 code implemented in IFEFFIT programs suite based on ningyoite [CaU^{IV}(PO_4)_2] (Dusausoy et al., 1996), autunite [(Ca,U^{VI})(PO_4)] (Locock and Burns, 2003), uraninite [UO_2] (Wyckoff, 1963) and liebigite [Ca_2UO_2(CO_3)_3·11H_2O] (Mereiter, 1982). Shells were only included, when they reasonably improved the fit, assessed by the F-test (Downward et al., 2007), and were considering at all times not to use more than a third of the total number of available independent points. Results using XAS techniques are presented in Chapters 7 and 9.2.

### 3.5.3. Environmental Scanning Electron Microscopy

Environmental Scanning Electron Microscopy (ESEM) was used to obtain images of the cells and biominerals formed in enrichment cultures. ESEM produces images by scanning a sample with a focused beam of electrons (Figure 3.5). By interaction of the electrons with atoms in the sample two signals are produced, which comprise a secondary electron (SE) and a backscattered electron (BSE) signal that are collected by different detectors. Most commonly images are collected in SE mode which is an inelastic scattering of low-energy electrons from the first few nanometers of a sample surface, creating a high resolution image of the surface. The BSE mode is useful in gathering information about the chemical information of a sample. This signal is obtained from the interaction of high-energy electrons with atoms resulting in
elastic scattering, whereby the heavier an element is (denser in atoms), the brighter the reflected signal will be.

![Diagram of ESEM principle](http://www.technoorg.hu/news-and-events/articles/high-resolution-scanning-electron-microscopy-1/)

Samples were prepared in two ways: For mineralogical observations, sample aliquots were washed in 30 mM bicarbonate buffer. In contrast, when cell associations with minerals were investigated, microbial cells were preserved prior to observation in a 2.5% glutaraldehyde phosphate buffered saline (PBS) buffer (8 g NaCl, 0.2 g KCl, 1.42 g Na₂HPO₄, 0.27 g KH₂PO₄, 1 L H₂O) overnight. The next day, samples were washed in diluted buffer of 60% and 30% PBS (diluted with deionised water) for 30 min each, followed by a dehydration step for 30 min in deionised water with increasing concentration of ethanol until 100% ethanol was reached. After sample preparation, a precipitate was coated onto an aluminum pin stub (Zeiss, Ø12.7 mm top) and dried anaerobically. A carbon coating was applied to enhance the sample conductivity. Imaging was performed on two different instruments. The first one was a FEI XL30 ESEM-Field Emission Gun (ESEM-FEG) and an EDAX Gemini EDS system was used for elemental analysis which provided results for Chapter 5. The other instrument was a FEI
Quanta 650 FEG from which images are presented in Chapters 6, 7, and 9.2. Both instruments were operating at 15 kV in high vacuum mode \((10^{-5} \text{ to } 10^{-6} \text{ mbar})\).

After acquisition of initial images (Chapter 4) with support by Dr H Bagshaw, training on both instruments including carbon coating allowed the author to produce the images presented in Chapters 5, 6, 7, 9.1 and 9.2.

### 3.5.4. Transmission electron microscopy

Transmission electron microscopy (TEM) has a higher resolution than ESEM and was used to obtain images of biominerals precipitated by bacteria and the microbe-mineral interface. Selected area electron diffraction (SAED) patterns were collected to identify the biomineral crystal structure.

Electron microscopy relies on the interaction of a source of X-rays that excites a sample and will be collected by a collector behind the sample. The fundamental principle of this technique uses the characteristic atomic structure of each element that allows a unique set of peaks to be emitted in an electromagnetic emission spectrum. As the electron beam passes through the sample, X-rays will be also diffracted on crystal planes, producing diffraction patterns (SAED) which are again characteristic for each crystal. The SAED patterns can be analysed for the \(d\) spacing of a crystal, which can be compared to XRD values and reference standards from the International Centre for Diffraction Data (ICDD) database.

Sample aliquots from the end of experiments were spun down \((5,000 \text{ g}, 20 \text{ min})\) and washed in a bicarbonate buffer \((30 \text{ mM})\) to remove any salts and re-dissolved in some buffer. A drop of the washed sample was layered onto a TEM copper grid with a holey carbon film coating and left to dry anaerobically. Grids were examined over a range of magnifications \((5,000 \text{ to } 200,000x)\) on two different TEM instruments. In Chapters 5 and 6, results are presented from the Philips CM200 field emission gun TEM (FEGTEM) fitted with an Oxford Instruments X-Max 80 mm\(^2\) SDD EDS system running Aztec software, and a Gatan Orius SC200 CCD camera running GMS 2 software based at the University of Leeds EPSRC Nanoscience and Nanotechnology Facility (LENNF). Imaging was performed with Dr Z Aslam and Dr M Ward. In Chapters 7, and 9.2, results are presented from the Philips FEI Technai T20 (200kV LaB6) instrument fitted with an Oxford Instruments X-Max 80 mm2 SDD EDX system operating Aztec software, and a Gatan...
Orius SC200 CCD camera operating GMS 2 software which is based in Manchester. Imaging was performed with Dr H Bagshaw.

3.5.5. PHREEQC modelling

Geochemical modelling of solution chemistry was done using PHREEQC version 3.1.2.8538, coupled with the ThermoChimie database version 9.0. Input data used were chemical media compositions, pH, eH, measured ion concentrations and known mineral phases (from Mineralogical analyses 3.5). Modelling was performed before and after ISA degradation. Results for this method are presented in Chapters 6 and 7. Modelling was performed with the support of Dr N Bryan and Dr K Smith.

3.6. References


4. Microbial degradation of isosaccharinic acid under conditions representative for the far field of radioactive waste disposal facilities (research article)

Gina Kuipers¹, Naji M. Bassil¹, Christopher Boothman¹, Nicholas Bryan², Jonathan R. Lloyd¹*

¹Research Centre for Radwaste Disposal & Williamson Research Centre for Molecular Environmental Science, School of Earth, Atmospheric and Environmental Sciences, University of Manchester, Oxford Road, Manchester M13 9PL, UK

²National Nuclear Laboratory Limited, Chadwick House, Warrington Road, Birchwood Park, Warrington, WA3 6AE, UK

*Corresponding author e-mail: Jon.lloyd@manchester.ac.uk

Status: The following pages present the original work as published in Mineralogical Magazine, November 2015, Vol. 79(6), pp. 1443–1454.
4.1. Abstract
It is UK Government policy to dispose of higher activity radioactive waste through geological disposal into an engineered deep underground geological disposal facility (GDF; DECC, 2014). Those wastes include low-level (LLW) and intermediate-level (ILW) radioactive wastes that are very heterogeneous, containing a range of inorganic and organic materials, the latter including cellulose items. After closure of the GDF, eventual resaturation with groundwater is expected, resulting in the development of a hyperalkaline environment due to the proposed use of a cementitious backfill. Under these high pH conditions, cellulose is unstable and will be degraded chemically, forming a range of water-soluble, low molecular weight compounds, of which the most abundant is isosaccharinic acid (ISA). As ISA is known to form stable soluble complexes with a range of radionuclides, thereby increasing the chance of radionuclide transport, the impact of microbial metabolism on this organic substrate was investigated to help determine the role of microorganisms in moderating the transport of radionuclides from a cementitious GDF. Anaerobic biodegradation of ISA has been studied recently in high pH cementitious ILW systems, but less work has been done under anaerobic conditions at circumneutral conditions more representative of the geosphere surrounding a GDF. Here we report the fate of ISA in circumneutral microcosms poised under aerobic and anaerobic conditions; the latter with nitrate, Fe(III) or sulfate added as electron acceptors. Data are presented confirming the metabolism of ISA under these conditions, including the direct oxidation of ISA under aerobic and nitrate-reducing conditions and the fermentation of ISA to acetate, propionate and butyrate prior to utilization of these acids during Fe(III) and sulfate reduction. The microbial communities associated with these processes were characterized using 16S rRNA gene pyrosequencing. Methane production was also quantified in these experiments, and the added electron acceptors were shown to play a significant role in minimizing methanogenesis from ISA and its breakdown products.

4.2. Introduction
It is UK Government policy to dispose of LLW and ILW radioactive waste, comprising the largest volume of radioactive waste of the national inventory, into an engineered deep underground geological disposal facility (GDF). The majority of the ILW and a small portion of the LLW containing long-lived radionuclides (DECC, 2014), will be encapsulated in grout within steel
drums, and then encased in concrete in vaults excavated into a suitable geological formation. The LLW and ILW vaults will become reducing after closure of the GDF, and a hyperalkaline environment will be created when groundwater penetrates the engineered barrier system of a cement-dominated GDF.

ILW and LLW wastes are very heterogeneous, and contain substantial amounts of cellulosic material, including paper, filters and cotton. These cellulosic compounds are known to be degraded under the anoxic hyperalkaline repository conditions that are expected in situ, leading to the production of a range of soluble organic compounds (Glaus et al., 1999). During this alkaline hydrolysis, glucose units are eliminated from the cellulose chain in a stepwise process, known as a ‘peeling-reaction’ (e.g. Van Loon et al., 1999; Pavasars et al., 2003), which is initiated at the reducing end group (a reactive aldehyde carbonyl). In contrast, the β-1,4 glycosidic linkages are alkali-stable and cannot be reduced (Askarieh et al., 2000). The peeling reaction competes with another abiotic reaction, the so called ‘stopping-reaction’, which is either a chemical transformation taking place at the end of the cellulose molecule or a physical termination which takes place at highly crystalline regions (e.g. Van Loon et al., 1999; Vercammen et al., 1999; Pavasars et al., 2003). Since the reaction can be reinitiated by the so-called ‘midchain scission’, which is a random cleavage of glycosidic bonds within the polysaccharide chain, all cellulose may be degraded over prolonged timescales (Glaus and van Loon, 2008).

In cementitious pore waters, as they are expected in a GDF, isosaccharinic acid (ISA, C₆H₁₂O₆) has been identified as the main product of the alkaline degradation of cellulosic material (Whistler & BeMiller, 1958; van Loon & Glaus, 1998; Glaus et al., 1999; Knill & Kennedy, 2003), supported by further results from a long-term study by Pavasars et al. (2003) and also by Glaus & van Loon (2008). In the presence of divalent cations, such as Ca²⁺, common in the cementitious backfill of a GDF where calcium concentrations are expected to be high, ISA binds to calcium ions to form the divalent salt Ca(ISA)₂ (Vercammen et al., 1999).

Various studies have shown that ISA can also form stable soluble complexes with metals and radionuclides, including those from the lanthanide and actinide series, particularly Am(III) (Tits et al., 2005), Eu(III) (Vercammen et al., 2001; Tits et al., 2005), Ni(II) (Warwick et al., 2003), Np(IV) (Rai et al., 2003; Gaona et al., 2008), Th(IV) (Vercammen et al., 2001; Tits et al., 2005),
and U(IV) (Warwick et al., 2004). Since ISA forms such strong soluble complexes with radionuclides, there is concern that ISA may increase radionuclide migration when the groundwater resaturates the GDF, thereby increasing the likelihood of transport from the engineered barrier system and through the geosphere. Conservative assumptions in modeling, estimate that considerable amounts of up to 0.1 M ISA can be produced (Bradbury & Sarott, 1995), which are considered chemically stable under alkaline conditions (Bradbury & Sarott, 1997), and show minor sorption on cement (Bradbury & Sarott, 1995; Van Loon et al., 1997), increasing the interest in the potential biodegradation of ISA by subsurface microorganisms.

Recent studies have demonstrated that the geochemical conditions in a GDF may not be sufficiently harsh to prevent microbial metabolism, with a range of aerobic and anaerobic microorganisms surviving highly alkaline (up to pH 12; Rizoulis, 2012; Williams et al., 2014) and/or radioactive conditions (Booth, 1987; Brown et al., 2014). Therefore microbial metabolism may have the potential to control the mobility of priority radionuclides, via a range of mechanisms (reviewed in Lloyd & Macaskie, 2000) moderating their transport after GDF closure. The utilization of ISA by aerobic organisms is well known (Strand et al., 1984; Bailey, 1986), but has not been reported widely under anaerobic conditions, although a recent study has demonstrated that ISA can serve as an electron donor for anaerobic metabolism (e.g. nitrate reduction) under alkaline conditions (pH 10; Bassil et al., 2014). However, if ISA degradation processes are incomplete and ISA should escape from the alkaline near-field of a cementitious GDF, there is little information on its fate in the largely anoxic circumneutral far-field geosphere, dominated in UK groundwaters by the potential electron acceptor sulfate (Bond & Tweed, 1995). It should be noted that at pH 10 and above, sulfate reduction using a range of electron donors (Rizoulis, et al., 2012), including ISA (Bassil et al., 2014) is minimal over the extended periods (several months) tested thus far.

In this study, an enrichment-cultivation approach was applied to study the fate of ISA under such circumneutral conditions. Sediments from a lime workings site near Buxton (Rizoulis et al., 2012; Williamson et al., 2013) which has areas with elevated pH values and high calcium concentrations, consistent with those of an ILW radioactive waste disposal facility (pH ~12.5, Ca$^{2+}$ ~20 mM; NDA, 2010), and is known to contain microorganisms able to degrade ISA (Bassil et al., 2014) were retrieved. Microcosms (pH 7.0) were inoculated with the limeworkings site
sediments, simulating conditions of the surrounding geosphere of a GDF under different possible redox conditions, using NO₃⁻, Fe(III), SO₄²⁻ or CO₂ as electron acceptors. A multidisciplinary approach was adopted to elucidate the fate of ISA, including geochemical, microbiological and microscopy techniques.

4.3. Material & Methods

Sediment acquisition: Surface sediment samples were collected from a depth of approximately 20 cm, at a site contaminated by legacy lime workings at Harpur Hill, Buxton in Derbyshire, UK. The study site showed a pH ranging from ca. 6.5 to 11 with high calcium and silicate concentrations, and for this study samples were collected from a marginal area and a pH of 6.8. The samples were stored in the dark at 4°C until analysis.

Ca(ISA)₂ preparation: Ca(ISA)₂ was prepared from α-lactose monohydrate and Ca(OH)₂ following the protocol of Vercammen et al. (1999).

Enrichment cultures: Enrichment cultures were grown by incubating a sediment inoculum (1% vol/vol) in a minimal medium containing 30 mM NaHCO₃, 4.7 mM NH₄Cl, 4.4 mM NaH₂PO₄•H₂O, 1.3 mM KCl, and 0.3 mL of mineral and vitamin stock solutions (Lovley et al., 1984). The pH of the medium was adjusted to 7 with NaOH then de-aerated with a N₂/CO₂ (80:20) gas mixture for 30 min. Ca(ISA)₂ was added to a final concentration of 1.5 mM as sole carbon source and electron donor. The medium was distributed over four different anaerobic experiments using the following terminal electron acceptors (TEAs) either 24 mM NaNO₃, Fe(III) oxyhydroxide added as a slurry to a concentration of 20 mmol L⁻¹, 12 mM Na₂SO₄ or no added electron acceptor (CO₂ served as the sole electron acceptor in these experiments). For each TEA three different controls were prepared in triplicate, comprising (i) a test control containing ISA, the respective TEA and an active inoculum, (ii) a control with no added electron donor (no ISA) and (iii) an “abiotic” sterile control that contained ISA and the appropriate TEA, but was autoclaved to kill any active microorganisms. All microcosms were prepared in serum bottles sealed with butyl stoppers that were flushed with an N₂/CO₂ (80:20) gas mixture for 5 min prior to autoclaving and later inoculation and incubation in the dark at 20°C. Aerobic experiments were also set up the same way, using the same medium (but lacking 30 mM NaHCO₃), but here the bottles were closed with a foam bung to facilitate oxygen transfer.
Samples were collected aseptically and frozen immediately at -20°C until further analysis. After microbial activity had reached a steady state, monitored by turbidity and pH, the cultures were re-subcultured into fresh medium, using a 1% (vol/vol) inoculum, and incubated for another incubation period. Data presented were obtained from this subculture of the sediment incubation experiments.

**Analytical techniques:** The pH of samples from the microcosms was measured with a calibrated Denver Instrument digital meter (pH 4, 7 and 12). Bacterial growth was monitored over the length of incubation by optical density (OD) measurements at 600 nm compared to a blank, containing only growth medium and Ca(ISA)₂. The Ferrozine spectrophotometric assay was used to quantify Fe(II) and total Fe (after reaction with hydroxylamine) by comparison to known standards at a wavelength of 562 nm (Lovley & Phillips, 1987). In addition, light microscopy was performed on key samples to check for microbial growth under both transparent light and fluorescent light using a Zeiss Axioscope Microscope fitted with an 100x ACHROPLAN lens (No: 440081), the latter using Hoechst 33342 nucleic acid stain.

**GC-TCD:** The gases oxygen and methane were analysed in the headspace of the sediment incubation experiments with an Agilent 7890A Gas Chromatograph, fitted with a 7697A headspace autosampler, connected to an Agilent 7890 Thermal Conductivity Detector (TCD). The chromatograph was equipped with a HP Molesieve column (30m x 25 μm x 0.53 mm). The TCD was heated to 180°C and the split/splitless inlet was heated to 250°C with a backpressure of 1.36 psi and argon as carrier gas was used at a flow rate of 104 mL min⁻¹. For analysis, the autosampler was set to a constant injection of 1 mL with pressure equilibration, and a split ratio of 1:100. After injection into a loop of 1 mL volume the sample was forwarded to the oven in an isothermal run at 50°C which took 12 min. The column flow was of 6 mL min⁻¹ together with a reference flow at 9 mL min⁻¹.

**Ion-Exchange Chromatography:** Sulfate, nitrate, nitrite, organic acids and ISA were analysed using ion exclusion high performance liquid chromatography (IE-HPLC), using a Dionex ICS5000 Dual Channel on Chromatograph, fitted with a Dionex AS-AP auto sampler, connected to a CD20 conductivity detector. The chromatograph was equipped with a Dionex Capillary (50 x 0.4 mm) AG11-HC 4 μm guard column and Dionex Capillary (250 x 0.4 mm) AS11-HC 4 μm
analytical column. For background reduction a Dionex ACES300 Chemical Suppressor was used.

For analysis, frozen aliquots were thawed, vortexed, centrifuged and the supernatant transferred to microcentrifuge tubes at a 1:50 sample/DIW ratio. The mobile phase (eluent) used comprised of a gradient of concentrated KOH which was mixed with high purity water at a flow rate of 0.015 mL/min and a backpressure of 3,200 psi. 0.4 µL was injected to a run that started at 1 mM KOH for 10 minutes, increased then to 38 mM up to minute 25 and then re-equilibrated to the initial value until 40 minutes.

**DNA extraction & pyrosequencing:** Bacterial community structure was examined by extraction of DNA from 200 µL of the sediment inoculum for the initial community and subcultured microcosms for the enrichment cultures using the MoBio PowerLyzer™ UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA). PCR for amplicon pyrosequencing was performed using tagged fusion bacterial primers 27F (Lane, 1991) and 907R (Muyzer et al., 1995), targeting the V1-V5 hypervariable region of the bacterial 16S rRNA gene. Pyrosequencing PCR was performed using Roche’s ‘Fast Start High Fidelity PCR system’ as described previously (Bassil et al., 2014 or Williamson et al., 2013). The pyrosequencing run was performed at the University of Manchester sequencing facility, using a Roche 454 Life Sciences GS Junior system. The 454 pyrosequencing reads were analysed using Qiime 1.8.0 release (Caporaso et al., 2010), and de-noising and chimera removal was performed in Qiime during OTU picking (at 97% sequence similarity) with usearch (Edgar, 2010). Taxonomic classification of all reads was performed in Qiime using the Ribosomal Database Project (RDP) at 90% confidence threshold (Cole et al., 2009), 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. In addition, Qiime was used to prepare rarefaction curves for the samples.
4.4. Results & Discussion

Microcosm experiments were conducted to determine the impact of a range of terminal electron accepting processes on the biodegradation of ISA at circumneutral pH as expected in the surrounding geosphere of a GDF. Results presented here focus on data collected from stable enrichment cultures that were prepared from the primary microcosms (i.e. the first subculture), with exception for the headspace gas results that were obtained from the primary enrichment cultures. The analysis of these subcultured experiments was considered more robust, being separated from the geochemically more complex primary cultures by the biogeochemical selection pressures imposed by the highly selective media used, making the biogeochemical and microbiological data from these experiments easier to interpret. ISA degradation was recorded under all the conditions tested, ranging from the aerobic incubations (Figure 4.1), to anaerobic incubations with either nitrate (Figure 4.2 A-C), Fe(III) (Figure 4.2 D-F) or sulfate (Figure 4.3 A-C) as electron acceptor or no added electron acceptor (Figure 4.3 D-E). In all cases, ISA was not degraded in the sterile controls, confirming a microbiological mechanism.

Biodegradation of ISA commenced immediately under aerobic and nitrate-reducing conditions, while there was a time lag of about seven days in the experiments under (Fe(III))-reducing and sulfate-reducing conditions. There was also pronounced ISA degradation, again after a lag period, in the no added electron acceptor incubation, indicative of fermentation processes, in this case leading to the production of acetate, butyrate and minor levels of propionate and formate. After an initial lag phase, ISA oxidation was complete within about 21 days under all conditions, apart from incubations with added Fe(III), where ISA was depleted after 28 days.

Figure 4.1: Biodegradation of ISA by aerobic microbial cultures at a pH of 7. ISA concentration: (□) test condition with active microbial inoculum; (●) sterile control (autoclaved).
Under aerobic conditions, the rate of ISA degradation was consistent from the start of the incubation until almost complete depletion of all ISA in the test incubation by 14 days, while concentrations remained stable in the sterile control (Figure 4.1). ISA degradation was accompanied by a drop in pH from 7 to 6 and a drop in turbidity from OD$_{600nm}$ = 0.42 to 0.13 (data not shown). The drop in pH is a result of equilibration with the atmosphere, which was
possible since the aerobic microcosms were non-sealed. In the IC analysis no organic acids were detected, consistent with complete oxidation of ISA to CO$_2$.

Under nitrate-reducing conditions, rapid ISA degradation was again noted until day 14, and then slowed (Figure 4.2 A) accompanied by a decrease in the rate of nitrate reduction, which was converted to nitrite (Figure 4.2 B). During this time period, about 82% of both the ISA and nitrate were removed, while only an additional 6% of these substrates were removed over the next 28 days. Incomplete ISA degradation indicated toxication due to elevated nitrite concentrations, which are considered to inhibit microbial metabolism (e.g. Shen et al., 2003). Only low amounts of the degradation product acetate were detected, along with very minor amounts of n-butyrate and propionate (Figure 4.2 C), indicating that ISA was degraded via denitrification to CO$_2$ and another denitrification endpoint, for example nitrogen. The pH in the denitrifying cultures remained stable, no methane was produced (Figure 4.4) and, similar to the aerobic experiment, a drop in turbidity from 0.58 to 0.18 over 42 days of incubation was measured.

In the Fe(III)-reducing enrichment cultures, ISA degradation commenced after a lag phase of seven days and accelerated from day 14. Concomitantly with a decline in ISA, spectrophotometric monitoring showed Fe(II) ingrowth, without production of VFAs, indicating that Fe(III) oxyhydroxide may have been used as TEA during the first stage of oxidation of ISA (Figure 4.2 D, E). The pH remained stable over the whole incubation period, whilst the turbidity could not be analysed because of interference from the Fe(III) oxyhydroxide added. After ca. 28 days, almost all of the ISA added had been metabolized, and approximately 50% of the carbon (7.2 mM of the 15.7 mM C added) was accounted for as acetate, n-butyrate and propionate (Figure 4.2 F), possibly reflecting fermentative pathways. However, Fe(III) was continually reduced throughout the extended time course, even when ISA was depleted, and therefore Fe(III) reduction could have been coupled to either (1) the direct oxidation of ISA (especially early on in the incubation when a slow rate of Fe(III) reduction was linked to modest ISA degradation) or (2) to the oxidation of fermentation products that formed from this substrate and were detected after 21 days of incubation, when Fe(III) reduction was maximal. A minor fraction of the carbon removed was recovered in the gas phase as methane after ISA depletion, which increased steadily to ca. 14% of the headspace volume after a prolonged incubation time of 200 days (Figure 4.4). The missing ca. 8.1 mM C (ca. 52%) may have been transformed into other
carbon products and/or resulted in biomass production, which could not be detected in our analyses.

Figure 4.3: Biodegradation of ISA by sulfate-reducing cultures (A-C) and in the absence of an added electron acceptor (D-E) at a pH of 7. A) ISA concentration: (■) test condition with active microbial inoculum; (●) sterile control (autoclaved). B) Sulfate concentration: (●) sterile; (■) test. C) Ions in test condition: (■) ISA; (●) acetate; (●) propionate. D) ISA concentration: (■) test condition with active microbial inoculum; (●) sterile control (autoclaved). E) Ions in test condition: (■) ISA; (●) acetate; (●) propionate; (●) formate.
Under sulfate-reducing conditions, ISA degradation started after a lag phase of three to seven days and then continued quickly until all the ISA (2.72 mM ISA equivalent to 16.1 mM C) was consumed after about 21 days (Figure 4.3 A). After ca. 14 days there was a strong smell of sulfide, confirming microbial sulfate-reducing activity and the experiment turned from beige to a grey/black colour which was indicative of the formation of iron-sulfide phases. This was accompanied by the steady depletion of sulfate in the cultures (Figure 4.3 B). Ca. 40% of the carbon from the ISA was converted to acetate, which was metabolized after the ISA had been depleted (Figure 4.3 C) presumably via sulfate reduction, with another 0.85 mM sulfate reduced over this time. The pH of this sulfate-reducing experiment remained stable at ca. 7, and the turbidity also fell from OD_{600nm} = 0.49 to 0.40. Given the geochemical profiles noted in this experiment, it is likely that both ISA and fermentation products from ISA were used as electron donors for sulfate reduction. Methane was detected (2.8% of the headspace after an extended incubation period of 200 days), but at lower levels than noted in the Fe(III)-reducing cultures.

![Figure 4.4: Gas evolution during the biodegradation of ISA in sediment enrichment slurries containing 2 mM Ca(ISA)_2 under different biogeochemical conditions as follows: (■) sterile control sterile (autoclaved); (●) 24 mM NaNO_3; (▲) 20 mmol L^{-1} Fe(III) oxyhydroxide; (▲) 12 mM Na_2SO_4; (×) no added electron acceptor.](image)

Finally, the fermentative pathways that were implied in the Fe(III)- and sulfate-reducing cultures, were explored in the no electron acceptor control experiment. Here, after an initial lag of about seven days, ISA was degraded efficiently and depleted after about 21 days, despite the absence of an added electron acceptor (apart from the CO_2 added in the headspace of the bottles). Of the ca. 16.3 mM C added as ISA, about 54% (8.1 mM C) was converted into VFAs, of which 4.7 mM C was converted to acetate and 3.2 mM to n-butyrate, and smaller amounts to
formate and propionate (Figure 4.3E). Besides the fermentation of ISA to VFAs, larger quantities of methane were detected in comparison to all other experiments. Especially after the time point of ISA depletion, relatively high amounts of methane were formed, resulting in about 25% CH$_4$ of the headspace volume after 96 days which increased only slightly after another 100 days of incubation by 4% (Figure 4.4), even though it still represented a small fraction of the total carbon metabolized. The main metabolic driving force in this incubation was clearly fermentation, while it is possible that methanogenesis could become more pronounced after long incubation times.

Figure 4.5: Molecular ecology (16S rRNA gene) analysis of microbial ecology of sediment inoculum and microbial enrichments. A) Alpha rarefaction plot showing number or observed species (distinct DNA sequences) in the sediment at day 0 ( ), and after aerobic incubation at day 21 ( ), nitrate reduction at day 21 ( ), Fe(III) reduction at day 28 ( ) and 50 ( ), sulfate reduction at day 21 ( ) and day 58 ( ), and with no added electron acceptor at day 21 ( ) and day 58 ( ). B) Microbial Community composition by phylogenetic classes in the corresponding samples.
Attempts were made to monitor microbial growth by quantifying changes in turbidity, used successfully in previous studies on ISA biodegradation before (e.g. Strand et al., 1986; Bassil et al., 2014), but in all experiments increases in turbidity were minimal, and in many cases fell over time, in some experiments by up to 70%. Interestingly, the turbidity of the sterile controls remained stable. Microscopic analyses, using the Hoechst 33342 dye to identify microbial cells, demonstrated that aggregates varying in size, and of up to 100 µm in diameter, formed in the microbiologically active experiments. These were surrounded by viable planktonic rod-shaped cells of approximately 2-4 µm in length (data not shown), which were clearly external to the aggregates and floating or actively moving in the medium. The clumping noted in these experiments makes the accurate measurement of microbial growth by turbidity impossible. However, DNA profiling experiments were used to identify key changes in the microbial communities that dominated under the biogeochemical conditions imposed. Rarefaction curves (Figure 4.5 A) showed a dramatic decrease in microbial diversity, from the raw sediment sample (>500 species), to the oxic samples incubated for 21 days (just over 100 species), while the anaerobic cultures contained only approximately 50-100 discrete gene sequences. Such a change in relative microbial abundance is common when selective conditions are imposed in microcosm experiments, for example with the addition of ISA as an electron donor, and an excess of electron acceptor such as nitrate or Fe(III) under anoxic conditions (e.g. Thorpe et al. 2012; Williamson et al., 2013, Bassil et al., 2014. Figure 4.5 B shows the broad phylogenetic affiliations of the organisms detected. In the sediment inoculum, no single species from these broad phylogenetic groups comprised more than 3% of the sequences detected. However, after 21 days incubation under aerobic conditions, when the ISA had been degraded fully, the microbial community was dominated by a Betaproteobacterium most closely related (97% sequence match) to Hydrogenophaga palleronii (Willems et al., 1989), a Gram-negative bacterium known to use hydrogen as an energy source, and also to oxidize organics (presumably ISA in this case) with oxygen as the TEA. In contrast, bacteria implicated in ISA degradation in the nitrate-reducing cultures included close relatives (>98% sequence homology) to known Betaproteobacteria, including a member of the Comamonadaceae (34% of sequences detected) and a close relative of Rhodoferax ferrireducens (15% of sequences). The microbial community in the Fe(III)-reducing culture at 21 days was dominated by a novel organism most closely related (96% sequence match) to an uncultured organism in the Gram-positive class.
Clostridia (83% of genes detected). Interestingly, organisms most closely related to known Fe(III)-reducers were in low relative abundance (e.g. <1% of species affiliated with Fe(III)-reducing Geobacter species (Lovley, et al., 1987; Lloyd, 2003) at 21 days. By day 50, this culture was dominated (40% of sequences) by a close relative (99% sequence similarity) to Dechlorosoma suillum, a perchlorate-reducing Betaproteobacterium. The selection for organisms not normally associated with the reduction of insoluble Fe(III) oxyhydroxide, may be due to the inclusion of the strong chelating agent ISA, in these experiments which could change dramatically the bioavailability of Fe(III), and hence the mechanism of reduction. It is also possible that Fe(III) reduction could have been linked directly to co-factor regeneration associated with fermentation of ISA. It should be noted that the majority of the microbial community in the fermentation experiments (no added electron acceptor), were also affiliated with Gram-positive Clostridia. In sharp contrast, the sulfate-reducing cultures were dominated by organisms well known to respire sulfate at neutral pH; approximately 50% of the community was affiliated with the Deltaproteobacterium Desulfovibrio idahonensis after 21 and 50 days, and the role of this organism in ISA biodegradation clearly warrants investigation.

4.5. Conclusion

In this study, we have confirmed that the microbial degradation of ISA occurs under a range of biogeochemical conditions at circumneutral pH, representative of the geosphere surrounding a potential GDF. In keeping with a previous study conducted at pH 10 (Bassil et al., 2014) microorganisms were shown to degrade ISA under aerobic, nitrate-, and Fe(III)-reducing conditions. However, in this study, ISA was also degraded under sulfate-reducing conditions, in sharp contrast to the high pH system, where the diminishing energy yield under alkaline conditions prevented measurable sulfate reduction over the time-scale of the experiments. The precise mechanisms of ISA degradation in these pH 7 experimental systems clearly warrant further attention, including fermentative pathways implicated in the Fe(III)- and sulfate-reducing experiments, in addition to direct coupling of ISA oxidation to anaerobic metabolism, e.g. during nitrate reduction. Subsequent conversion of the initial ISA degradation products to methane was also confirmed in these experiments, and the addition of electron acceptors that could compete with microbially derived CO₂ during methanogenesis (i.e. nitrate, Fe(III) and sulfate) had a dramatic impact on the yield of methane. The impact of a range of biogeochemical processes
on the end products of ISA biodegradation is clearly an area where further research would be valuable, as is the controlling role of microbial metabolism on the mobility of priority radionuclides during ISA metabolism, especially in sulfidic systems. Sulfate-reduction may eventually dominate in and around a GDF, as sulfate in cements and groundwaters can be present at around 9 mM in brackish saline waters, and up to 50 mM in other UK groundwaters (Metcalfe et al., 2007), resulting in the formation of poorly soluble sulfide phases that could immobilize a broad range of radionuclides.

4.6. Acknowledgements
This work was supported by the Radioactive Waste Management (RWM). We would like to thank Alastair Bewsher and Paul Lythgoe (The University of Manchester) for analytical support.

4.7. References


5. Microbial reduction of Fe(III) coupled to the biodegradation of isosaccharinic acid (ISA) (research article)

Gina Kuipers¹, Christopher Boothman¹, Heath Bagshaw¹, Rebecca Beard²*, Nicholas Bryan³, Jonathan R. Lloyd¹*

¹Research Centre for Radwaste Disposal & Williamson Research Centre for Molecular Environmental Science, School of Earth and Environmental Sciences, The University of Manchester, Oxford Road, Manchester M13 9PL, UK
²Radioactive Waste Management Limited, Building 587, Curie Avenue, Harwell Oxford, Didcot, Oxfordshire OX11 0RH, UK.
³National Nuclear Laboratory, Chadwick House, Birchwood, Warrington WA3 6AE, UK
⁴National Nuclear Laboratory Limited, Chadwick House, Warrington Road, Birchwood Park, Warrington, WA3 6AE, UK
*Corresponding author e-mail: Jon.lloyd@manchester.ac.uk

Key words: Isosaccharinic acid, biodegradation, nuclear waste disposal, Clostridia, Geobacter

Status: Submitted to Applied Environmental Microbiology (Date: 16/06/2017).
5.1. Abstract
Isosaccharinic acid (ISA), formed through alkaline hydrolysis of cellulose in intermediate level nuclear waste ILW, is a strong complexant of metals and has the potential to mobilize priority radionuclides in an underground geological disposal facility. In this study, microbial ISA degradation was studied under anaerobic conditions with Fe(III) oxyhydroxide as the terminal electron acceptor at pH 7 to 10, representative of conditions in the geosphere surrounding a geological disposal facility. A multidisciplinary approach was used including mineralogical analyses (XRD, ESEM and TEM), alongside geochemical profiling of ISA and its biodegradation products, and microbial community analysis by 16S rRNA gene pyrosequencing. Under the conditions imposed, ISA degradation was constrained to a pH limit of ≤9 and was degraded during Fe(III) reduction and fermentation. For the first time, biominerals resulting from ISA degradation were analysed, showing two Fe(II) minerals, siderite [FeCO₃] and vivianite [(Fe₃PO₄)₂·8H₂O], not normally observed in natural environments, possibly as a result of chelation of ISA with Fe(III) that made the electron acceptor more soluble. A complex microbial consortium dominated by Clostridia and a close relative of the known Fe(III)-reducing bacterium Rhodoferax ferrireducens was associated with these activities. The results are discussed in the context of GDF safety case development, including the potential impact on priority radionuclides such as U(VI), Np(V), and Tc(VII), which are highly susceptible to reductive immobilization when in contact with Fe(II)-bearing biominerals.

5.2. Importance
The deep geological disposal of nuclear waste with lower radioactive content, involves the co-disposal of other materials, such as cellulose. For the construction of such a disposal facility, cement will be used, which will mix with incoming groundwater forming an alkaline solution that is chemically aggressive. This alkaline solution will degrade the cellulose chemically to isosaccharinic acid (ISA). ISA is a soluble complex-forming degradation product that can transport radioactive compounds into the surrounding geosphere and thus there exists interest in studying its fate. Since ISA is an organic carbon, the study of microbial metabolism can have
implications on the removal of ISA, and can thus control its fate under a range of GDF-relevant geochemical conditions. The results presented in this study, demonstrate the importance of microbial processes in safety case development for geodisposal of nuclear waste.

5.3. Introduction
In the UK, and many other nuclear economies worldwide, it is Government policy to dispose of long-lived higher activity radioactive waste into an engineered deep underground geological disposal facility hosted within a suitable geological formation (GDF) (DECC, 2014). Nuclear waste materials are classified by their activity, with Intermediate Level Waste (ILW) and High Level Waste (HLW) destined for deep geological disposal. The dominant waste form by mass in the UK is ILW, of which the majority will be immobilized in a cementitious grout placed within stainless steel containers, that are placed into excavated vaults and in turn are surrounded by cementitious backfill (RWM, 2010c). After closure of the GDF, resaturation with groundwater will create a high pH, reducing environment intended to limit radionuclide mobility from the ILW through the surrounding geosphere. The resulting wasteform will be very heterogeneous and include a high concentration of cellulosic items, such as paper, filters and cotton, leading to potential generation of organic breakdown products via abiotic alkaline reactions. One of the most stable degradation products expected is isosaccharinic acid (ISA) (Whistler and BeMiller, 1958; van Loon and Glaus, 1998a; Glaus et al., 1999a; Knill and Kennedy, 2003), a polyhydroxy carboxylic acid that is highly soluble in water (Bradbury and Sarott, 1995; van Loon et al., 1997). Under alkaline pH conditions, ISA is expected to form strong bidentate complexes with a range of metals and radionuclides (Bradbury and Sarott, 1995; Bontchev and Moore, 2004; Gaona et al., 2008), including Ni(II) (Warwick et al., 2003), Am(III) (Tits et al., 2005), Eu(III) (Vercammen et al., 2001; Tits et al., 2005), Np(IV) (Rai et al., 2003; Gaona et al., 2008), Th(IV) (Vercammen et al., 2001; Tits et al., 2005), U(VI)/U(IV) (Baston et al., 1994b; Rao et al., 2004; Warwick et al., 2004). These radionuclide-ISA complexes show decreased sorption to cement, and thus ISA may potentially enhance radionuclide transport and facilitate migration from the highly alkaline, engineered barrier system to the surrounding geosphere, raising concern about the fate of radionuclide-ISA complexes. There is however the potential for microbial attenuation that can potentially use ISA as microbial growth substrate when the pH values become sufficiently low for microbes to survive (Kuipers et al., 2015; Rout et al., 2015b).
Recent studies have already demonstrated the potential for anaerobic biodegradation of ISA under high pH conditions, representative of the chemically disturbed zone of a GDF (Bassil et al., 2015b; Charles et al., 2015; Rout et al., 2015a), with fermentation and the reduction of nitrate or iron sustaining ISA biodegradation. It should be noted, that in cementitious ILW systems at pH 10 and above, sulfate reduction using a range of electron donors (Rizoulis et al., 2012), including ISA (Bassil et al., 2015b; Charles et al., 2015), is minimal over the extended time periods (several months) tested thus far. However, under neutral pH conditions representative of the “far-field” geosphere surrounding a GDF, complete ISA degradation has been observed under aerobic, \( \text{NO}_3^- \), Fe(III) and \( \text{SO}_4^{2-} \) reducing conditions (Rout et al., 2014, 2015c; Kuipers et al., 2015). In particular, insoluble Fe(III) oxides and oxyhydroxides will be present as electron acceptors in and around a GDF that occur naturally in the geosphere or are produced \textit{in situ} from the corrosion of steel present in ILW and engineering materials used in GDF construction (Lovley and Phillips, 1986a; Konhauser, 1997; Duro et al., 2014). This complex mix of organic growth substrates and Fe(III) oxides has the potential to support the development of anaerobic microbial communities, which will in turn can exert control on the biogeochemistry of the GDF. The extent of Fe(III) reduction is of particular interest, as it can influence the pathways of organic matter decomposition (Lovley and Phillips, 1988), while reduced Fe(II) phases can provide new biomineral surfaces that are capable of reducing several priority radionuclides including Tc(VII) (Lloyd et al., 2000b; McBeth et al., 2011; Thorpe et al., 2014) or Np(V) (Law et al., 2010) to insoluble tetravalent forms. Dissimilatory Fe(III)-reducing bacteria can also enzymatically reduce soluble U(VI) to insoluble U(IV) (Lovley et al., 1991a). Finally, Fe(III) reduction could also help utilize hydrogen gas generated in the GDF, in competition with methanogenesis (Lovley and Phillips, 1986a, 1987), and could therefore impact on gas production and management in a GDF.

Consistent with results from recent ISA biodegradation studies (Bassil et al., 2015b; Charles et al., 2015), there exists potential that microbial metabolism within the highly alkaline “near-field” GDF may not sufficiently degrade ISA produced in the ILW. Therefore we explore here pH-boundaries for ISA degradation, more representative of the surrounding “far field” subsurface geosphere. Anaerobic enrichment cultures, able to couple the oxidation of ISA to the reduction of Fe(III) oxyhydroxides, were obtained using sediment inocula, that were collected from a
legacy lime workings site near Buxton (Rizoulis et al., 2012; Williamson et al., 2013). This site has areas of elevated pH and high calcium concentrations, consistent with those of an ILW radioactive waste disposal facility (pH ~12.5, Ca$^{2+}$ ~20 mM; NDA, 2010), and is known to contain microorganisms able to degrade ISA (Bassil et al., 2015b; Kuippers et al., 2015; Rout et al., 2015c). Enrichment cultures were set up across a pH range of 7 to 10 to explore pH boundaries for ISA metabolism. In the second part of this study, a circumneutral pH ISA-degrading, Fe(III)-reducing culture, representative of geosphere groundwater conditions, was transferred seven times into fresh medium to enrich for a stable microbial community for further experimentation. From this experiment key microorganisms, potentially important in ISA degradation, were identified using 16S rRNA pyrosequencing, while changes in geochemistry and Fe mineralogy were characterized, to help identify the potential fate of iron and other metals and radionuclides in these systems.

5.4. Materials and methods

Materials

Shallow subsurface sediment samples (pH of 6.8) were acquired from a marginal area at a location contaminated by legacy lime workings at Harpur Hill, Buxton in Derbyshire, UK from a depth of approximately 20 cm. The samples were stored in the dark at 4°C until use. Ca(ISA)$_2$ was prepared from α-lactose monohydrate and Ca(OH)$_2$ following the protocol of Vercammen et al. (1999a). Ferrihydrite was prepared by hydrolysis of 0.6 M Fe(III) chloride in 18Ω de-ionized water (DIW). The pH was continually adjusted to 7 by addition of 10 M NaOH solution whilst stirring. The resultant precipitate was washed 6 times by centrifugation at 5000 g for 20 min, and re-suspended in DIW. X-ray diffraction analysis of the material confirmed it to be amorphous 2-line ferrihydrite (Eggleton and Fitzpatrick, 1988; Drits et al., 1993).

ISA biodegradation experiments

**Microbial cultures pH 7-10:** Fe(III)-reducing enrichment cultures were prepared using an inoculum (1% vol/vol) of sediment from Harpur Hill, Buxton, which was added to 30 mL minimal medium containing 30 mM NaHCO$_3$, 4.7 mM NH$_4$Cl, 4.4 mM NaH$_2$PO$_4$·H$_2$O, 1.3 mM KCl, and 10 mL L$^{-1}$ of mineral and vitamin stock solutions (Lovley et al., 1984). Ca(ISA)$_2$ was added to a final concentration of 4 mM as the sole carbon source and electron donor and 25 mmol L$^{-1}$ of
Fe(III) oxyhydroxide was added as the sole TEA. Triplicate incubations were set up at pH 7, 8, 9 or 10 (adjusted by adding respective amounts of concentrated NaOH), in 100 mL serum bottles, sealed with butyl stoppers. Prior to inoculation, the bottles were autoclaved and de-aerated with a N₂/CO₂ (80:20) gas mixture to provide anaerobic conditions and if necessary the pH was re-adjusted by adding NaOH. Sterile controls (heat-treated) were also prepared as appropriate. All enrichment cultures were incubated in the dark at 20°C with periodic sampling. After measuring the redox potential (Eh; Denver Instrument Accumet digital meter with a Mettler Toledo Inlab Redox Micro ORP) and pH (Mettler Toledo FEP20 digital meter with a Fisherbrand FB68801 electrode), the samples were frozen at -20°C until further analysis. Geochemical analyses and X-ray diffraction measurements were carried out on these cultures.

**Stable enrichment culture:** In addition to the primary enrichment cultures described above, a stable enrichment culture was maintained over 7 subcultures (with a 1% vol/vol inoculum) at pH 7 and then used to inoculate the following; (i) a test culture, comprising 2 mM Ca(ISA)₂ as the sole carbon source and 25 mmoles L⁻¹ Fe(III) oxyhydroxide, alongside controls of the same set up but where (ii) the electron donor was omitted (no ISA), or (iii) the inoculum was sterilized (autoclaved). Parallel, a stable enrichment fermenting culture was maintained as in (i) over 7 consecutive transfers, but Fe(III) as an alternative electron acceptor was omitted; analyses for this control included IC and 16S rRNA sequencing measurements.

**Geochemical characterization**

Microbial Fe(III) reduction was monitored using the ferrozine spectrophotometric assay (Stookey, 1970). Biogenic Fe(II) was determined by digestion in 0.5 N HCl for 1 h and total bioavailable Fe was determined by digestion in 0.5 N HCl with 0.25 N hydroxylamine-HCl overnight, followed by the ferrozine assay (Lovley and Phillips, 1986b).

Organic acids and ISA were analyzed using ion exchange high performance liquid chromatography (IE-HPLC). A Dionex ICS5000 Dual Channel system, fitted with a Dionex AS-AP auto sampler was used, equipped with a Dionex Capillary (50 x 0.4 mm) AG11-HC 4 µm guard column and Dionex Capillary (250 x 0.4 mm) AS11-HC 4 µm analytical column, a Dionex ACES300 Chemical Suppressor for background suppression, and a CD20 conductivity detector. The eluent comprised a gradient of concentrated KOH, which was continually mixed with high purity water at a flow rate of 0.015 mL/min and a backpressure of 3,200 PSI. For analysis,
frozen aliquots were defrosted, centrifuged and the supernatant was diluted at a ratio of 1/50 sample/deionized water. Samples were injected onto a run which started at 1 mM KOH for 10 min, increased to 38 mM after 25 min and then re-equilibrated to 1 mM KOH, until the 40 min run-time was completed.

**Microbial community analysis**

Changes of the microbial community composition as a result of the applied incubation conditions were studied using 16S rRNA gene sequencing. This analysis was performed with the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a Roche ‘Fast Start High Fidelity PCR System’ (Roche Diagnostics Ltd, Burgess Hill, UK). Primers used were 515F (5′-GTG YCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′), targeting the V4 hyper variable regions for 2×150-bp paired-end sequencing (Caporaso et al., 2011, 2012). PCR reactions were performed with 50 µL volume. The cycle started with denaturing at 95°C for 2 min, followed by 36 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension step of 5 min at 72°C. PCR products were cleaned up and normalized to ~20 ng each using the SequalPrep Normalisation Kit (Fisher Scientific, Loughborough, UK). PCR amplicons were pooled in equimolar ratios, using a 4 pM sample library spiked with 4 pM PhiX, to a final concentration of 10% (Kozich et al., 2013). Sequence analysis was performed using an Agilent BioAnalyzer at the Centre for Musculoskeletal Research, Manchester (Kozich et al., 2013). Raw sequences were divided into samples by barcodes (up to one mismatch was permitted) using a sequencing pipeline. Quality control and trimming was performed using Cutadapt (Martin, 2011), FastQC (Andrews, 2010), and Sickle (Joshi and Fass, 2011). MiSeq error correction was performed using SPADes (Nurk et al., 2013). Forward and reverse reads were assembled to full-length sequences with PANDAseq (Masella et al., 2012). With Chimera Slayer the sequences were screened for chimeras and these were removed subsequently (Haas et al., 2011). OTU’s were generated using UPARSE (Edgar, 2013) and classified by USEARCH at the 97% similarity level (Edgar, 2010). In this step, singletons were removed. The taxonomic assignment was performed by RDP classifier (Wang et al., 2007) and α-rarefaction curves were generated in Qiime (Caporaso et al., 2010).
Mineralogical characterization

Powder X-ray diffraction (XRD) analysis was used for mineral phase identification. Sample aliquots were centrifuged anaerobically (14,000 g, 10 min) and coated onto a glass slide. The slides were analyzed in an anaerobic holder fitted with an internal knife-edge using a Bruker D8 Advance instrument, fitted with a Gobel Mirror and a Lynxeye detector. Cu Kα X-rays were used in a scan from 5-70 degrees with step size 0.02 degrees and count time of 0.5 sec per step. Eva v14 software was used to match patterns to standards from the ICD (International Centre for Diffraction Data) database. Relative proportions of minerals detected were estimated using Topas v4.2 software. Transmission Electron Microscopy (TEM) was used to image Fe(II)-bearing minerals and associated microbial cells. Sample aliquots were spun down and the wet pellet was layered onto a TEM copper grid with a holey carbon film coating and dried anaerobically. Grids were examined on a Philips CM200 field emission gun TEM (FEGTEM) fitted with an Oxford Instruments X-Max 80 mm² SDD EDS system running Aztec software, and a Gatan Orius SC200 CCD camera running GMS 2 software. Environmental Scanning Electron Microscopy (ESEM) was also used to image the samples in backscattered electron (BSE) and secondary electron (SE) modes. Prior to observation, sample aliquots were washed in 30 mM bicarbonate buffer, placed onto an aluminum pin stub (Zeiss, Ø12.7 diameter top) and dried anaerobically. Carbon coating was applied to enhance the sample conductivity. Imaging was performed using a FEI XL30 ESEM-Field Emission Gun (ESEM-FEG) operating at 15 kV in high vacuum mode (10-5 to 10-6 mbar) and an EDAX Gemini EDS system was used for elemental analysis. Microbial cells and associated minerals were also imaged regularly using light microscopy. Here, the cells in sample aliquots were stained using a Hoechst 33342 nucleic acid stain and spread on a glass slide. Samples were viewed immediately using a Zeiss Axioscope Microscope fitted with a 100x ACHROPLAN lens (No: 440081), using conventional light, and then fluorescent light with a filter Fs 02 (ex/em 365/420).

5.5. Results

Fe(III)-reducing primary enrichment cultures; pH 7 to 10

Sediments from an ISA-containing lime-kiln site (Williamson et al., 2013; Bassil et al., 2015b; Rout et al., 2015c) were used to inoculate a minimal growth medium amended with ISA as the sole electron donor and Fe(III) oxyhydroxide as the terminal electron acceptor (TEA) at pH
values between 7 and 10. Ion chromatographic analysis of ISA in solution (Figure 5.1 A) showed complete removal at pH 7 over 14 days of incubation followed by acetate production (peaking at 2.5 mM day 28) and the reduction of around 90% of the Fe(III) present after 80 days (Figure 5.2 B and C). ISA decreased at slower rates at pH 8 and 9, but was fully depleted after 77 days with a higher yield of acetate produced (Figure 5.3 A & B), peaking at 5.5 mM (pH 8) and 8.8 mM (pH 9), and lower overall yields of Fe(II) (Figure 5.4 C). In addition, butyrate was detected in some of the samples at pH 7 and 8, but was removed after ISA was depleted (Figure 5.9). In contrast, there was no significant metabolism of ISA, production of volatile fatty acids (VFAs) or reduction of Fe(III) in the pH 10 experiment as well as in the sterile controls, confirming that ISA degradation and Fe(III) reduction were microbially-mediated in the pH range 7 to 9. The onset of Fe(III) reduction in these experiments occurred after ISA degradation was

![Graphs showing ISA consumption, acetate production, and Fe(II) production over time at different pH values.](image)

Figure 5.5. Primary sediment enrichments for ISA-degrading, Fe(III)-reducing bacteria at pH values between 7 to 10. A) ISA consumption, B) acetate production and C) Fe(II) production. Symbols are: sterile control (autoclaved cells) dashed line (●); and active microbial cultures with solid line at (●) pH 7 (△) pH 8; (●) pH 9; and (●) pH 10.
initiated and continued after ISA had been depleted, indicating that Fe(III) reduction was not fully dependent on the oxidation of ISA, but coupled to the metabolism of ISA degradation products. Microbial metabolism in these cultures resulted in an increase of about 0.5 pH units and a decrease in $E_h$ to between -230 and -350 mV, consistent with the development of more reducing conditions.

XRD crystallography identified changes in the Fe mineralogy in the cultures (Figure 5.10). Calcite, quartz, albite, and anhydrite were identified at all pH values and in the sterile control, confirming that they were present in the sediment inoculum and not produced biologically. In the microbially active incubations (pH 7 to 9), two other sharp peaks were detected at $2\Theta = 13.2^\circ$ and at $2\Theta = 31.9^\circ$ at the end of the experiment, and identified as siderite [FeCO$_3$] and vivianite [Fe$_3$(PO$_4$)$_2$·8H$_2$O], respectively. Since these two peaks were absent under sterile and high pH (10) conditions where no Fe(III) reduction was observed, it was clear that microbial Fe(III) reduction led to the production of soluble Fe(II) which reacted with carbonate and phosphate in the medium to form these two biominerals. In the siderite diffraction pattern a shift of approximately 0.2º to a lower $2\Theta$ angle was noted, indicating partial substitution of Fe$^{2+}$ by a smaller cation, most likely Ca$^{2+}$, which was abundant, resulting in a mixed phase of (Ca,Fe)CO$_3$ (Wajon et al., 1985; Patterson et al., 1994). The replacement was estimated to be around 7.5% ±0.01. A quantitative analysis in the bioreduced samples using Topas v4.2 software showed a dominance of siderite at low pH compared to vivianite, while the ratio of vivianite increased at higher pH values and was the sole Fe(II) biomineral detected at pH 9 (Figure 5.11).

**Stable enrichment culture**

**Geochemical characterization**

A stable enrichment culture that was able to degrade ISA under Fe(III)-reducing conditions at pH 7, was maintained over seven consecutive transfers (Figure 5.2) and compared to a parallel incubation with no-added electron acceptor (CO$_2$ only) (Figure 5.9). For the stable enrichment culture the pH was chosen to be at 7, as it is the most representative condition for neutral groundwaters surrounding the GDF. Following ISA degradation, fermentation products, dominated by acetate and butyrate, accumulated (Figure 5.2A), as observed in the initial sediment incubations described above. During these processes, the pH also increased significantly in the microbially active culture, compared to the sterile control and the no electron
donor control. The redox potential also dropped from +120 mV and levelled off at around -270 mV when ISA had been depleted (Figure 5.2D).

Approximately 30% of the carbon from ISA was recovered as VFAs in these experiments. The remaining 70% was most likely metabolized to other fermentation products such as alcohols not detected by IC and also to CO₂. Consistent with a previous study where methane production was minimal after the addition of Fe(III) oxyhydroxide as a competing electron acceptor for CO₂, and posed a sink for reducing equivalents generated during ISA oxidation (Kuipers et al.,

![Graphs](image)

**Figure 5.6.** Geochemical results obtained from the stable enrichment for ISA-degrading, Fe(III)-reducing bacteria at pH 7. Results are shown for an enrichment set up with ISA, Fe(III) and an active inoculum: A) Ion concentrations [mM] for (■) ISA, (◆) acetate, (▲) n-butyrate, (▲) propionate and (Φ) ISA in sterile control added as comparison; B) Fe(II) ingrowth [mmoles L⁻¹]; C) pH and D) Eₐ [mV] with symbols in B)-D) (■) test condition; (Φ) sterile control; (▲) no ISA.
methanogenesis was seen as a minor metabolic pathway. Interestingly after ISA depletion at 35 days, Fe(II) production continued until approximately 85% of the total iron was reduced after 42 days incubation. This suggests the use of an intermediate degradation product as an electron donor, such as acetate or butyrate. Comparing these results to an ISA fermentation experiment without an added TEA (Figure 5.12) confirmed the accumulation of fermentation products, which were not removed from solution over a period of 113 days. This suggests that Fe(III) played a role in oxidation of these intermediates produced during ISA degradation.

**Mineralogical characterization**

After microbial activity ceased, the color of the mineral phases in the enrichment cultures changed from a brick-red (characteristic of ferrihydrite) to a grey color. Precipitates were subjected to mineralogical analyses, including XRD, ESEM and TEM, before and after incubation (Figure 5.3). The precipitate at the start showed a sharp edge in the TEM SAED pattern (insert Figure 5.3E) with d-spacings of 1.52 and 2.55 nm, commonly observed for 2-line ferrihydrite (Eggleton and Fitzpatrick, 1988; Drits et al., 1993; Janney et al., 2000; Guo and Barnard, 2013). XRD identified the end point materials to comprise primarily of siderite and vivianite (Figure 5.14), as noted in the sediment microcosms above. Quantification of the end point mineralogy gave a relative ratio of 64% siderite to 36% vivianite, +/-1.2%, which was slightly more enriched for vivianite than in the sediment incubation. This ratio was probably controlled by phosphate availability, which was depleted to below the limit of detection by the end of the incubation, and the pH. The formation of Fe(II) phosphate minerals has been observed previously in sediments with very high biological activity and high phosphate concentrations (Konhauser, 1997; Bae and Lee, 2013; Azam and Finneran, 2014). Ca$^{2+}$ incorporation into the siderite crystal structure was also indicated by a change in the 2θ value, replacing approximately 8% of the Fe$^{2+}$, in line with the data obtained in the sediment incubations.

TEM and ESEM imaging were used to confirm the identity of the biominerals detected by XRD. Poorly crystalline 2-line ferrihydrite (Figure 5.3A; Figure 5.15) changed to minerals of higher crystallinity after incubation (Figure 5.3B, C, D). Agglomerations of crystals with high peaks of C, O and Fe in the TEM EDS profile Figure 5.3B, F) and lattice d-spacings of (Å) 3.40 (012), 2.73
(104), 1.91 (202) and 1.43 (214), were consistent with siderite (Bartelmehs and Downs, 1998a; Kim et al., 2004). However, small peaks of Ca and P and other unidentified faint rings from SAED patterns were noted in the same region of the sample, suggesting the presence of vivianite. In ESEM images, siderite appeared as globular aggregates that consisted of individual hexagonal tabular mineral plates varying between 5-90 µm in size (crystal “L” in Figure 5.3K). The partial substitution of Fe$^{2+}$ with Ca$^{2+}$ suggested from XRD analysis was also consistent with Ca peaks in the EDS profiles (Figure 5.3F, L). Crystals of radial-fibrous morphology were also seen using TEM, with dominant EDS peaks of Ca, P, O and Fe (Figure 5.3C, G) and a high degree of crystallinity (SAED insert Figure 5.3C), with visible lattice fringes ($d$-spacings of 6.63 Å, 2.68 Å, 2.19 Å and 1.67 Å) that were consistent with vivianite (Sameshima et al., 1985; Bartelmehs and Downs, 1998b; Fuller et al., 2014). In ESEM images the vivianite crystals exhibited hexagonal prismatic crystals with angular edges of approximately 20-40 µm width and

Figure 5.7. TEM (A-J) and ESEM (K) images with corresponding EDS analyses (E, F, G, H, L, M) after incubation. Sterile control shows unaltered ferrihydrite (A, E). In the microbiologically active enrichments, the mineral phases included crystal aggregates rich in C, O, Ca, Fe with $d$-spacings matching siderite (F, I), radial ordered crystals rich in Fe-Ca-P (C, G) with $d$-spacings matching vivianite and an amorphous Fe-S phase (D, H), and bacterially associated iron (arrows in J pointing at cells). ESEM imaging (K) revealed two crystalline structures in an amorphous matrix: (L) comprising Fe, Ca, O and a spherulitic shape identified as siderite and (M) comprising Fe, P, O and a prismatic shape identified as vivianite. X-axis in EDS images is in keV.
40-150 µm length, aggregated as radial rosettes (crystal “M” in Figure 5.3K) with high peaks of P, Fe and O by EDS.

TEM images (e.g. Figure 5.3D, H) showed needle-shaped aggregates of lower crystallinity that appeared to be comprised of mainly Fe and S in EDS analysis, although sulfide minerals were not detected with XRD. It should be noted, that small quantities of sulfate (0.13 mM) were present in the medium, and this was removed by day 42, possibly via sulfate reduction (Figure 5.13). The production of minor levels of iron sulfide was supported by SAED analysis, where faint rings indicated a poorly crystalline structure (SAED insert Figure 5.3D), with a dominant ring at a d-spacing 5.48 nm consistent with disordered mackinawite (FeS) in the 001 orientation (Wolthers et al., 2003). Biogenic iron-sulfides are often of poorly crystalline structure (Berner, 1964; Cornwell and Morse, 1987) and are well documented in previous TEM studies (e.g. Ohfuji and Rickard, 2006; Veeramani et al., 2013). Poorly crystalline mackinawite is regarded as metastable precursor to formation of more crystalline pyrite (Wolthers et al., 2003).

Rod-shaped microbial cells of length 1.0-2.5 µm and a diameter of approximately 0.4 µm were clearly visible in the Fe(III)-reducing cultures using light and fluorescence microscopy, in tandem with TEM and ESEM imaging (Figure 5.16). They were closely attached to bioreduced iron minerals, indicating their involvement in Fe(III) reduction (Figure 5.3J).

**Microbial community analysis**

16S rRNA gene profiling was used to quantify microbial community changes from the initial soil inoculum to the 7th subculture of the stable ISA-degrading/Fe(III)-reducing enrichment and to the 7th culture of an ISA-fermenting enrichment, at 35 days when ISA was fully depleted. Rarefaction curves (Figure 5.17) confirmed a dramatic decrease in microbial diversity, from the raw sediment sample, comprising approximately 600 distinct phylotypes (Kuippers et al., 2015), to approximately 90 discrete gene sequences in the enrichment cultures with Fe(III) and under fermenting conditions after incubation with ISA.
Both enrichment samples were dominated by sequences affiliated with Gram-positive Clostridia with 30.4% of genes detected in the Fe(III)-reducing culture and 38.6% in the fermenting culture (Figure 5.4). Species identified within this Class included close relatives to Ruminicoccaceae (16.2% in the Fe(III)-reducing experiment and 35.5% in the fermenting control). Another important phylogenetic Class in the Fe(III)-reducing experiment comprised sequences most closely affiliated with Deltaproteobacteria (16.6% of sequences), of which most sequences belonged to species of Geobacter (10.6% abundance and >98% sequence similarity) and Desulfovibrio (5.2% abundance and >97% sequence similarity). Next to the well-known Fe(III)-reducing Geobacter spp. (Lovley and Phillips, 1986a; b) and sulfate-reducing Desulfovibrio spp. (e.g. Devereux et al., 1990), another known Fe(III)-reducing organism most closely related to Rhodoferax ferrireducens (5.2% of sequences and 98% sequence similarity) was detected. This species was shown to couple the oxidation of acetate to Fe(III) reduction (Finneran et al., 2003). This was in contrast to the ISA fermenting sample, where sequences most closely related to the Class Betaproteobacteria comprised 21.7% of the total sequences, followed by sequences belonging to Sphingobacteriia (14.5%).

Figure 5.8. Molecular community (16S rRNA gene) analysis of stable enrichment cultures at pH 7, showing a sterile control; the ISA degrading, Fe(III)-reducing experiment after ISA degradation at 35 days and an ISA-fermenting control experiment after ISA depletion at 35 days.
5.6. Discussion

In this study, we have explored the biogeochemical fate of ISA in soils enrichment cultures provided with Fe(III) oxides. ISA degradation was explored at a pH gradient from 7 to 10, representative for the wider geosphere adjacent to the alkali-disturbed zone surrounding an ILW GDF. The rate and extent of ISA degradation was significantly increased at pH 7 to 9 in this study, when compared to data from a previous study conducted at pH 10 (Bassil et al., 2015b). Furthermore, fermentation was more pronounced at higher pH values, whereas the rate of Fe(III) reduction decreased. This is in keeping with the higher energy yields associated with fermentative processes, compared to Fe(III) reduction (Flynn et al., 2014), which would be expected to utilize short chain volatile fatty acid fermentation products as electron donors, but become energetically unfavorable at high pH (Rizoulis et al., 2012).

In this study we focused on the metabolism of poorly ordered Fe(III) oxyhydroxides, which although highly bioavailable to Fe(III)-reducing bacteria (Lentini et al., 2012), are frequently reduced to a mixed valence iron oxide magnetite [Fe$_3$O$_4$] in laboratory incubations using similar growth media (e.g. Coker et al., 2008; Hansel et al., 2003; e.g. Lovley and Phillips, 1987). However, multiple factors have been shown to impact on the rate and end point of Fe(III) reduction in laboratory cultures, including not only the crystallinity of the iron oxide (Roden and Zachara, 1996; Liu et al., 1997; Fredrickson et al., 1998; Cutting et al., 2009), but also the pH and $E_h$ (e.g. Bell et al., 1987), electron donor supplied (Lentini et al., 2012) and the presence of chelating ligands (Dobbin et al., 1995, 1996; Islam et al., 2005; McBeth et al., 2011). In this study, Fe(III) reduction progressed further than the mixed valence Fe(II)/Fe(III) oxide magnetite, resulting in the formation of fully reduced Fe(II) carbonate and phosphate phases. This is most likely due to the presence of a cocktail of potential chelating agents, including ISA and organic acid fermentation products, which may have increased the bioavailability of the amorphous ferric substrate to Fe(III)-reducing bacteria, and circumvented the progression to magnetite via a goethite intermediate noted in other studies (Hansel et al., 2003; Coker et al., 2008). This led instead to the formation of siderite and vivianite biominerals, after coordination of the fully reduced Fe(II) with carbonate and phosphate present in the growth medium. It should also be noted that the high amount of Ca$^{2+}$ complexed to ISA was mineralized and substituted Fe in the structure of siderite.
In the stable enrichment culture at pH 7, known model Fe(III)-reducing species, such as *Geobacter* and *Rhodoferax* spp., were detected within the microbial community (at 4% and 10% relative abundance by 16S rRNA gene sequencing), which were completely absent in ISA fermenting control samples. However both enrichment incubations (with and without Fe(III)), were clearly dominated by Gram-positive *Clostridia* of which species were most closely affiliated with the *Ruminococcaceae*. *Ruminococcaceae* possess extracellular enzymes, which allow them to degrade complex organic material, such as cellulose (Pettipher and Latham, 1979; Flint *et al*., 1991). They have a fermentative metabolism, but are potentially able to reduce Fe(III) and thus could play a role in Fe(II) formation (Dobbin *et al*., 1999; Park *et al*., 2001; Scala *et al*., 2006). The selection for several Fe(III)-reducing organisms may be due to the inclusion of ISA as a strong chelating agent which could change the solubility and hence bioavailability of Fe(III), and therefore the mechanism of reduction. Even though the sulfate concentration in these enrichment microcosms was low (0.13 mM), sulfate reduction was noted and resulted in a modest enrichment of sequences most closely affiliated with *Desulfovibrio* spp. Sulfate-reducing bacteria are able to enzymatically reduce Fe(III) and any sulfide produced by these organisms is also able to mediate the formation of Fe(II) sulfide phases (Lovley *et al*., 1993a) such as the observed mackinawite.

The geochemical profile together with the microbial fingerprints of our experiments, indicated that initial ISA degradation occurred mainly via fermentation, with Fe(III) reduction being a minor electron sink. This is consistent with a higher energy yield from fermentation of organic molecules per electron transferred compared to oxidation using Fe(III) as TEA (Lovley *et al*., 1989):

\[ \Delta G' \text{ (Standard in situ)} \]

**Carbohydrate oxidation coupled to Fe(III) reduction:**

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 24 \text{Fe(OH)}_{3(s)} + 42 \text{H}^+ \rightarrow 24 \text{Fe}^{2+} + 6 \text{HCO}_3^- + 60 \text{H}_2\text{O} \quad -12 \quad -30
\]

**Carbohydrate fermentation:**

\[
\text{C}_6\text{H}_{12}\text{O}_6 + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{CH}_3\text{CH}_2\text{COO}^- + \text{HCO}_3^- + \text{H}_2 + 3 \text{H}^+ \quad -71 \quad -93
\]

As such, it is likely that fermentative pathways underpinned initial ISA degradation, whilst the onset of microbially-mediated Fe(III) reduction was probably coupled to the oxidation of ISA.
fermentation products, which would also explain why Fe(III) reduction continued under removal of acetate and butyrate after ISA had been depleted. When Fe(III) oxide was omitted as electron acceptor, ISA was still fermented to acetate and butyrate, but these volatile fatty acids accumulated in solution. Thus, the addition of Fe(III) oxide to ISA-degrading cultures resulted in a decrease in the levels of residual fermentation products, oxidizing organic degradation products further to CO₂ as commonly observed in anoxic model systems (Lehours et al., 2010).

In conclusion, this work has important implications for the transport of radionuclides complexed to ISA into the wider geosphere surrounding a GDF under mildly alkaline conditions and where Fe(III) oxides are present. Fe(III) reduction was shown to support the complete oxidation of ISA to CO₂. Additional benefits arising from Fe(III) reduction, are the immobilization of a range of a range of redox active priority radionuclides that are susceptible to reductive immobilization via Fe(II) biominerals, including siderite and vivianite e.g. mediating the reduction of soluble Tc(VII) (Lloyd et al., 2000b; McBeth et al., 2011; Thorpe et al., 2014) or Np(V) (Law et al., 2010) to insoluble tetravalent forms. Vivianite has also been shown to reduce soluble U(VI) to immobile U(IV) (Veeramani et al., 2011). Follow up studies should focus on biominerals from ISA degradation under more realistic subsurface in situ conditions, using a range of different Fe(III) minerals likely to be present in the surrounding geosphere of a GDF. Finally, modest levels of sulfate reduction were noted in our experiments, and the coupling of ISA biodegradation to sulfate reduction in the deep subsurface geosphere surrounding a GDF clearly warrants further attention. The formation of insoluble sulfide phases in high sulfate groundwaters that dominate in much of the UK (Metcalfe et al., 2007), and elsewhere at depth, could also have a controlling impact on the fate of a radionuclide-ISA complex during ISA degradation.

Overall this work adds further weight to the concept of a microbial buffer zone that may develop around an ILW GDF, minimizing the release of radionuclides into the surrounding geosphere and thus acting as a “bio-barrier” zone (Lloyd, 2015).

5.7. Acknowledgements
The authors thank the Radioactive Waste Management (RWM) and the NERC BIGRAD consortium for financial support. We would like to thank Michael Ward of the University of Leeds LENNF facilities for TEM support. Further thank goes to Alastair Bewsher and Paul Lythgoe at the University of Manchester for analytical support. JRL also acknowledges the financial support
of the Royal Society and the EU MIND programme under “Euratom2014–2015” and the call “NFRP-06-2014: Supporting the implementation of the first of a kind geological repositories”.

5.8. Associated Content

Appendix

The appendix contains XRD data and fitting results, solution chemistry of ISA fermentation experiments, additional ESEM and light microscopy images and α-rarefaction curves.

5.9. Author information

Corresponding Author: *jon.lloyd@manchester.ac.uk.

Notes

The authors declare no competing financial interest.

5.10. References


Wajon, J.E., Ho, G.-E. and Murphy, P.J. (1985) Rate of precipitation of ferrous iron and formation of mixed iron-calcium carbonates by naturally occurring carbonate materials. Water Research, 19, 831–837.


5.11. Supplementary information

Title: Microbial reduction of Fe(III) coupled to the biodegradation of isosaccharinic acid (ISA)

Author list: Gina Kuipers, Christopher Boothman, Heath Bagshaw, Rebecca Beard, Nicholas Bryan, Jonathan R. Lloyd

Pages: 143-146

Figure 5.9. Butyrate concentration in primary sediment enrichments for ISA-degrading, Fe(III)-reducing bacteria at pH values between 7 to 10. Symbols are: sterile control (autoclaved cells) dashed line (●); and active microbial cultures with solid line at (●) pH 7 (▲) pH 8; (●) pH 9; and (●) pH 10. Note that high error bars are as a result that butyrate was not detected in all triplicate bottles.
Figure 5.10. XRD patterns showing iron mineral biotransformations at pH 7-10. Graphs from top to bottom: Sterile control, pH 7, pH 8, pH 9 and pH 10.

Figure 5.11. Relative percentages of vivianite and siderite in the sediment incubations from pH 7 to 10, containing active microbial inocula. Sediment-derived minerals (quartz, anhydrite and albite) are not shown as they were expected to remain the same.
Figure 5.12. Solution chemistry of a stable (7 consecutive transfers) ISA-fermenting enrichment control sample at pH 7 without [no Fe(III)], grey dashed line is ISA concentration of sterile control.

![ISA Fermentation](image)

Figure 5.13. Sulfate concentrations in a stable ISA-degrading enrichment culture at pH 7, supplemented with 25 mM Fe(III) (red line). Grey dotted line shows the sterile control.

![Sulfate](image)

Figure 5.14. XRD pattern of a precipitate collected from the seventh subculture of an ISA-degrading, Fe(III)-reducing culture at pH 7. Peaks are matched to those of vivianite (red) and siderite (blue) OCDD standards.

![XRD Pattern](image)
Figure 5.15. ESEM image of amorphous Fe(III) oxyhydroxide before the addition of an Fe(III)-
reducing inoculum.

Figure 5.16. Light microscopic images of the stable enrichment culture of ISA-degrading, Fe(III)-
reducing bacteria at pH 7 after 35 days. Cells were stained using Hoechst stain (DNA shown in blue or cyan). Images are showing: A) partially reduced fine grained Fe(III) oxyhydroxide; B) mainly hexagonal siderite crystals with vivianite crystal in top right corner; C) radial large vivianite crystal; D) two siderite crystals.

Figure 5.17. α-Rarefaction curves showing the species diversity from stable enrichment cultures
at pH 7. Blue line: sterile sample; red line: ISA degrading, Fe(III)-reducing experiment;
yellow line: ISA fermenting control experiment.
[This page is left intentionally blank]
6. The biogeochemical fate of nickel during microbial ISA degradation; implications for nuclear waste disposal (research article)

Gina Kuippers¹, Christopher Boothman¹, Heath Bagshaw¹, Michael Ward², Rebecca Beard³#, Nicholas Bryan⁴, Jonathan R. Lloyd¹*

¹Research Centre for Radwaste Disposal & Williamson Research Centre for Molecular Environmental Science, School of Earth, Atmospheric and Environmental Sciences, University of Manchester, Oxford Road, Manchester M13 9PL, UK
²Leeds Electron Microscopy and Spectroscopy Centre, School of Chemical and Process Engineering, University of Leeds, Leeds LS2 9JT, UK
³Radioactive Waste Management Limited, Building 587, Curie Avenue, Harwell Oxford, Didcot, Oxfordshire OX11 0RH, UK
⁴National Nuclear Laboratory Limited, Chadwick House, Warrington Road, Birchwood Park, Warrington, WA3 6AE, UK

*Corresponding author e-mail: Jon.lloyd@manchester.ac.uk

Key words: Nickel, immobilization, isosaccharinic acid, biodegradation, Fe(III)-reducing microorganisms, mackinawite, nuclear waste management

Status: Manuscript submitted to Scientific Reports (28/08/2017).
6.1. Abstract

Intermediate level radioactive waste (ILW) generally contains a heterogeneous range of organic and inorganic materials, encapsulated in cement. Of particular concern are cellulosic waste items, which will chemically degrade under the conditions predicted during waste disposal, forming significant quantities of isosaccharinic acid (ISA), a strongly chelating ligand. ISA therefore has the potential to increase the mobility of a wide range of radionuclides, including Ni-63 and Ni-59, which are known to form stable Ni-ISA complexes. Although ISA is known to be metabolized by anaerobic microorganisms, the biodegradation of metal-ISA complexes remains unexplored. This study investigates the fate of a Ni-ISA complex in Fe(III)-reducing enrichment cultures, representative of a microbial community in the subsurface. After initial sorption of Ni onto Fe(III) oxyhydroxides, microbial ISA biodegradation resulted in >90% removal of the remaining Ni from solution when present at 0.1 mM, whereas higher concentrations of Ni proved toxic. The microbial consortium was dominated by close relatives to Clostridia and Geobacter species associated with ISA degradation. Nickel was preferentially immobilized with trace amounts of biogenic amorphous Fe-sulfides. This study highlights the potential for microbial activity to help remove chelating agents and radionuclides from groundwaters in the subsurface geosphere surrounding a geodisposal facility.
6.2. Introduction

The policy of the UK Government (and those of other nuclear nations) is to dispose of long-lived intermediate level waste (ILW) via engineered deep underground geological disposal facilities (GDFs) (DECC, 2014). Post-closure, a cementitious GDF will become saturated with groundwater, which, through interaction with the cementitious materials that make up the engineered barrier components, will result in the development of a highly alkaline plume. These high pH conditions are expected to minimize the mobility of toxic or hazardous radionuclides and inhibit most microbiological processes. However, organic ligands have the potential to impact on the solubility of radionuclides in these wastes. Ligands can be either present in ILW, for example the decontamination agents ethylenediaminetetraacetic acid (EDTA) or nitrilotriacetic acid (NTA), or they may form under GDF conditions from the chemical degradation of organic materials. Of particular interest are cellulosic materials, which are present at high loadings and have been shown to undergo alkaline hydrolysis at elevated pH and calcium concentrations, resulting in the production of small organic acids in the pore fluids (NDA and DECC, 2014). Under GDF conditions, the main stable end product is expected to be isosaccharinic acid (ISA) (Whistler and BeMiller, 1958; van Loon and Glaus, 1998b; Glaus et al., 1999a; Knill and Kennedy, 2003; Pavasars et al., 2003; Glaus and van Loon, 2008). Even though ISA is known to sorb to cement (Bradbury and Sarott, 1995; van Loon et al., 1997), it can form strong water-soluble complexes with some priority radionuclides, especially divalent cations, such as nickel (Ni) (Bradbury and Sarott, 1995; Baker et al., 2003). Ni-based steel alloys typically comprise approximately 95% Fe and 5% Ni, and are chiefly valuable to minimize corrosion of steel (Platt et al., 1997). Under repository conditions, Ni-63 (half-life 9.9 x 10^1 years) and Ni-59 (half-life 7.6 x 10^4 years) are activation products formed by irradiation of stable Ni in nuclear reactor steel components (NDA, 2010f, 2014), and their fate needs to be considered as part of the safety assessment of a GDF. Upon groundwater resaturation of the GDF, steel corrosion will release Ni, which should be poorly soluble in the alkaline porewaters (pH 11.5 to 13.5) and sorb readily onto cement (Bradbury and Sarott, 1995; Scheidegger et al., 2000). However, the formation of aqueous complexes between ISA and Ni (at a 2:1 stoichiometry) (Warwick et al., 2003) may significantly enhance the transport of Ni in the groundwater. Thus, there is concern regarding the fate of Ni, alongside other priority radionuclides that may complex with ISA, in and around a cementitious GDF.
Microbially-mediated processes have been shown to control the fate of ISA, which can be used as an organic carbon source and electron donor for microbial metabolism, under a range of biogeochemical conditions, including nitrate and Fe(III) reduction at high pH (Bassil et al., 2015a; b; Charles et al., 2015; Rout et al., 2015a) and neutral pH (Kuipers et al., 2015; Rout et al., 2015c). Furthermore, the biodegradation of radionuclide-ISA complexes, such as Ni-ISA, could lead to sorption of the radionuclide, or its incorporation into new biominerals. Under GDF conditions, the presence of Fe(III) oxyhydroxides is expected to be significant from both, the corrosion of steel alloys, and naturally occurring minerals in the wider circumneutral pH geosphere (or “far field”). These Fe(III)-bearing minerals may therefore play a significant role in sustaining ISA metabolizing anaerobes in and around a GDF. However, bioavailable Fe(III) minerals such as Fe(III) oxyhydroxide not only serve as electron acceptors, but are also effective scavengers for metals and radionuclides, which could release Ni upon reduction to Fe(II) (Howell et al., 1998; Waychunas et al., 2005). The microbial reduction of Fe(III) minerals in the subsurface and associated secondary mineral transformations could therefore have a profound influence on the fate of Ni and other priority radionuclides in the geosphere. In this study, we have used a microbial inoculum obtained from an alkaline, Ca$^{2+}$-rich lime kiln site, which is an analogue for a cementitious GDF and known to contain ISA-degrading microorganisms (Bassil et al., 2015b; Kuipers et al., 2015). This inoculum was used to investigate the fate of Ni during ISA biodegradation under Fe(III)-reducing conditions relevant to the wider geosphere of a GDF, including identifying the role of key Fe(II)-bearing biominerals in Ni immobilization.
6.3. Results

Nickel solubility

In this study the fate of non-radioactive Ni was explored in ISA-degrading Fe(III)-reducing microbial cultures. Prior to the study, abiotic factors in the system that could control the solubility of Ni were considered, e.g. the presence of Fe(III) oxyhydroxide that exhibits high adsorptive capacities for metal ions (Filip et al., 2011). The solubility of Ni (at 0.1 mM Ni and 1 mM Ni) was tested in freshwater minimal medium (FWM) at neutral pH, containing (i) medium only, (ii) medium plus ISA, (iii) medium plus Fe(III) or (iv) medium plus ISA and Fe(III) (Figure 6.5). The biosorption of Ni onto cells was not specifically tested, as it is considered to be low (Holan and Volesky, 1994; Tsezos et al., 1995). In the presence of the medium only, approximately 90-91% Ni remained in solution, whilst in the presence of ISA the solubility of Ni was increased to approximately 100%, regardless of the initial Ni concentration. In the medium with Fe(III) oxyhydroxide, the solubility of Ni dropped to around 64% at 0.1 mM Ni and to 15% at 1 mM Ni, but was increased by approximately 10% in the medium containing Fe(III) and ISA at 1 mM Ni, but remained unchanged at 0.1 mM Ni.

These initial tests showed that Ni had a great solubility in the medium which was enhanced by ISA, and therefore has the potential to increase radionuclide mobility in groundwater systems. However, the presence of competing Fe(III) oxyhydroxides decreased the solubility of the Ni.

Development of an Fe(III)-reducing Ni-ISA degrading enrichment culture

Biogeochemistry. An anaerobic enrichment culture representative of Fe(III)-reducing bacteria present in the geosphere surrounding a GDF was developed using ISA complexed with Ni as the electron donor and Fe(III) oxyhydroxide as the electron acceptor. Microbial inocula were obtained from a lime workings site, with high calcium concentrations and pH values relevant to cementitious nuclear waste disposal (Bassil et al., 2015b), and were enriched to stable cultures with ISA as the sole carbon source at circumneutral pH. Medium inoculated with microbial enrichments without Ni and at 0.1 mM Ni concentration, there was an increase in pH from 7.0 to 7.3 while the $E_h$ fell to around -250 mV after approximately 14 days (Figure 6.1A, B). This coincided with a drop in ISA levels, which was no longer detected after approximately 28 days (Figure 6.1C), while volatile fatty acids (VFAs) comprising acetate and butyrate accumulated,
peaking at 28 days (Figure 6.1E, F). Thereafter, acetate remained stable in solution during an extended incubation time (62 days), whilst butyrate concentrations decreased after ISA was depleted. Fe(III) reduction, monitored using the ferrozine assay by Fe(II) ingrowth (Figure 6.1D), was also initiated during the period of ISA biodegradation, and continued after ISA was depleted until day 42, at which it plateaued at approximately 26 mmoles L\(^{-1}\) Fe(II). It should be noted that the medium also contained comparatively low levels of sulfate (0.13 mM), which decreased in the presence of 0.1 mM Ni and in its absence, alongside ISA removal, until it was fully depleted at day 42 (Figure 6.6). In contrast in the enrichment cultures supplemented with 1 mM Ni, pH, \(E_{h}\), and ISA concentrations all remained stable and Fe(III) and sulfate were not reduced. Abiotic influences, two control experiments were set up, containing ISA and Fe(III) oxyhydroxide, either
with an autoclaved inoculum or without the inoculum added. ISA concentration remained stable in these controls (Figure 6.1B), while the pH remained unchanged, the $E_h$ showed only a minor drop over the incubation time (Figure 6.1A, D) and Fe(II) levels remained constant, all consistent with abiotic influences playing a negligible role in these experiments (Figure 6.1E).

**Fate of nickel.** The concentrations of Ni in the supernatants of these experiments was also monitored by ICP-AES, to help identify the fate of the metal during ISA biodegradation and Fe(III) reduction (Figure 6.2). At the start of incubation, soluble Ni concentrations were 64% (64 µM) of the 0.1 mM Ni added, and 12% (115 µM) of the 1 mM Ni added to the microbially-active cultures. These results were in agreement with data from the initial Ni solubility tests and were most likely a result of sorption to the surface of the Fe(III) oxyhydroxides that were added.

![Figure 6.2. ICP-AES analysis of Ni in solution [mM] in the Ni-ISA biostimulation, Fe(III)-reducing experiment. All bottles contained 4 mM ISA and 25 mM Fe(III) oxyhydroxide plus (●) 1 mM Ni only; (♦) a sterile (autoclaved) microbial inoculum and 1 mM Ni; a microbially active inoculum with (■) 0.1 mM Ni or (▲) 1 mM Ni.

Over the course of the experiment, the soluble Ni concentration remained stable in the control incubations containing an autoclaved inoculum or no added inoculum, confirming that sorption or abiotic precipitation reactions were negligible. Similarly, no decrease in soluble Ni concentrations was observed in the microbially active enrichment culture containing 1 mM Ni, where ISA degradation was absent. In contrast, in the microbially active 0.1 mM Ni incubation, the soluble Ni concentration decreased from day 14 from 64 µM (presumably after sorption to Fe(III)) to 8.5 µM (9%) at the end of the incubation (Figure 6.2) and remained at this value throughout an extended incubation time of 200 days (data not shown). Thus, following Ni
sorption onto Fe(III) oxyhydroxides (36% removal), the oxidation of ISA and reduction of Fe(III) led to the almost complete removal (90%) of the Ni added.

**Molecular Ecology.** A stable microbial community was enriched from lime kiln soil samples over seven consecutive transfers in ISA/Fe(III)-containing medium, with the last transfer used in the Ni-ISA biostimulation experiments discussed above. DNA was extracted from these final cultures at selected time points and the 16S rRNA genes present were amplified and sequenced, to identify changes in microbial community composition that may have controlled the fate of the Fe and Ni. The chosen profiles were from the 0 mM Ni and 0.1 mM Ni experiments at 0 hours, at 28 days, the point at which ISA was depleted, and at 63 days, the point where Fe(III) reduction levelled off (Figure 6.3). α-rarefaction curves showed a dramatic decrease in microbial diversity from the original soil inoculum (approximately 600 distinct sequences detected; Kuipers et al., 2015) to the seventh transfer of the enrichments (approximately 190 distinct sequences detected at the start of this experiment; Figure 6.7). A further modest decrease in microbial diversity was noted over the duration of this experiment, whereby the 0 mM Ni and 0.1 mM Ni supplemented enrichments contained approximately 160 distinct sequences. The decrease in community microbial diversity was confirmed by calculating the Shannon H indices (values are shown above the columns in Figure 6.3).

![Figure 6.3](image-url)

Figure 6.3. 16S rRNA gene sequencing data from the Fe(III)-reducing Ni-ISA degrading enrichment, sowing phylogenetic classes at selected time points. Shannon H biodiversity index on top of columns is a measure of microbial community diversity.
Analysis of 16S rRNA gene sequences obtained from the 0.1 mM Ni experiment after ISA depletion (28 days) showed enrichment in organisms most closely affiliated with the Gram-positive class Clostridia, representing approximately 56% of the total abundance of bacteria detected. This class comprised mainly of members of the family Ruminococcaceae (53% of genes detected), known to possess a multi-enzyme cellulosome complex that could play a central role in carbohydrate metabolism (Berg Miller et al., 2009). Another important class detected was the Gram-negative Deltaproteobacteria comprising approximately 18% of sequences detected. This class was dominated by sequences affiliated most closely (97% to 99% match) with the Fe(III)-reducing family Geobacteraceae (16% of sequences detected). Despite representing less than 2% of the sequences, detection of members of the Desulfovibrionaceae was considered important, as these organisms were affiliated with the sulfate-reducing bacteria Desulfovibrio putealis B7-43 (1% of sequences, 98% match) and Desulfovibrio L7 sp.(0.65% of sequences, 100% match). A second 16S rRNA gene profile was obtained from the 0.1 mM Ni experiment when Fe(III) reduction had finished (day 63). Here there was a higher percentage of sequences affiliated with Betaproteobacteria (26% of sequences), of which most of the sequences (25%) were related to Dechlorosoma suillum PS (100% match) from the genus Azospira, an anaerobic, perchlorate-reducing organism (Byrne-Bailey and Coates, 2012). The second most abundant class detected was again most closely affiliated with Clostridia (20% of sequences) followed by the Bacteroidia (17% of sequences), of which most sequences were affiliated with an uncultured bacterium from the vadinBC27 wastewater-sludge group (11% of sequences), from the genus Rikenellaceae, which are described as anaerobic, mesophilic carbohydrate-fermenting organisms (Su et al., 2014).

**Biomineralogy.** To help define the fate of Ni in the ISA-degrading, Fe(III)-reducing cultures, the mineralogical end-products in these experiments were assessed using X-ray Diffraction (XRD), Transmission Electron Microscopy (TEM) and Environmental Scanning Electron Microscopy (ESEM).

At the start of the Ni-ISA biostimulation experiment, a brick-red precipitate was observed, which remained unchanged in the sterile and no electron donor controls, and also the incubations with 1 mM Ni added. This mineral phase was converted to a dark grey precipitate where ISA metabolism was observed (microbially active experiment with 0 mM Ni and 0.1 mM Ni). TEM
analysis of the red precipitate in the sterile control showed the presence of fine grained material, while selected area electron diffraction (SAED) revealed two broad rings at about 1.49 Å and 2.5 Å, all consistent with 2-line ferrihydrite that is of irregular, poorly ordered nanoparticulate dimensions (2-4 nm) (Eggleton and Fitzpatrick, 1988; Drits et al., 1993; Janney et al., 2000; Guo and Barnard, 2013). The XRD patterns (Figure 6.8) obtained for the bioreduced samples in the absence of Ni and with 0.1 mM Ni added, revealed two sharp peaks at 2Θ = 13.2° and at 2Θ = 31.9°, corresponding to siderite (FeCO₃) and vivianite (Fe₃(PO₄)₂·8H₂O) (Figure 6.8). The relative abundance of vivianite:siderite was very similar at both Ni concentrations with 40%-60% at 0 mM Ni and 35%-65% at 0.1 mM Ni, respectively. Fe(II) in the siderite structure was partially replaced by Ca(II), as previously noted (Kuipers et al., submitted).

![Image](image_url)

Figure 6.4. ESEM (A, B) and TEM (C) images of biominerals from the Fe(III)-reducing Ni-ISA degrading enrichment with 0.1 mM Ni with representative EDS profiles. A and B: Biominerals identified were (1) siderite, (2) vivianite and (3) an amorphous phase, comprising S, Fe, and Ni. C: (4) a crystalline area without Ni (4) and (5, 6) weakly crystalline areas with Fe, S and Ni.
ESEM imaging of the post-reduction precipitates (with 0.1 mM Ni; Figure 6.4A and B), confirmed the identity of (1) siderite and (2) vivianite crystals, which did not show Ni in the corresponding EDS profile. Precipitates of less crystalline morphology were also noted, comprising relatively high amounts of Fe, S and Ni, (EDS 3 in Figure 6.4B); peaks of P, Ca and O were probably background signal from the underlying vivianite crystal (EDS 1 and 2 in Figure 6.4A). Similarly, in TEM images, distinct crystals of angular shape that contained Ca, Fe, P and O (60-70 nm length; 12-16 nm diameter; area 4 in Figure 6.4C) had matching lattice d spacings for siderite in the corresponding diffraction pattern: 2.72 (104), 2.17 (113), 1.47 (122), 1.41 (214) and 1.27 Å (0210) (Bartelmehs and Downs, 1998a; Kim et al., 2004), but did not contain Ni. In contrast, a weakly crystalline area (area 5 in Figure 6.4C), deduced from very faint rings in the diffraction pattern with d spacings at 5.18 Å, 2.59 Å, 1.78 Å and 1.68 Å in the diffraction pattern, had high peaks of Fe, S, and Ni, consistent with literature values for nanocrystalline mackinawite (Evans et al., 1964; Lennie et al., 1995; Wolthers et al., 2003; Ohfuji and Rickard, 2006; Gramp et al., 2010a). Another area was amorphous, with no rings in the diffraction pattern, but consisted of mainly Fe, S and Ni (area 6 in Figure 6.4C). Finally, TEM mapping suggested close association of Ni and S areas, whilst P, Ca and O were accumulated in separate locations, and Fe was detected throughout the mapped area (Figure 6.9). Additional PHREEQC modelling was carried out, to estimate the Ni solubility. Results from the modelling suggested that Ni was undersaturated in solution under anoxic conditions and before reduction (Figure 6.10) while after bioreduction precipitation of millerite and mackinawite was predicted (Figure 6.11).

6.4. Discussion

Ni is an important constituent in steel alloys used for nuclear reactor components. Irradiation of stable Ni leads to the formation of Ni-63 and Ni-59 activation products, hence there is great interest in understanding the fate of Ni during the (bio)geochemical evolution of a GDF and the surrounding geological environment.

Previous studies on metal-microbe interactions have focused on a range of microorganisms, including anaerobic metal- and sulfate-reducing bacteria, for their ability to immobilize radionuclides via mechanisms including bioreduction, bioaccumulation, biosorption or biomineralisation (Macaskie, 1991; Francis et al., 1994, 1996; White et al., 1998; Lloyd and Macaskie, 2000, 2002; Lloyd and Renshaw, 2005; Field et al., 2010). To this date, studies on Ni
removal relevant to nuclear disposal have looked at Ni intercalation into biominerals, such as hydrogen uranyl-phosphates (Basnakova and Macaskie, 1996, 1997; Bonthrone et al., 1996), and the biodegradation of organic ligands with subsequent Ni precipitation, such as Ni-citrate complexes (Francis et al., 1992, 1996; Joshi-Tope and Francis, 1995). However, no studies to date have addressed the fate of Ni-ISA-complexes, which could potentially form under repository conditions, and have the potential to influence the performance of a GDF (Warwick et al., 2003; Almond et al., 2016).

Here we studied the fate of a Ni-ISA complex in circumneutral enrichment cultures containing Fe(III) as the main terminal electron acceptor, approximating conditions in the “far field” surrounding a GDF. It should be noted, however, that to facilitate mineralogical investigations that underpin this study, significantly higher concentrations of soluble Ni were used, than those expected in a GDF environment for ILW, which will range around $3.8 \times 10^{-7}$ M within $10^6$ years of GDF evolution (Bradbury and Sarott, 1995). In these incubations, ISA biodegradation coincided with Fe(III) reduction, and led to a significant decrease in the concentration of Ni when supplied at 0.1 mM. At 1 mM Ni, no ISA removal occurred, highlighting the strongly inhibitory impact of higher Ni concentrations on microorganisms. No ISA removal or Fe(III) reduction were observed in the control incubations (sterile control or no electron donor), indicating a governing role of microbial activity responsible for ISA removal, biogenic Fe(II) formation and Ni removal. In the microbially active experiment at 0.1 mM Ni, fermentation played a significant role as approximately 60% of the carbon from ISA degradation was recovered as acetate and butyrate.

Other end-products identified included the crystalline Fe(II) biominerals, siderite (Fe(II)-carbonate) and vivianite (Fe(II)-phosphate). Although ferrihydrite is considered highly bioavailable to Fe(III)-reducing bacteria (Lentini et al., 2012), the production of fully reduced Fe(II) minerals from this substrate, rather than a mixed Fe(II)/Fe(III) valence iron oxide, such as magnetite, is not commonly observed in laboratory studies (e.g. Lovley and Phillips, 1988; Lovley et al., 1994), but has been previously noted when ISA was supplied as an electron donor (Kuipers et al., 2015; Kuippers et al., in press). Chelating ligands are known to enhance microbial Fe(III) reduction rates (Dobbin et al., 1995, 1996; Islam et al., 2005; McBeth et al., 2011), and since ISA has been shown to complex with Fe(III) minerals (Glaus and van Loon, 2008), ISA may have increased the solubility and hence bioavailability of ferrihydrite to microbial
Fe(III) reduction in our experiments. We conclude therefore, that chelation of ISA with Fe(III) will result in the production of Fe(II) biominerals not normally observed in natural environments.

The negative standard reduction potential of the Ni(II)/Ni(0) couple \((E_0 = -0.26\, \text{V})\) makes Ni highly resistant to microbially-mediated reduction under pH-neutral conditions (Tsezos et al., 1995). Therefore, capture of Ni via interaction with biomineralogical products is a far more feasible endpoint under anoxic ISA-degrading conditions. In these Fe(III)-reducing experiments, the endpoint minerals included Fe(II) carbonate phase siderite and Fe(II)-phosphate phase vivianite. Siderite is known to play a role in trace metal immobilization because cations of similar size to Fe(II), such as Co(II), Cr(III), Ni(II), can be readily incorporated into the crystal structure in place of ferrous iron (Fredrickson et al., 2001; Roh et al., 2003). In these experiments only Ca(II) was incorporated into the siderite structure upon metabolism of Ca(ISA)\(_2\), whereas Ni(II) was not found to be associated with the biomineral, nor was Ni associated with vivianite. However it should be noted that Fe(II)-bearing minerals, including siderite and vivianite, are known to mediate the reduction of soluble Tc(VII) (Lloyd et al., 2000b; McBeth et al., 2011; Thorpe et al., 2014) or Np(V) (Law et al., 2010) to insoluble tetravalent forms. Additionally vivianite has also been shown to reduce soluble U(VI) to immobile U(IV) (Veeramani et al., 2011). Thus siderite and vivianite may decrease the mobility of this and other priority radionuclides in the wider geosphere.

Regarding the fate of Ni, EDS profiles from TEM and ESEM images showed that Ni accumulated together with Fe and S in amorphous precipitates, consistent with mackinawite (FeS), although this mineral could not be detected with XRD. Mackinawite is a common metastable iron-sulfide mineral in anoxic sediments and a possible precursor to greigite and pyrite formation (Takeno et al., 1970; Schoonen and Barnes, 1991; Morse and Arakaki, 1993; Lennie et al., 1995). Furthermore it is known to substitute Ni for Fe, into the sulfide mineral structure (Evans et al., 1964; Clark, 1970; Gramp et al., 2007; Kwon et al., 2015). The identification of Ni-containing iron-sulfide phases in the ISA biostimulated experiment, accompanied by an enrichment of Desulfovibrio sp., is consistent with the production of sulfide by sulfate-reducing bacteria that can act as a precursor to precipitate Ni. This is in agreement with PHREEQC modelling that suggested formation of mackinawite (FeS) and millerite (NiS) in these experiments. The PHREEQC database does not include data for mixed phases, such as
Fe\({\text{1-}}\text{x}\),Ni,S, and so it predicts the formation of these separate phases. However, since pure Ni-sulfides, such as heazelwoodite (Ni\(_3\)S\(_2\)) or millerite (NiS), are generally only observed in systems that are low in Fe (Gramp et al., 2007), and (Fe,Ni)S has a greater stability over pure FeS (Clark, 1970; Takeno et al., 1970; Kwon et al., 2015), a mixed Fe\({\text{1-}}\text{x}\),Ni,S phase is most likely, in agreement with TEM and ESEM imaging, following the equation:

\[
(1-x)\text{Fe}^{2+\text{aq}} + x\text{Ni}^{2+\text{aq}} + H_2\text{S}_{\text{aq}} \rightarrow \text{Fe}_{1-x}\text{Ni}_x\text{S}_{(s)} + 2\text{H}^{+\text{aq}}
\]

The immobilization of Ni precipitated as a mixed (Fe,Ni)S phase is thus a result of combined microbial activity by Fe(III)- and sulfate-reducing bacteria.

16S rRNA gene profiling of the enrichment identified key soil microorganisms that may have been involved in the degradation of ISA and thus supported Ni immobilization. The 16S rRNA gene profiles of the 0.1 mM Ni and without Ni experiments were very similar, being dominated by fermenting organisms associated with members from the family Clostridia during ISA degradation (day 28), whilst Betaproteobacteria increased in later stages of the incubation, when Fe(III) reduction became more important (day 63). Although close relatives to well-known Fe(III)-reducing bacteria such as Geobacter species were detected in these cultures, we cannot discount the involvement of other more abundant organisms in mediating this process. Finally, although Ni is an essential trace element for several enzymes (Nielsen and Ollerich, 1974; Babich and Stotzky, 1983; Pümpel et al., 2003; Madigan et al., 2015), at higher concentrations it poses a toxic threat to microorganisms (Poulson et al., 1997; Ruggiero et al., 2005). Such an inhibitory effect of Ni was observed at 1 mM concentration, where no ISA biodegradation (or Fe(III) reduction) was recorded.

In summary, it was shown that the solubility of Ni in ISA-degrading Fe(III)-reducing enrichment cultures was influenced by two dominant factors; the mobilization of Ni by complexation with ISA, and the competing immobilization of Ni by sorption to Fe(III) oxyhydroxides. The overall fate of Ni in the system was defined by microbial metabolism, which was driven by biodegradation of ISA. The combined activity of Fe(III)- and sulfate-reducing microorganisms resulted in >90% Ni incorporation into FeS sulfides, presenting a stable sink for the Ni. Given the high abundance of sulfate as electron acceptor in deep subsurface groundwaters in the UK (Metcalf et al., 2007) and elsewhere, the fate of Ni and other radionuclides in the deep geosphere surrounding a GDF, is likely to be governed by sulfidation reactions. These
processes will be potentially mediated by Fe(III)- and sulfate-reducing bacteria in the deep surface that have been stimulated by the release of organics such as ISA, that may migrate in the alkaline groundwater plume from a cementitious GDF. Since ISA oxidation with Fe(III) and sulfate reduction is energetically unfavourable in the alkaline GDF “near field” (Rizoulis et al., 2012), precipitation of Ni and other radionuclides is expected to occur in the geological “far field” of a GDF. It should be noted that also direct abiotic interactions within the geosphere are possible once the strong complexant ISA has been biodegraded, e.g. with clay minerals, known to adsorb cationic Ni due to their negatively charged surfaces, and thus are able to retard the movement of Ni (Babich and Stotzky, 1983; Carvalho et al., 2008; Suresh et al., 2009). Additionally to the trace levels of FeS formed, ISA degradation in our experiments supported the microbial conversion of ferrihydrite to the Fe(II) minerals siderite and vivianite, and these products of dissimilatory Fe(III) reduction are known to form at moderately high pH (Williamson et al., 2013), and abiotically mediate the reduction of soluble Tc(VII) (Lloyd et al., 2000b; McBeth et al., 2011; Thorpe et al., 2014), Np(V) (Law et al., 2010) or U(VI) (Veeramani et al., 2011) to insoluble tetravalent forms. Thus, it is likely that microbial metabolism in the biosphere surrounding a GDF will play a significant role in minimizing the transport of mobile radionuclide-ISA complexes, through the fermentation of ISA at high pH (Bassil et al., 2015a), and further coupling of biodegradation reactions to the reduction of electron acceptors such as Fe(III) and sulfate in and around the GDF, extending to the circumneutral biosphere. Consequently it is likely that a “bio-barrier” will develop, which could play a key role in defining the long-term fate of priority radionuclides, including Ni-63 and Ni-59. Clearly further research is warranted in this area, including studies to define the impact of microbial metabolism on ISA complexed to other radionuclides including Am(III), Th(IV) and U(VI). There exists also the need to focus on more realistic subsurface in situ conditions to better quantify the efficiency of metal and radionuclide immobilisation upon ISA degradation. Although it is clearly a significant challenge to quantify the impact of microbial metabolism in GDF scenarios, over long time periods within highly complex biogeochemical gradients, it is reasonable to expect that such studies would support a reduction in conservatisms in future GDF safety cases.
6.5. Methods

Experimental conditions

Fresh water minimal (FWM) medium preparation. FWM was prepared in deionised water and contained 30 mM NaHCO$_3$, 4.7 mM NH$_4$Cl, 4.4 mM NaH$_2$PO$_4$·H$_2$O, 1.3 mM KCl, and 0.3 mL of mineral and vitamin stock solutions (Lovley et al., 1984).

Ca(ISA)$_2$ preparation. α-lactose monohydrate and Ca(OH)$_2$ was used for preparation of Ca(ISA)$_2$ following the protocol of (Vercammen et al., 1999a).

Fe(III) oxyhydroxides preparation. 0.6 M FeCl$_3$ were hydrolyzed in six washing steps with 18Ω de-ionized water (DIW), whilst the pH was continually adjusted under stirring to 7 by addition of 10 M NaOH solution. With the ferrozine assay the final Fe(III) concentration of the resultant precipitate was determined.

Nickel stock preparation. Stock solutions of 0.1 M and 0.01 M NiCl$_2$·6H$_2$O (n = 237.7 g/mol) were prepared with deionised water and filter-sterilized prior to use (0.22 µm, diam. 33 mm, Millex-GP, Sigma-Aldrich).

Nickel solubility. To help determine the impact of the growth medium on Ni solubility, Ni was added at 0.1 mM Ni or 1 mM Ni to 100 mL serum bottles, containing 30 mL (i) FWM, (ii) FWM plus 20 mmole L$^{-1}$ Fe(III), (iii) FWM plus 4 mM ISA, or (iv) FWM plus 20 mmole L$^{-1}$ Fe(III) plus 4 mM ISA. The solutions were incubated for 7 days and then sampled for analysis by ICP-AES.

Fe(III)-reducing Ni-ISA degrading enrichment. Anaerobic Fe(III)-reducing microbial communities were enriched from shallow subsurface sediments that were collected from a site contaminated by legacy lime workings at Harpur Hill, in Buxton, Derbyshire, UK (Kuipers et al., 2015). Cultures were prepared with a 1% (v/v) inoculum added to 100 mL serum bottles containing 30 mL FWM. ISA (as Ca(ISA)$_2$) was added to 4 mM as sole carbon source and 25 mmole L$^{-2}$ of poorly soluble Fe(III) oxyhydroxide was added as the terminal electron acceptor (TEA) to support anaerobic growth. The bottles were sealed with butyl stoppers and flushed with a N$_2$/CO$_2$ (80:20) gas mixture for 5 min to provide anaerobic conditions and to adjust a pH of 7 and incubated in the dark at 20°C. After Fe(III) reduction had reached a plateau, a 1% vol/vol inoculum was transferred to fresh medium, and the seventh consecutive transfer was used to prepare five tests in triplicate: three biologically active treatments with ISA,
Fe(III) oxyhydroxide, an active inoculum, and (i) 0 mM Ni, (ii) 0.1 mM Ni, or (iii) 1 mM Ni, alongside (iv) a sterile control containing Ca(ISA)$_2$, Fe(III) oxyhydroxide, 1 mM Ni and a sterile (autoclaved) inoculum, and (v) a no-inoculum control as (iv) but without a microbial inoculum. Time-based samples were collected aseptically and frozen immediately at -20°C until further analysis.

**Geochemical analyses**

The pH was monitored throughout the experiment with a calibrated Mettler Toledo FEP20 digital meter equipped with a Fisherbrand FB68801 electrode and the redox potential with a with a Denver Instrument Accumet digital meter equipped with a Mettler Toledo Inlab Redox Micro ORP. Microbial Fe(III) reduction was monitored by the ferrozine spectrophotometric assay quantifying Fe(II) following the protocol from Stookey (1970). Total bioavailable Fe was determined at the beginning and end of the experiment by digesting 20 µL of sample in 980 µL of 0.5 N HCl and 0.25 N hydroxylamine-HCl, and biogenic Fe(II) was determined by digestion of 20 µL of sample in 980 µL of 0.5 N HCl, followed by the ferrozine assay (Lovley and Phillips, 1986b).

**Ion-Exchange Chromatography.** ISA and organic acids were analyzed using ion exchange high performance liquid chromatography (IE-HPLC), using a Dionex ICS5000 Dual Channel on Chromatograph, fitted with a Dionex AS-AP auto sampler that was connected to a CD20 conductivity detector. The columns were a Dionex Capillary (50 x 0.4 mm) AG11-HC 4 µm guard column and Dionex Capillary (250 x 0.4 mm) AS11-HC 4 µm analytical column. A Dionex ACES300 Chemical Suppressor was used for background reduction. The mobile phase was concentrated KOH mixed with high purity water at a flow rate of 0.015 mL/min. Background suppression was hold at 13 mA.

**Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES).** ICP-AES was used to quantify total Ni in solution. Samples were prepared by centrifugation to remove any suspended particles and the supernatants were diluted to below <10 ppm Ni in 2% nitric acid. Samples were analyzed together with standards (from 10 ppm stocks obtained from VWR) on a Perkin-Elmer Optima 5300 DV.

**Microbial analyses**
16S rRNA gene sequencing. 16S rRNA gene sequencing was performed with the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a Roche ‘Fast Start High Fidelity PCR System’ (Roche Diagnostics Ltd, Burgess Hill, UK). The primers used were 515F (5′-GTG YCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′), targeting the V4 hyper variable regions for 2 × 150-bp paired-end sequencing (Caporaso et al., 2011, 2012). PCR was performed in 50 µL reactions, starting with the denaturation at 95°C for 2 min, followed by 36 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension step of 5 min at 72°C. PCR products were cleaned up and normalized to ~20 ng each using the SequalPrep Normalisation Kit (Fisher Scientific, Loughborough, UK). PCR amplicons were pooled in equimolar ratios, using a 4 pM sample library spiked with 4 pM PhiX, to a final concentration of 10% (Kozich et al., 2013). Sequence analysis was performed using an Agilent BioAnalyzer at the Centre for Musculoskeletal Research, Manchester (Kozich et al., 2013). Raw sequences were divided into samples by barcodes (up to one mismatch was permitted) using a sequencing pipeline. Quality control and trimming was performed using Cutadapt (Martin, 2011), FastQC (Andrews, 2010), and Sickle (Joshi and Fass, 2011). MiSeq error correction was performed using SPADes (Nurk et al., 2013). Forward and reverse reads were assembled to full-length sequences with PANDAseq (Masella et al., 2012). Using ChimeraSlayer the sequences were screened for chimeras which were removed subsequently (Haas et al., 2011). OTU’s were generated with UPARSE (Edgar, 2013) and classified by USEARCH (Edgar, 2010) at the 97% similarity level, and singletons were removed. The taxonomic assignment was performed by the RDP classifier (Wang et al., 2007) and α-rarefaction curves were generated in Qiime (Caporaso et al., 2010).

Mineralogical analyses

X-Ray Diffraction (XRD) crystallography. XRD was used for mineral phase identification by centrifugation of sample aliquots and coating wet pellets onto a XRD glass slide which was left to dry anaerobically. Samples were analyzed in an anaerobic holder fitted with an internal knife-edge on a Bruker D8 Advance, fitted with a Gobel Mirror and a Lynxeye detector. Cu Kalpha1 X-rays were used from 5-70 degrees with step size 0.02 degrees and count time of 0.5 sec per step. Eva v14 was used to match crystal patterns to standards from the ICDD (International
Centre for Diffraction Data) database and a semi-quantitative analysis of relative crystallite proportions was achieved with Topas v4.2 software.

Transmission Electron Microscopy (TEM) and Environmental Scanning Electron Microscopy (ESEM). TEM and ESEM were used to characterize biominerals formed during ISA degradation and to trace the fate of Ni in the solid phase. For TEM analysis, samples were centrifuged and the pellet was added to a TEM copper grid with a holey carbon film coating and let to dry anaerobically. Samples were viewed over a range of magnifications (5,000-200,000x) on a Philips FEI Technai T20 (200 kV LaB6) instrument fitted with an Oxford Instruments X-Max 80 mm² SDD EDS system operating Aztec software, and a Gatan Orius SC200 CCD camera operating GMS 2 software. For ESEM imaging sample aliquots were washed in a bicarbonate buffer and dried anaerobically on an aluminium pin stub (Zeiss, Ø12.7 diameter top) before coating with carbon to enhance conductivity. The instrument used was a FEI XL30 ESEM-Field Emission Gun (ESEM-FEG) operating at 15kV in high vacuum mode (10⁻⁵ to 10⁻⁶ mbar) secondary electron (SE) mode fitted with an EDAX Gemini EDS system for elemental analysis.

6.6. Supplemental material
Additional information provides nickel solubility data, sulfate concentration, XRD data, TEM maps and α-rarefaction curves.

6.7. Acknowledgements
This work was supported by Radioactive Waste Management (RWM). We thank the University of Leeds EPSRC Nanoscience and Nanotechnology Facility (LENNF) (EPSRC EP/K023853/1) for access to TEM. Further thank goes to Alastair Bewsher, Paul Lythgoe and John Waters at the University of Manchester for analytical support. JRL also acknowledges the financial support of the Royal Society and the EU MIND programme under “Euratom2014–2015” and the call “NFRP-06-2014: Supporting the implementation of the first of-a-kind geological repositories”.

6.8. Author contributions
GK – primary investigator, planning, preparation and monitoring of experiments, data acquisition, analysis and interpretation, manuscript drafting; CB – sample analysis for microbial ecology (sequencing); HB – training and support on the ESEM instrument; MW – support with
TEM analysis; RB – industrial sponsor and manuscript review; NB – manuscript review; JRL
Planning experiments with GK, data interpretation, review and editing of manuscript.

6.9. Competing financial interests
The authors declare no competing financial interest.

6.10. References


Caporaso, G.J., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer,


complexes by Pseudomonas fluorescens. *Journal of Bacteriology*, **177**.


Kuipers, G., Boothman, C., Bagshaw, H., Rebecca, B., Bryan, N. and Lloyd, J.R. (no date) Microbial reduction of Fe(III) coupled to the biodegradation of isosaccharinic acid (ISA). *Applied and Environmental Microbiology*.


6.11. Supplementary information

**Title:** The biogeochemical fate of nickel during microbial ISA degradation; implications for nuclear waste disposal

**Author list:** Gina Kuipers, Christopher Boothman, Heath Bagshaw, Rebecca Beard, Nicholas Bryan, Jonathan R. Lloyd

**Pages:** 182-188

Figure 6.5. Pre-test for Ni solubility in freshwater minimal medium (FWM). Tests at 0.1 mM and 1 mM concentrations include (i) FWM only; (ii) Fe(III), (iii) ISA, (iv) Fe(III) and ISA.

![Nickel solubility graph](image)

Figure 6.6. Sulfate concentration in Ni-ISA biostimulation, Fe(III)-reducing experiment. Symbols are: ( ) sterile control, (▲) 0 mM Ni, and ( ▼) 0.1 mM Ni.

![Sulfate graph](image)

Figure 6.7. α-rarefaction curves 16S rRNA gene sequencing data from Fe(III)-reducing Ni-ISA degrading enrichments showing maximum of distinct species per sample.

![α-rarefaction curves graph](image)
Figure 6.8. XRD patterns of Fe(III)-reducing Ni-ISA degrading enrichments showing: A) Sterile control, 1.0 mM Ni; microbially active with B) 0 mM Ni, C) 0.1 mM Ni, or D) 1.0 mM Ni; E) Abiotic control without microbial inoculum and with 1.0 mM Ni.
Figure 6.9. TEM mapping of a precipitate at the end of the Fe(III)-reducing Ni-ISA degrading enrichment at 0.1 mM Ni. Detected elements are displayed in maps showing O, Fe, P, Ca, Ni and S.
Figure 6.10. PHREEQC modelling output file showing Ni-ISA biostimulation, Fe(III)-reducing experiment with 0.1 mM Ni at incubation start (before bioreduction). The system is in equilibrium with ferrihydrite. Saturation indices show that vivianite, siderite and apatite will precipitate (positive value), but no Ni-containing form is expected to precipitate.
Figure 6.11. PHREEQC modelling output file showing Ni-ISA biostimulation, Fe(III)-reducing experiment with 0.1 mM Ni when bioreduced (negative pe). The system is in equilibrium with vivianite and millerite. Saturation indices show supersaturation with mackinawite. Thus the simulation indicates millerite and mackinawite will form, which will under natural conditions be a mixed form.

Reading input data for simulation 3.

SOLUTION 1

| Temp | 25 |
| PH  | 7.29 |
| Pe  | -7.93 |
| Redox Pe | |
| Units | mmol/kgw |
| Density | 1 |
| Water | 1 | kg |
| Na  | 24.15 |
| C4  | 29.8 |
| Cl  | 6.04 |
| P   | 4.35 |
| Mg  | 0.079 |
| S   | 0.249 |
| Ca  | 3.95 |
| Fe2 | 0 |
| Fe(2) | 28.44 VIVIANITE 0 |
| N  | 0.1 MILLERITE 0 |

Beginning of initial solution calculations.

Initial solution 1.

<table>
<thead>
<tr>
<th>Elements</th>
<th>Molality</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(4)</td>
<td>2.980e-002</td>
<td>2.980e-002</td>
</tr>
<tr>
<td>Ca</td>
<td>3.950e-003</td>
<td>3.950e-003</td>
</tr>
<tr>
<td>Cl</td>
<td>6.040e-003</td>
<td>6.040e-003</td>
</tr>
<tr>
<td>Fe2</td>
<td>1.417e-005</td>
<td>1.417e-005</td>
</tr>
<tr>
<td>Mg</td>
<td>2.490e-004</td>
<td>2.490e-004</td>
</tr>
<tr>
<td>Na</td>
<td>3.415e-002</td>
<td>3.415e-002</td>
</tr>
<tr>
<td>Ni</td>
<td>2.160e-002</td>
<td>2.160e-002</td>
</tr>
<tr>
<td>NaCl</td>
<td>7.900e-005</td>
<td>7.900e-005</td>
</tr>
<tr>
<td>P</td>
<td>4.350e-003</td>
<td>4.350e-003</td>
</tr>
<tr>
<td>S</td>
<td>2.480e-004</td>
<td>2.480e-004</td>
</tr>
</tbody>
</table>

Equilibrium with Vivianite

Equilibrium with Millerite

---

Description of solution

PH = 7.290
Pe = -7.930
Activity of water = 0.988
Ionic strength = 4.406e-002
Mass of water (kg) = 1.000e+000
Total alkalinity (equ) = 3.518e-002
Total CO2 (mol/kg) = 2.980e-002
Temperature (deg c) = 25.000

Electrical balance (equ) = 1.129e-003
Percent error, 100*(Ca2+(H2)|/Ca2+(H2O)) = 1.44
Iterations = 19
Gamma iterations = 5
Osmotic coefficient = 0.94826
Total H = 1.110456e+002
Total O = 5.881204e+003

---

Saturation indices

<table>
<thead>
<tr>
<th>Phase</th>
<th>SI</th>
<th>log IAP</th>
<th>log KT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anapatite</td>
<td>5.56</td>
<td>10.58</td>
<td>5.02</td>
</tr>
<tr>
<td>Aragonite</td>
<td>0.71</td>
<td>-7.60</td>
<td>-8.31</td>
</tr>
<tr>
<td>Calcite</td>
<td>15.48</td>
<td>89.26</td>
<td>51.88</td>
</tr>
<tr>
<td>Ca(HPO4)2(s)</td>
<td>1.02</td>
<td>1.31</td>
<td>0.30</td>
</tr>
<tr>
<td>Ca3(PO4)2(sulf)</td>
<td>4.03</td>
<td>14.31</td>
<td>10.22</td>
</tr>
<tr>
<td>CaH(PO4)2(sulf)</td>
<td>3.03</td>
<td>15.62</td>
<td>12.30</td>
</tr>
<tr>
<td>Calcite</td>
<td>0.88</td>
<td>-7.60</td>
<td>-8.48</td>
</tr>
<tr>
<td>CH4</td>
<td>26.49</td>
<td>157.80</td>
<td>120.02</td>
</tr>
<tr>
<td>Chlorapatite</td>
<td>12.80</td>
<td>17.71</td>
<td>12.11</td>
</tr>
<tr>
<td>Fe3(PO4)3(s)</td>
<td>410.98</td>
<td>8.66</td>
<td>-402.32</td>
</tr>
<tr>
<td>Mackinawite</td>
<td>0.42</td>
<td>-3.16</td>
<td>-3.80</td>
</tr>
<tr>
<td>Magnesite(not)</td>
<td>0.93</td>
<td>-8.82</td>
<td>-8.51</td>
</tr>
<tr>
<td>Millorite</td>
<td>0.90</td>
<td>-10.33</td>
<td>-10.13</td>
</tr>
<tr>
<td>Pyrite</td>
<td>0.21</td>
<td>-50.87</td>
<td>-58.79</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>2.12</td>
<td>-9.97</td>
<td>-11.18</td>
</tr>
<tr>
<td>Troilite</td>
<td>2.13</td>
<td>-8.18</td>
<td>-8.21</td>
</tr>
<tr>
<td>Vivianite</td>
<td>-0.00</td>
<td>3.12</td>
<td>3.12</td>
</tr>
</tbody>
</table>

---
7. Biomineralization of uranium-phosphates fuelled by the microbial degradation of isosaccharinic acid (ISA) (research article)

Gina Kuipers¹, Kurt Smith¹, Pieter Bots², Katherine Morris¹, Christopher Boothman¹, Samuel Shaw¹, Nicholas Bryan³, Jonathan R. Lloyd¹*

¹Research Centre for Radwaste Disposal & Williamson Research Centre for Molecular Environmental Science, School of Earth, Atmospheric and Environmental Sciences, University of Manchester, Oxford Road, Manchester M13 9PL, UK
²Civil and Environmental Engineering, University of Strathclyde, Glasgow, UK
³National Nuclear Laboratory Limited, Chadwick House, Warrington Road, Birchwood Park, Warrington, WA3 6AE, UK
*Corresponding author e-mail: Jon.lloyd@manchester.ac.uk

Key words: isosaccharinic acid, biodegradation, isosaccharinic acid, biodegradation, ningyoite, uranyl-phosphate, nuclear waste management

Status: Manuscript in preparation for submission to Environmental Science & Technology.
Intermediate level radioactive waste (ILW) generally contains a heterogeneous range of organic and inorganic materials encapsulated in cement. Of particular interest is the chemical degradation of cellulosic waste items that can produce significant quantities of isosaccharinic acid (ISA), a strong chelating compound that can form stable complexes with key radionuclides, such as uranium. Microbial break-down of ISA has been demonstrated recently, however uncertainties remain regarding how this will impact on the fate of U(VI) and U(IV) under geodisposal conditions. Therefore, this study looked at cultures enriched on ISA and under anoxic conditions (with Fe(III) oxide or CO₂ only) to which U(VI) was added. At incubation start, U(VI) formed insoluble uranyl-phosphates, which remained unchanged in ISA fermentation experiments, whilst under Fe(III)-reducing conditions, U(VI) was remobilized and reduced that resulted in precipitation of a U(IV)-phosphate biomineral. 16S rRNA sequencing data indicated the enrichment of mechanism-dependent microbial communities, which were dominated in both experiments by Clostridia species, but additionally contained Deltaproteobacteria under Fe(III)-reducing conditions. This study highlights the importance of microbial metabolism in the surrounding geosphere of a GDF and the immediate influence geochemical parameters can have on biomineral formation, including the fate of uranium. This work emphasizes the potential for a microbially-mediated “bio-barrier” extending into the geosphere surrounding a GDF, capable of attenuating radionuclide transport to the surface biosphere.
7.2. Introduction

UK Government policy (and that of other nuclear nations) is to dispose of long-lived intermediate level waste (ILW) via an engineered deep underground geological disposal facility (GDF). This will use a multi-barrier containment system, intended to isolate and contain the waste for sufficient time to allow the majority of the radioactivity to decay, although for longer lived radionuclides transport away from the barrier system cannot be ruled out over very long timescales (Askarieh et al., 2000; DEFRA and NDA, 2008; Morris et al., 2011; DECC, 2014). In general, the waste will be packaged, grouted into steel containers, and then placed into a geological disposal facility (GDF). Depending on the geological environment in which a GDF is built, the GDF will be closed and backfilled with a material such as cement (Crossland and Vines, 2001; Nirex, 2007a; b). This would provide beneficial chemical conditions for the retardation of most radionuclides by providing a high pH and reducing environment that enhances sorption and promotes low radionuclide solubility (Crossland and Vines, 2001).

In ILW, uranium (U) will typically be the dominant radionuclide by mass (RWM and NDA, 2015). Due to the long half-life of $^{238}\text{U}$ and its daughter isotopes, it is important to understand the behavior of U in the engineered barrier system to support the post closure safety case for a GDF. Redox cycling of U exerts a major control on its mobility, which is mainly governed by its speciation and redox state. Under oxic conditions U(VI) can form stable carbonate complexes which enhances its mobility in the environment (Clark et al., 1995). The formation of stable colloids at elevated pH may also promote migration of uranium in the geosphere (Bots et al., 2014; Smith et al., 2015). In contrast, under anoxic conditions sparingly soluble U(IV) dominates, precipitating as U(IV) oxide phases (Lloyd et al., 2005). The imposed hyperalkaline conditions in a GDF can reduce the mobility of U(VI) by formation of uranyl silicates and uranate phases that sorb to the cement phase (Wellman et al., 2007). The transport behavior of U can, however, be altered by the presence of ligands, such as the decontamination agents ethylenediaminetetraacetic acid (EDTA) or nitriloacetic acid (NTA) (Francis, 1998; Hummel et al., 2005; Suzuki and Suko, 2006). Of particular interest with respect to the disposal of ILW are cellulosic items present in the waste, which exist in large quantities; approximately 2,800 t (or 1%) of ILW (NDA and DECC, 2014). Under alkaline conditions, cellulosic wastes are expected to undergo abiotic alkaline degradation (Machell and Richards, 1957; Greenfield et al., 1992;
Glaus et al., 1999a; Knill and Kennedy, 2003). This will result in the production of a range of small organic degradation products, of which isosaccharinic acid (ISA), a polyhydroxy ligand, is predicted to dominate (Whistler and BeMiller, 1958; van Loon and Glaus, 1998a; Knill and Kennedy, 2003; Pavasars et al., 2003; Glaus and van Loon, 2008). ISA sorbs weakly to surfaces within the cementitious wastes (Bradbury and Sarott, 1995; van Loon et al., 1997) and is known to form stable complexes with radionuclides, such as U(VI) or U(IV), which could in turn significantly increase their mobility (Baston et al., 1994b; Rao et al., 2004; Warwick et al., 2004). Due to the complexing ability of ISA, understanding the geochemical cycling of U-ISA complexes under repository conditions is important in predicting the mobility and fate of this radionuclide in the geosphere.

Previous studies that have focused on the removal of U(VI) from solution used the addition of an electron donor to stimulate microbial U(VI) reduction and the formation of insoluble U(IV) (Lovley et al., 1991a; Newsome et al., 2015a). ISA is a significant organic substrate and may play a pivotal role in stimulating microbially-mediated processes that could control the fate of U(VI) in and around the GDF. Previously, microorganisms were investigated that can use ISA as an electron donor and carbon source for their metabolism under a range of biogeochemical conditions and pH values. Incomplete ISA degradation occurred at high pH (=10) due to the diminishing energy yield when coupled to electron acceptors such as sulfate (Bassil et al., 2015a; b; Rout et al., 2015a). In contrast, ISA was observed to be completely degraded under neutral pH conditions, representative of the geological environment surrounding an ILW GDF (Kuipers et al., 2015; Rout et al., 2015b). However to date, there have been no studies that look at the fate of radionuclides, such as U(VI), when complexed to ISA during microbially-mediated respiratory processes that are expected in and around a GDF.

This work focuses on microbial processes in the anaerobic circumneutral geosphere surrounding a GDF and their effect on the fate of U(VI). Under these conditions oxygen is likely to be depleted and other alternative electron acceptors, such as Fe(III) oxyhydroxides, can be reduced coupled to the oxidation of organics, including ISA. Fe(III) minerals can occur naturally in the geosphere or might be produced in situ from the corrosion of steel present in ILW and engineering materials used in GDF construction (Lovley and Phillips, 1986a; Konhauser, 1997; Duro et al., 2014). In addition, in the absence of Fe(III) as a terminal electron acceptor,
fermentation of ISA may play a significant role for the biogeochemistry of the GDF and surrounding geosphere, unless microorganisms will reduce U(VI) as another alternative electron acceptor, another hypothesis to be tested. This study investigates the fate of U(VI) in pH neutral enrichment cultures with ISA under both Fe(III)-reducing and fermenting conditions to approximate potential conditions in the “far field” surrounding a GDF.

7.3. Materials and methods

Sediment acquisition. The experiments used microbial inocula obtained from an alkaline legacy lime workings site in Buxton, U.K., a model system representative of the far field geosphere of a GDF (Williamson et al., 2013; Bassil et al., 2015b; Kuippers et al., 2015). Shallow subsurface sediment samples (pH of 6.8; 20 cm depth) were collected and kept in the dark at 4°C until use.

Fe(III) oxyhydroxide preparation. For the production of Fe(III) oxyhydroxides 0.6 M FeCl₃ were hydrolyzed in six washing steps with 18Ω de-ionized water (DIW), whilst the pH was continually adjusted under stirring to 7 by addition of 10 M NaOH solution. The ferrozine assay was used to determine the final Fe(III) concentration of the resultant precipitate.

ISA-biodegrading enrichment cultures. Enrichment cultures were grown by adding a sediment inoculum (1% vol/vol) to anoxic minimal medium, containing 30 mM NaHCO₃, 4.7 mM NH₄Cl, 4.4 mM NaH₂PO₄·H₂O, 1.3 mM KCl, and 10 mL L⁻¹ of mineral and vitamin stock solutions (Lovley et al., 1984). Ca(ISA)₂ was prepared from α-lactose monohydrate and Ca(OH)₂ following the protocol of Vercammen et al. (1999a) and was added to 3.5 mM ISA as sole carbon source and electron donor. The bottles were de-aerated for 10 min with an N₂/CO₂ (80:20) gas mixture to support anoxic growth conditions, resulting in a final pH of 7. To stimulate Fe(III)-reducing, ISA-degrading microbial communities, the bottles were supplemented with Fe(III) oxyhydroxide. For ISA-fermenting conditions, the experiment was prepared as above, but Fe(III) oxyhydroxide was omitted. Selection for microorganisms degrading ISA occurred via periodic re-subculturing of an inoculum (1% vol/vol) to fresh medium after metabolic activity leveled off (indicated by stable redox potential). A stable enrichment culture was obtained for experimentation after 12 consecutive transfers.
ISA biodegradation and U(VI) immobilization experiment (with added Fe(III)). The experimental medium was prepared as above. The experiments (30 mL) were purged with a N₂/CO₂ (80:20) gas mixture for 10 minutes, whilst adjusting the pH to 7. Serum bottles were prepared in triplicate as (a) 3.5 mM ISA, 30 mmoles L⁻¹ Fe(III) and 0.1 mM U(VI) and (b) 3.5 mM ISA, 30 mmoles L⁻¹ Fe(III) and 1 mM U(VI). After autoclaving, the bottles were de-aerated for 10 min with an N₂/CO₂ (80:20) gas mixture to ensure anaerobic conditions, and U(VI) was added as a spike of uranyl chloride (UO₂Cl₂) in pH 3 HCl. The bottles were then inoculated with 1% vol/vol of the 12th consecutive transfer of the ISA-degrading, Fe(III)-reducing enrichment culture obtained as described above. Controls were also prepared, containing the same medium with (c) 3.5 mM ISA, 30 mmoles L⁻¹ Fe(III), 1 mM U(VI) and an autoclaved (sterile) inoculum and (d) 3.5 mM ISA, 30 mmoles L⁻¹ Fe(III), 1 mM U(VI) and no inoculum. All serum bottles were incubated in the dark at room temperature with periodic shaking and sampling.

ISA fermentation, U(VI) immobilization experiment. Serum bottles for this experiment were prepared as above, containing (a) 3.5 mM ISA and 0.1 mM U(VI) and (b) 3.5 mM ISA and 1 mM U(VI). The microbial inoculum used was 1% vol/vol of the 12th consecutive transfer of the ISA-fermenting enrichment culture. Controls were also prepared, containing the same medium with (c) 3.5 mM ISA, 1 mM U(VI) and an autoclaved (sterile) inoculum and (d) 3.5 mM ISA, 1 mM U(VI) and no inoculum. All serum bottles were incubated in the dark at room temperature with periodic shaking and sampling.

Geochemical characterization. Samples were withdrawn, using anaerobic and aseptic techniques, and pH, Eₘ, Fe(II) and U(VI) were measured before freezing at -20°C for further analysis. Microbial Fe(III) reduction was measured by adding an aliquot to 0.5 M HCl for analysis of Fe(II) or to 0.25 M hydroxylamine-HCl for analysis of total bioavailable Fe by the ferrozine assay (Lovley and Phillips, 1987). U(VI) in solution was quantified spectrophotometrically with 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol at 578 nm (Johnson and Florence, 1971). ISA and organic acids were analyzed by ion exclusion high performance liquid chromatography (IE-HPLC), using a Dionex ICS5000 Dual Channel system, fitted with a Dionex AS-AP auto sampler that was connected to a CD20 conductivity detector. A Dionex Capillary (50 x 0.4 mm) AG11-HC 4u guard column and a Dionex Capillary (250 x 0.4 mm) AS11-HC 4u analytical column were used for these analyses. The mobile phase was a
concentrated KOH that was mixed with high purity water at a flow rate of 0.015 mL/min and a backpressure of 3,200 PSI. Background suppression was set at 13 mA. Samples (0.4 µL) were injected to a run starting at 1 mM KOH for 10 minutes, which was increased to 38 mM up to minute 25 and re-equilibrated until 40 minutes. For sulfate and phosphate measurements, the instrument was operated with a Dionex Capillary (50 x 2 mm) AG18 guard column and a Dionex Capillary (250 x 2 mm) AS18 analytical column. The mobile phase was used as above at a flow rate of 0.025 mL/min and a backpressure of 2,100 PSI. Background suppression was set at 23 mA. Samples (0.1 µL) were run at 38 mM KOH for a run-time of 10 minutes.

16S rRNA gene sequencing. 16S rRNA gene sequencing was performed with the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a Roche ‘Fast Start High Fidelity PCR System’ (Roche Diagnostics Ltd, Burgess Hill, UK). The primers utilized were 515F (5′-GTG YCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′), targeting the V4 hyper variable regions for 2 × 150-bp paired-end sequencing (Caporaso et al., 2011, 2012). For PCR, 50 µL reactions were denatured at 95°C for 2 min, followed by 36 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension step of 5 min at 72°C. PCR products were cleaned and normalized to ~20 ng mass each using the SequalPrep Normalisation Kit (Fisher Scientific, Loughborough, UK). PCR amplicons were pooled in equimolar ratios, using a 4 pM sample library spiked with 4 pM PhiX to a final concentration of 10% vol/vol (Kozich et al., 2013). Raw sequences were divided into samples by barcodes (up to one mismatch was permitted) using a sequencing pipeline, followed by quality control and trimming using Cutadapt (Martin, 2011), FastQC (Andrews, 2010), and Sickle (Joshi and Fass, 2011). MiSeq error correction was performed using SPADes (Nurk et al., 2013). Forward and reverse reads were assembled to full-length sequences with PANDAseq (Masella et al., 2012). The sequences were screened for chimeras using ChimeraSlayer, and removed (Haas et al., 2011). OTU’s were generated with UPARSE (Edgar, 2013) and classified by USEARCH (Edgar, 2010) at the 97% similarity level, and singletons were removed. The taxonomic assignment was performed by the RDP classifier (Wang et al., 2007) and α-rarefaction curves were generated in Qiime (Caporaso et al., 2010).

Mineralogical characterization. All mineralogical analyses were performed under anaerobic conditions. Mineral phase identification was done with powder X-ray diffraction (XRD)
crystallography on a Bruker D8 Advance, fitted with a Gobel Mirror and a Lynxeye detector. Samples, fitted in an anaerobic holder, were scanned with Cu K alpha1 X-rays in a mode from 5-70 degrees with step size 0.02 degrees and count time of 0.5 sec per step. Crystal patterns were matched to standards from the ICDD (International Centre for Diffraction Data) database with Eva v14 and curve fitting of the matched standards was done with a Rietveld refinement in Topas v4.2 software. Imaging of precipitates was done using both Environmental Scanning Electron Microscopy (ESEM) and Transmission Electron Microscopy (TEM) with selected area electron diffraction (SAED). TEM images were collected on a Philips CM200 equipped with a field emission gun TEM (FEGTEM) fitted with an Oxford Instruments X-Max 80 mm$^2$ SDD EDS. Samples were centrifuged (14,000 g, 10 min) and the wet pellet was layered onto a TEM copper grid with a holey carbon film coating and dried anaerobically. ESEM scanning was performed on a FEI Quanta 650 FEG ESEM. Prior to observation, sample aliquots were washed in 30 mM bicarbonate buffer, placed onto an aluminium pin stub (Zeiss, Ø12.7 mm top) and dried anaerobically. A carbon coating was applied to enhance the sample conductivity. Imaging was performed operating at 15 kV in high vacuum mode ($10^{-5}$ to $10^{-6}$ mbar) and an EDAX Gemini EDS system was used for elemental analysis.

**U speciation and coordination analysis.** X-ray Absorption Spectroscopy (XAS) was used to determine speciation and coordination of uranium at the end of the biodegradation experiments. XAS data were collected for the M$_{IV}$-edge and L$_{III}$-edge of U.

For studies of the U M$_{IV}$-edge High-Energy Resolution Fluorescence Detection X-Ray absorption near edge structure (HERFD XANES), aliquots from experiments containing 1 mM U(VI) were spun down (5,000 g, 20 min) and a wet paste was layered in a sample holder. These spectra were collected on the ID26 beamline at the European Synchrotron Radiation Facility (ESRF) in Grenoble (Gauthier et al., 1999) using a Si(111) monochromator and a X-ray emission spectrometer (Glatzel et al., 2013). The intensity of the Mβ emission line (3336.0 eV) was recorded using five spherically bent Si crystal analysers aligned at 75° Bragg angle within a helium atmosphere (Kvashnina et al., 2013, 2014).

For U L$_{III}$-edge XANES and Extended X-ray Absorption Fine Structure (EXAFS) spectroscopy, moist sediment pellets were collected by centrifugation (5,000 g, 20 min) and dried anaerobically. The dry material was diluted by adding cellulose to approximately 1% U w/w and
creating a 2 mm thick pellet with final weight of 1.5 g. All samples were prepared under strictly anaerobic conditions and stored under an Ar atmosphere at -80 °C until analysis on B18 XAS beamline at the Diamond Light Source, Harwell, UK. EXAFS spectra were collected for the U L_{III} edge at room temperature using a 9-element solid-state Ge detector in fluorescence mode (Dent et al., 2009). Standard spectra were also collected in transmission mode for U(VI) (as schoepite, UO₂·2H₂O) and U(IV) (as uraninite, UO₂). Spectra were merged and energy calibration was completed by parallel measurement of a Y-foil calibrated to 17038 eV. Background subtraction was performed using Athena software (Ravel and Newville, 2005). Resulting EXAFS spectra were k³-weighted and spectra were Fourier transformed (FT) over the k-range of between 3 to 12 Å⁻¹ and compared with standard spectra. For modelling EXAFS data the Demeter software suit (0.9.25) was used (Ravel and Newville, 2005). Theoretical phases and amplitudes were calculated with FEFF6 using crystal structures for ninygoite [CaU⁴⁺(PO₄)₂] (Dusausoy et al., 1996), autunite [(Ca,U⁴⁺)PO₄] (Locock and Burns, 2003), and uraninite [UO₂] (Wyckoff, 1963). Shells and multiple scatterers (MS) were only included in the final fit, when they improved the fit as assessed by the F-test (Downward et al., 2007). Parameterization of the EXAFS models used no more than two thirds of the total number of available independent points.

7.4. Results

The impact of microbial ISA metabolism on the fate of uranium, was investigated in minimal medium containing U(VI) at either 0.1 mM U or 1 mM U concentration and ISA, and that were inoculated with a microbial enrichment from a lime kiln site. The enrichments were grown with ISA as the sole electron donor, and either with Fe(III) as an electron acceptor, or with no Fe(III) (i.e. under fermentation conditions). Incubations were set up under anoxic conditions at neutral pH, to approximate “far field” geosphere conditions surrounding a GDF.

Geochemistry. The biogeochemical fate of ISA was quantified by Ion Chromatographic (IC) analysis. In samples with microbial inoculum, and at either 0.1 mM U(VI) or 1 mM U(VI), ISA decreased after a lag phase until it was fully depleted by 14 days (Figure 7.1). There was no difference in the experiments with Fe(III) or without added electron acceptor. The ISA removal was accompanied by a dramatic decrease in redox potential to -200 mV in systems with Fe(III) and to -110 mV in systems without added electron acceptor (Figure 7.7). In the Fe(III)-reducing
experiment a modest increase in pH by 0.2 units was noted, whereas in the experiment without Fe(III) very modest acidification of the medium was observed (Figure 7.8). In addition, there was an increase in the concentration of volatile fatty acids (VFAs) in all experiments, which mainly comprised acetate and smaller amounts of butyrate. In the systems with Fe(III), concentrations of acetate and butyrate levels reached approximately 2.9 mM and 0.6 mM, respectively, at the point when ISA was depleted and at both U concentrations (14 days). In the experiments without added electron acceptor, VFA production was slightly higher (3.8 mM acetate and 0.7 mM butyrate at both U concentrations). In experiments with added Fe(III), butyrate concentrations decreased slightly after ISA was depleted, whilst in the experiments without Fe(III) it continued to increase slightly until the end of the incubation. In the incubations with added Fe(III) oxyhydroxides, Fe(II) ingrowth was monitored by the ferrozine assay. Fe(II) production was only detected in microbially active experiments after 7 days and plateaued in the experiment with 1 mM U at day 14 (Figure 7.1A) and in the experiment with 0.1 mM U at day 21 (Figure 7.1B) (at 56% and 59% of the total HCl-extractable Fe fraction, respectively). In
addition, the total HCl-extractable Fe fraction decreased to about 40% at 0.1 mM U and to 75% at 1 mM U compared to the starting concentration at day 90, indicating stable Fe mineral formation (Figure 7.11). An inoculated control incubation with Fe(III) but without added U was set up alongside (Figure 7.6), and showed similar ISA removal rates compared to the incubation experiments with U.

U(VI) concentrations in the supernatant were monitored spectrophotometrically (orange lines in Figure 7.1). When the incubations were initiated, the concentration of U(VI) was around 86% at 0.1 mM U and 18% at 1 mM U in experiments with Fe(III), and dropped steeply until day 2 before ISA removal had commenced. When ISA was removed from solution at day 14, U was remobilized to approximately 23% at 0.1 mM U and 13% at 1 mM U, and remained at a similar level over an extended incubation time up to day 35. In contrast, in experiments without Fe(III) the starting soluble U(VI) concentrations were 11% at 0.1 mM U and 7% at 1 mM U and thus lower than in the experiment with added Fe(III). When fermentation of ISA had begun, U(VI) levels were below the level of detection. Upon ISA removal in the experiment with 1 mM U, approximately 7% of U(VI) was remobilized at day 21, which dropped again to below the level of detection over the remaining duration of the experiment.

To help identify possible abiotic influences during ISA biodegradation, control experiments were set up, containing U(VI) and ISA, and a sterile microbial inoculum (grey dashed lines in Figure 7.1) or the inoculum was omitted (Figure 7.9). In these controls, ISA and Fe(II) levels (Figure 7.10), redox (Figure 7.7) and pH values (Figure 7.8) remained stable, consistent with abiotic influences playing a minor role in these experiments. The fate of U(VI) in the abiotic control experiments with ISA, and either with a sterilized microbial inoculum or without the inoculum, showed that after initial equilibration, the soluble U(VI) fraction remained at approximately 1% U(VI), confirming oversaturation of U-species (Figure 7.12).

**Molecular Ecology.** Stable enrichment cultures were prepared using inocula from sediments collected from a well characterized lime workings site at Harpur Hill, Buxton (Williamson *et al.*, 2013). Cultures were maintained by consecutively transferring inocula to fresh medium to select for microorganisms using ISA as the sole electron donor and carbon source, with either Fe(III) as terminal electron acceptor or in a system without additional terminal electron acceptors (only CO₂). In the 12th transfer, U was added to a final concentration of either 0.1 or 1 mM U(VI). DNA
was extracted from these cultures at the end of the incubation and 16S rRNA genes were amplified and sequenced using Illumina sequencing protocols to evaluate the impact of U(VI) on the microbial community composition, and to identify possible ISA-oxidizing and/or U(VI)-reducing microorganisms.

Alpha-rarefaction curves showed a significant decrease in species diversity from approximately 600 species in the original soils (Kuipers et al., 2015) to approximately 150 species in the ISA biodegradation/Fe(III) reduction experiments and to approximately 135 species in the ISA fermentation experiments (Figure 7.13). The species diversity was more alike to each other, when comparing genetic profiles from the same electron acceptor at different U concentrations, than when comparing profiles from same U concentration with different electron acceptor.

The microbial enrichment culture used to inoculate the U(VI)-ISA incubation experiment with added Fe(III), comprised predominantly of sequences most closely related to species from the family *Clostridia* (43.6%), *Betaproteobacteria* (26.2%) and *Deltaproteobacteria* (7.6%) (Figure 7.2). The microbial community remained similar after incubation with U(VI), but the sequences belonging to the *Deltaproteobacteria* doubled at both U concentrations (to 14.7% of the total number of sequences at both U concentration). Affiliates of the Class of *Deltaproteobacteria* comprised two families, of which approximately 11% of total sequence abundance at 0.1 mM U
concentration and 11.8% at 1 mM U were most closely affiliated with members from the 
Geobacteraceae (97% match) and approximately 3.7% of total sequence abundance at 0.1 mM 
U concentration and 3% at 1 mM U were affiliated with Desulfovibrionaceae (98% match).
Sequences belonging to the Coriobacteria (99% match) were slightly enriched (~4% of total 
sequence abundance from previously <1%) after ISA biodegradation, as well as sequences 
belonging to the Negativicutes (from 2% to 7% of total sequence abundance), which were 
associated with members from the family Veillonellaceae (≥98% match).

The microbial community of the U(VI)-ISA experiment without added Fe(III) was characterized 
by sequences dominantly associated with Clostridia (41.3% of the total number of sequences at 
0.1 mM U; 46.8% at 1 mM U) and Betaproteobacteria (21.5% at 0.1 mM U; 18.7% at 1 mM U; 
Figure 7.2). However, these fermenting experiments also contained an enrichment of 
sequences affiliated with family members from the Veillonellaceae (98% match; 10.4% at 
0.1 mM U; 12.6% at 1 mM U) belonging to the class Negativicutes. Another significant Class 
was the Bacteroidetes (12.4% at 0.1 mM U; 12.5% at 1 mM U), of which most sequences were 
associated with the genus vadinBC27 wastewater sludge group (approximately 68% at 0.1 mM 
U; 73% at 1 mM U) from the Family Rikenellaceae.

**Figure 7.3.** Uranium M_{IV}-edge high resolution (HR) XANES data showing in green: U(IV) 
standard, yellow: U(VI) standard, blue: ISA degradation, Fe(III)-reducing experiment at 
1 mM U and red: ISA-fermenting experiment at 1 mM U.
**Bio-mineralogy.** To help identify the impact of microbial ISA metabolism on the GDF mineralogy, end-point mineralogical products were characterized using XRD, TEM and ESEM analyses. XAS was used to characterize the U coordination environment. In addition, U M\text{IV}-edge (Figure 7.3) and U L\text{III}-edge (Figure 7.16) XANES spectra were collected to identify the oxidation state.

**Uranium fate; ISA-fermenting experiment with no Fe(III)**

Figure 7.4. Mineralogical analysis of ISA fermentation experiment at 1 mM U(VI) concentration: (A) ESEM image; (B), C) TEM images; D) TEM EDS; E) non-phase shift corrected U L\text{III}-edge EXAFS data and F) corresponding k\(^3\) weighted Fourier transform of EXAFS data. ESEM and TEM images show spherulitic U-containing bioprecipitates which were fitted (dashed line) as uranyl-phosphate from the autunite group.

In the U(VI)-ISA fermenting system, ISA was removed within 14 days of incubation regardless of the starting U-concentration (0.1 mM or 1 mM). ICP-MS analysis showed a decrease in U concentrations in solution at the start of the incubation, before ISA was removed. This was despite that up to 8% U was briefly remobilized to solution upon ISA degradation. At the experimental end point, U was fully precipitated. A precipitate from the start of the incubation, which contained the bulk of the U, was identified with XRD analysis as a uranyl phosphate [(K,Na)(UO\(_2\))PO\(_4\)] belonging to the autunite group (Figure 7.14). After bioreduction, the mineral end products were characterized again (Figure 7.15), and showed evidence for a similar uranyl phosphate phase. In addition, in the 0.1 mM U experiment, a Ca-phosphate mineral which was identified as whitlockite [Ca\(_{10}\)Mg\(_2\)H\(_2\)(PO\(_4\))\(_{14}\)] by comparison to a standard reference library was
identified. In the fermenting systems, both ESEM (Figure 7.4 A) and TEM imaging (Figure 7.4 B, C), revealed well defined spherulitic minerals with an average size of 0.8 µm ±0.014 µm, consisting of individual plates (15 to 40 nm) that aggregated to bigger spherules, after fermentation of the ISA. EDS analysis of the TEM images (Figure 7.4 D) identified peaks of Ca, P and U and some Na and Mg, consistent with XRD data. U M\text{IV}-edge spectra of the 1 mM U fermentation experiment revealed the dominance of U(VI) (Figure 7.3). Additional U L\text{III}-edge spectra, confirmed the presence of U(VI) in the solid phase from the 0.1 mM and 1 mM U experiments (Figure 7.16). The EXAFS analyses were informed by the relevant U(VI)-phosphate literature (Locock and Burns, 2003) and could be fitted with a shell of two axial oxygens at 1.77 Å, four equatorial oxygens at 2.27 Å and four monodentate phosphorus atoms at 3.62 Å (Table 7.1; Table 7.2). XRD and EDS analyses indicated the presence of a U(VI)-phosphate phase in the 0.1 mM U experiment, similar to the phase observed in the 1 mM U experiment, however XAS data could not be collected for this sample. Despite the U being present as U(VI), cells were observed in TEM images closely associated with external nodular clusters containing high amounts of U and P (Figure 7.18 7.20).

Uranium fate; ISA biodegradation experiment with Fe(III)

U-precipitates collected at the start of the incubation prior to ISA degradation, in the experiment supplemented with Fe(III) as an electron acceptor and 1 mM U(VI), were identified as uranyl-phosphates [(K,Na)(UO\text{2})PO\text{4}] by XRD, consistent with the data from the ISA-fermenting experiment. After bioreduction of the Fe(III), XRD patterns of the precipitates showed two sharp peaks at 2Θ = 13.2° and at 2Θ = 31.9° (Figure 7.15), corresponding to siderite (FeCO\text{3}) and vivianite (Fe\text{3}PO\text{4})\text{2}·8H\text{2}O, which have been observed previously in ISA biodegradation studies under Fe(III)-reducing conditions (Kuipers et al., n.d.). Both, TEM (Figure 7.5 A, B) and ESEM images (Figure 7.5 C) also showed U-containing minerals that appeared in sheets that were partially rolled up and agglomerated in clusters. EDS analyses from several different areas (Figure 7.5 E) showed Fe, P, U and Ca were present. Selected area electron diffraction patterns (SAED; Figure 7.5 D) matched the lattice d spacings for polycrystalline ningyoite [CaU(PO\text{4})\text{2}·H\text{2}O] (Muto et al., 1959; Khijniak et al., 2005). U M\text{IV}-edge XANES (Figure 7.3) and U L\text{III}-edge data (Figure 7.16) collected for the 1 mM U sample lacked the characteristic spectral
shoulder feature of U(VI), thereby confirming reduction to U(IV). The EXAFS fitting for the 1 mM U experiment was informed by published literature with eight oxygen atoms at 2.40 Å and two phosphorus shells of 1 atom at 3.16 Å and 1.5 atoms at 3.68 Å (Figure 7.5 F, G; Table 7.1), and was consistent with ningyoite (Dusausoy et al., 1996). Given the presence of Ca in the ningyoite structure (Dusausoy et al., 1996) and the fact that it was detected in EDS and XRD analyses, attempts were made to include Ca in the fit. The addition of a shell of 2 Ca atoms at 3.85 Å improved the fitting parameters but not with statistical significance. Given that Ca is a weakly scattering atom, the addition of Ca also resulted in larger errors of the Debye-Waller factor, wherefore it was not included in the final fit. The XANES region of the U L_{III}-edge spectrum from 0.1 mM U experiment was similar to data from to the 1 mM U experiment, suggesting reduction from U(VI) to U(IV) (Figure 7.16). The best fit for the EXAFS data was achieved with the same ningyoite-like structure with an oxygen shell at 2.40 Å with eight oxygens and one phosphorus shell containing one atom at 3.13 Å that was modelled with a satisfactorily F-test result (Figure

Figure 7.5. Mineralogical analysis of ISA biodegradation, Fe(III) reduction experiment at 1 mM U(VI) concentration showing A), B) TEM images; C) TEM electron diffraction pattern with rings identified at 2.4 Å, 2.2 Å and 1.62 Å; D) ESEM image; E) ESEM EDS analysis F) non-phase shift corrected Fourier transform of U L_{III}-edge EXAFS data and G) corresponding k^2 weighted EXAFS data. ESEM and TEM images show sheeted precipitates, partially folded up and containing U, which were fitted (dashed line) as a ningyoite.
Table 7.1. EXAFS fit parameters for U(VI)-ISA degradation experiments with Fe(III) as terminal electron acceptor and without Fe(III) added. The amplitude factor ($S_0^2$) was fixed as 1.0 for each sample. Indices are ax for axial atoms and eq for equatorial atoms.

7.5. Discussion

Since the complexation of ISA with radionuclides, such as U(VI), may significantly enhance their solubility and thus mobility, understanding their fate is crucial to understanding the performance of a geological disposal system for ILW (DECC, 2014). Microbial metabolism in the circumneutral “far field” geology of a GDF has been identified as a mechanism that may result in the degradation of such complexes, and thus retard the transport of radionuclides escaping into the biosphere. This study investigated the impact of ISA biodegradation on the fate of U(VI), and studied the organic degradation products associated ISA, and the fate of U(VI) in microbial enrichment cultures.

For this purpose, microbial enrichment cultures were prepared containing ISA as the sole carbon source and U(VI) at either a concentration of 0.1 mM or 1 mM. Fe(III) was also supplied as the electron acceptor, while “fermentation” conditions were stimulated by excluding an
additional electron acceptor. In all microbially active enrichment cultures, ISA was removed from solution and fermentation products accumulated, whereas in abiotic systems ISA remained in solution, demonstrating the importance of a microbial inoculum to remove the chelate from solution, as noted in previous studies on ISA biodegradation (Bassil et al., 2015b; Kuipers et al., 2015; Rout et al., 2015c). However, the production of fermentation products was more pronounced when no additional electron acceptor was provided, yielding approximately 50% carbon as fermentation products from ISA, whereas in the system with Fe(III) only 13% of the carbon was recovered as fermentation products. This is most likely due the oxidation of fermentation products to CO$_2$, coupled to the dissimilatory reduction of Fe(III).

16S rRNA gene sequencing showed that all enrichments cultures were dominated by Clostridia spp. The ISA-fermenting cultures with U(VI) were more heavily enriched in Clostridia and Betaproteobacteria, whereas the ISA-degrading, Fe(III)-reducing cultures also contained higher percentages of Deltaproteobacteria, implicated in Fe(III) reduction in previous studies (e.g. Coleman et al., 1993; Roden and Lovley, 1993; Lovley, 2000).

After complete biodegradation of the ISA in the Fe(III)-reducing system, there were clear differences in the microbial communities imposed by the addition of U. Regardless of the amount of added U(VI), sequences associated with Betaproteobacteria and Negativicutes were enriched and sequences affiliated with Gammaproteobacteria decreased, relative to the experiments that did not contain added U(VI). Similarly, in the fermentation experiments at both U(VI) concentrations, the sequences affiliated with Negativicutes increased and those of Bacteroidetes decreased. In both systems, the Negativicutes were most closely affiliated with members from the Family Veillonellaceae (>98%), which has been capable of U(VI) reduction in lab studies (Woolfolk and Whiteley, 1962; Boonchayaanant et al., 2009) and was moreover observed as most dominant Family in an in situ U(VI) bioreduction study (Gihring et al., 2011).

Even though U(VI) was only reduced in the system with added Fe(III), both systems showed the presence of bacteria known to be capable of U(VI) reduction. In the Fe(III)-reducing experiments, known Fe(III)-reducing bacteria from the Class of Deltaproteobacteria, such as Geobacter sp. (≥11%), were significantly enriched compared to the fermenting cultures (≤2%). Besides the reduction of Fe(III), Geobacter sp. are well known to reduce U(VI) (Lovley et al., 1991a; Holmes et al., 2002) and presumably played a role in reducing the U(VI) to U(IV) in
these experiments, in contrast to the fermenting system where U(VI) was not reduced. Other species belonging to *Deltaproteobacteria* were associated with *Desulfovibrio* species, were present at low percentages in both systems (at 5% of total abundance in the fermenting and 3% in the Fe(III)-reducing cultures), which are also known to reduce U(VI) enzymatically (Lovley and Phillips, 1992; Lovley *et al.*, 1993b; Pietzsch *et al.*, 1999). In addition to these Gram-negative microorganisms, Gram-positive *Clostridium* species have also been reported to reduce U(VI) (Francis *et al.*, 1994). However, it is unlikely that these organisms played a significant role in reducing U(VI) in these experiments, as they were abundant in the fermenting experiments, where the U remained as U(VI). It is more likely that the *Geobacter* and *Veillonellaceae* species that were exclusively enriched for in the presence of Fe(III), mediated the U(VI) reduction noted in these treatments.

Consistent with a different degradation pathway and a distinct microbial community evolution in the system with Fe(III) compared to with no Fe(III), the identified mineralogical end products in this experiment were significantly different. Despite using a medium containing carbonate, a strong complexant for U(VI), the solubility of the U(VI) was low in these experiments presumably due to the presence of significant levels of soluble phosphate, which is well known to form insoluble minerals when mixed with U(VI) (Langmuir, 1978). Indeed, XRD analyses at the beginning of the experiments revealed the presence of a U(VI)-phosphate, a member from the autunite group [(HPO$_4$)$_4$]. Consistent with studies of naturally formed autunite/meta-autunite that have been stable over geological timescales (Sato *et al.*, 1997; Jerden and Sinha, 2003), the U(VI)-phosphates observed in the fermentation experiment, are considered stable end products and a sink for U (Langmuir, 1978). Using solid phase characterization, including X-ray absorption analysis, we described the minerals that formed during biodegradation of ISA, which comprised U(VI)-phosphates in the fermentation system and U(IV)-phosphates in the Fe(III) reduction experiment. In the fermentation experiment at the 0.1 mM U concentration, whitlockite [Ca$_3$(PO$_4$)$_2$], a member from the apatite group, was also identified using XRD. This mineral has presumably formed as a result of ISA fermentation, liberating Ca$^{2+}$ into solution, forming the biomineral whitlockite. It should be noted, that apatites are considered suitable for the radioactive waste cleanup, because they can readily incorporate tri- and tetravalent actinides by double substitution for Ca$^{2+}$, and are very stable and resistant to radiation damage (Fuller *et al.*, 2003).
2002; Oelkers and Montel, 2008; Handley-Sidhu et al., 2011). Successful EXAFS fitting of this experiment was not achieved, however XRD results indicated a similar U(VI)-phosphate phase compared to the experiment at 1 mM U.

In contrast, in the ISA biodegradation experiment containing Fe(III) as an electron acceptor, initial rapid precipitation of U(VI) resulted in the formation of a U(VI)-phosphate, identified with XRD as metaankoleite. In the biotic incubations, remobilization of small quantities of U(VI) during ISA biodegradation and Fe(III) reduction, indicated that the microbial degradation also impacted on the fate of U, of which the bulk was precipitated at the end of the incubation experiment. XRD analyses of these bioprecipitates revealed crystalline Fe(II)-carbonate and Fe(II)-phosphate minerals, but no U-containing mineral phase, which could either be explained by a lack of crystallinity of the U-biominal or detection limits for U minerals. However, additional XANES and EXAFS studies identified the U mineral, as a reduced U(IV)-phosphate mineral, fitted with a ningyoite structure [CaU(PO$_4$)$_2$·H$_2$O] (Khijniak et al., 2005; Bernier-Latmani et al., 2010; Fletcher et al., 2010; Lee et al., 2010; Rui et al., 2013; Jones et al., 2015; Newsome et al., 2015a), whereas in abiotic control incubations XRD analyses confirmed the presence of U(VI)-phosphate as end point mineral. Thus, the transformation and reduction from metaankoleite to ningyoite was clearly connected to microbially driven ISA biodegradation coupled to Fe(III) reduction. Since this system selected for microorganisms that respire Fe(III) and the 16S rRNA gene sequences indicated an enrichment of such strains, these metal-reducing bacteria may have also reduced the U(VI), most likely in the solid and aqueous form. Focusing on U(VI) as an alternative electron acceptor, previous studies indicated that Ca in solution decreased the amount of microbial U(VI) reduction (Lee et al., 2010 and references therein), however the end point mineral siderite seemed to have incorporated Ca into the crystal structure, as seen before in ISA biodegradation studies (Kuippers et al., n.d.), and thus microbial U(VI) reduction in this experiment cannot be excluded. Thus, the mechanism of the microbially-mediated U(VI) reduction could have been either via direct biotic enzymatic reduction using U(VI) as an alternative electron acceptor (Lovley et al., 1991a; Gorby et al., 1992; Fredrickson et al., 2000), or via microbially-mediated indirect processes, whereby the Fe(III) is first reduced enzymatically to Fe(II), which in turn shows reducing capacity for abiotic U(VI) reduction (Jeon et al., 2005; O’Loughlin et al., 2010; Veeramani et al., 2011). This form of
microbially-mediated abiotic reduction of U(VI) might have directly competed with enzymatic reduction (Veeramani et al., 2011). In particular, the presence of vivianite was shown to reduce U(VI) to monomeric U(IV) coordinated to phosphate (Veeramani et al., 2011). Supporting the assumption of biotic enzymatic U(VI) reduction was the enrichment of *Geobacter* and *Desulfovibrio* spp., known metal- and U(VI)-reducing bacteria, which decreased in the U(VI)-ISA fermentation experiment compared to ISA fermentation without U(VI). To unravel the mechanism of U(VI) reduction, further EXAFS results are awaited in the near future.

Although early work on microbial U(VI) reduction noted uraninite (UO$_2$) as the dominant end point (Lovley et al., 1991b; Sharp et al., 2009; Veeramani et al., 2013), here the U(IV) product was found coordinated to a PO$_4$ group. U(IV)-phosphates have been observed as the products of microbial U(VI) reduction in previous studies, for example in the presence of aqueous phosphate (Bernier-Latmani et al., 2010; Lee et al., 2010) or after reduction of a U(VI) phosphate mineral phase (Khijniak et al., 2005; Rui et al., 2013). The presence of phosphate is also known to inhibit uraninite formation and favor the production of poorly crystalline U(IV) precipitates (Veeramani et al., 2011). Here a more crystalline form of U(IV) phosphate was identified, and their remarkable recalcitrance towards oxidation should be noted (Finch and Murakami, 1999; Jerden and Sinha, 2003; Pinto et al., 2012; Newsome et al., 2015a; b), compared to monomeric U(IV) species which can show complete remobilization under oxidizing conditions (Moon et al., 2007; Newsome et al., 2015b).

### 7.6. Conclusions

The addition of electron donors has been often considered as a strategy to stimulate microbial metabolism that in turn can remediate uranium contamination via U(VI) reduction (e.g. Williams et al., 2012; Newsome et al., 2015a). This study sheds light on self-attenuating processes by a subsurface microbial community that can effectively control the fate of U(VI) in solution during ISA biodegradation associated with the disposal of ILW. Microbial communities retrieved from sediments, representative of the geosphere surrounding a GDF, were capable of biodegrading ISA in the presence of U(VI) under both Fe(III)-reducing and fermenting conditions. In the case of ISA fermentation, the uranium was immobilized completely as a U(VI)-phosphate, whereas in the Fe(III)-reducing enrichment cultures, U(VI) was fully reduced to U(IV), present in stable U(IV)-phosphate biominerals.
In conclusion, ISA biodegradation at circumneutral pH has proven a self-attenuating process that has the potential to remove ISA escaping microbial metabolism in the alkaline near field of a GDF. For the long-term stability of a GDF, the bioprecipitation as U(VI)- or U(IV)-phosphates in the geosphere of a GDF would seem highly beneficial for the safe operation of such a facility, offering an additional attenuated ‘bio-barrier’ to augment the physical and chemical barriers that underpin the conventional GDF design principle.

7.7. Acknowledgements
This work was supported by a studentship to GK funded by Radioactive Waste Management (RWM). We thank both Diamond Light Source (SP13599) and European Synchrotron Radiation Facility (EV/192) for beamtime and EnvRadNet for travel and subsistence to ESRF. We would like to thank Dr Z Azlam of the University of Leeds LENNF facilities for TEM support. Further thank goes to Alastair Bewsher and John Waters at the University of Manchester for analytical support. JRL also acknowledges the financial support of the Royal Society and the EU MIND programme under “Euratom2014–2015” and the call “NFRP-06-2014: Supporting the implementation of the first of-a-kind geological repositories”.

7.8. Competing financial interests
The authors declare no competing financial interest.

7.9. Author contributions
GK – primary investigator, preparation and monitoring of experiments, data acquisition, analysis and interpretation, manuscript drafting; KS, KM, SS and PB – support with XANES & EXAFS data analysis and interpretation; CB – sample analysis for microbial ecology (sequencing); MW – support with TEM analysis; RB, KM, NB – manuscript review; JRL Planning experiments with GK, data interpretation, review and editing of manuscript.

7.10. Associated Content
Supporting Information
Details of medium, $E_n$, design of tagged fusion primers and PCR amplifications, sulfate and phosphate data, XANES, EXAFS fitting results, pyrosequencing data processing, and phylogenetic affiliations of the most abundant bacterial OTUs identified.
7.11. References


Suzuki, Y. and Suko, T. (2006) Geomicrobiological factors that control uranium mobility in the environment: Update on recent advances in the bioremediation of uranium-contaminated
The text on this page appears to be a list of references formatted in the style of academic literature. Here is the plain text representation of the document:


7.12. Supplementary information

**Title:** Biomineralization of uranium-phosphates fuelled by the microbial degradation of isosaccharinic acid (ISA)

**Author list:** Gina Kuipers, Kurt Smith, Pieter Bots, Katherine Morris, Christopher Boothman, Samuel Shaw, Nicholas Bryan, Jonathan R. Lloyd

---

**Figure 7.6.** ISA biodegradation control experiment with Fe(III).

**Figure 7.7.** Redox potential evolution in U(VI)-ISA enrichment experiments.

**Figure 7.8.** pH evolution in U(VI)-ISA enrichment experiments with and without added Fe(III).
Figure 7.9. Abiotic ISA control experiments in freshwater minimal medium (FWM) and with 1 mM U testing ISA solubility in experiments with microbial inocula and without the inoculum.

Figure 7.10. Relative Fe(II) ingrowth compared to total HCl-extractable iron measured with the ferrozine assay.

Figure 7.11. Total Fe in microcosms analyzed by the ferrozine assay after acidification in 0.5 N HCl or 0.25 N hydroxylamine-HCl (Lovley and Phillips, 1986a). Sterile control contains heat-killed microbial inoculum, 1 mM U and Fe.
Figure 7.12. Abiotic U control experiments in freshwater minimal medium (FWM) testing U solubility under experimental conditions.

Figure 7.13. α-rarefaction curves obtained from 16S rRNA gene sequencing after biodegradation of ISA showing maximum of distinct species per sample.
Figure 7.14. XRD patterns for abiotic experiments. First pattern showing U(VI)-ISA control with Fe(III) oxyhydroxide and a sterile inoculum, second pattern showing U(VI)-ISA control with a sterile inoculum.

Figure 7.15. XRD patterns for experiments after U(VI)-ISA biodegradation. Standards are whitlockite, metaankoleite, siderite, vivianite and ranciete.
Figure 7.16. Uranium L_{III}-edge XANES spectra for U(VI)-ISA experiments with and without Fe(III) after bioreduction collected at beamline B18, Diamond Lightsource, Harwell.

Figure 7.17. Mineralogical analysis of U(VI)-ISA fermentation experiment at 0.1 mM U(VI) concentration: (A) TEM image; B) corresponding EDS; C) ESEM image; D) non-phase shift corrected U L_{III}-edge EXAFS data and E) corresponding k^3 weighted Fourier transform of EXAFS data. ESEM and TEM images show spherulitic bioprecipitates containing Ca-P-Na-U which were fitted (dashed line) as liebigite [Ca_2UO_2(CO_3)_3·11H_2O], a uranyl-carbonate.
Figure 7.18. TEM images showing microbial association with biominerals observed in the U(VI)-ISA fermenting experiment, while in the ISA-U(VI) degradation experiment with Fe(III) such observations were not made, probably due to the high Fe concentrations. A) Clusters of fibrous structure comprising high amounts of U (1) compared to the cell envelope where U was absent (2); C) U-containing precipitates around cell which could be internal or external; D), E) and F) show clusters of round shape containing mainly P and U which seem to be externally.

Figure 7.19. Mineralogical analysis of U(VI)-ISA Fe(III) reduction experiment at 0.1 mM U(VI) concentration showing A) non-phase shift corrected Fourier transform of U L$_{III}$-edge EXAFS data and B) corresponding $k^3$ weighted EXAFS data. ESEM and TEM images show sheeted precipitates, partially folded up and containing U, which were fitted (red line) with one oxygen shell containing 8 atoms and a phosphorus shell containing 1 atom.
Figure 7.20. TEM images of ISA-U(VI)-Fe(III) experiment showing cell envelopes. A) cell coated in Fe (TEM EDS below) and B) cells surrounded by partially reduced Fe(II)-precipitates.
<table>
<thead>
<tr>
<th>Fit No.</th>
<th>118</th>
<th>119</th>
<th>120</th>
<th>121</th>
<th>123</th>
<th>124</th>
<th>127</th>
</tr>
</thead>
<tbody>
<tr>
<td>k min</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>k max</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
<td>13.5</td>
<td>13.5</td>
</tr>
<tr>
<td>R min</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>R max</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>NIDP</td>
<td>29.40</td>
<td>29.40</td>
<td>29.40</td>
<td>29.40</td>
<td>29.40</td>
<td>29.40</td>
<td>29.40</td>
</tr>
<tr>
<td>Variables</td>
<td>10</td>
<td>12</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>df</td>
<td>19.4</td>
<td>17.4</td>
<td>15.4</td>
<td>15.4</td>
<td>15.4</td>
<td>13.4</td>
<td>11.4</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>37916.7</td>
<td>11618.6</td>
<td>4571.8</td>
<td>4240.3</td>
<td>4791.0</td>
<td>4791.0</td>
<td>2058.3</td>
</tr>
<tr>
<td>Rd $\chi^2$</td>
<td>1954.5</td>
<td>667.7</td>
<td>296.9</td>
<td>275.3</td>
<td>311.1</td>
<td>253.1</td>
<td>180.6</td>
</tr>
<tr>
<td>R-Factor</td>
<td>0.322</td>
<td>0.074</td>
<td>0.033</td>
<td>0.031</td>
<td>0.033</td>
<td>0.024</td>
<td>0.016</td>
</tr>
<tr>
<td>$v$ R-factor</td>
<td>0.567</td>
<td>0.273</td>
<td>0.182</td>
<td>0.176</td>
<td>0.181</td>
<td>0.155</td>
<td>0.128</td>
</tr>
<tr>
<td>Amp</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>O ax</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>O eq1</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>O eq2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O ax ms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C biden</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P monodentate</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>MS OO</td>
<td>3x2 def</td>
<td>3x2 def</td>
<td>3x2 def</td>
<td>3x2 def</td>
<td>3x2 def</td>
<td>3x2 def</td>
<td>3x2 def</td>
</tr>
<tr>
<td>MS OP</td>
<td>1 def</td>
<td>1 def</td>
<td>1 def</td>
<td>1 def</td>
<td>1 def</td>
<td>1 def</td>
<td>1 def</td>
</tr>
<tr>
<td>MS OPO</td>
<td>2 guess</td>
<td>2 guess</td>
<td>2 guess</td>
<td>2 guess</td>
<td>2 guess</td>
<td>2 guess</td>
<td>2 guess</td>
</tr>
<tr>
<td>U</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha$</td>
<td>1.000</td>
<td>1.000</td>
<td>0.999</td>
<td>0.999</td>
<td>0.905</td>
<td>0.978</td>
<td>0.978</td>
</tr>
</tbody>
</table>

Table 7.2. F-test for U(VI)-ISA incubation experiment with no Fe(III) as an electron acceptor (fermentation) at 1 mM U. Fit No. 127 was chosen as best fit.

<table>
<thead>
<tr>
<th>Fit No.</th>
<th>359</th>
<th>361</th>
<th>362</th>
<th>363</th>
<th>364</th>
</tr>
</thead>
<tbody>
<tr>
<td>k min</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>k max</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>R min</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>R max</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Variables</td>
<td>3</td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>df</td>
<td>10.6</td>
<td>8.6</td>
<td>7.6</td>
<td>5.6</td>
<td>5.6</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>3639.2</td>
<td>2269.9</td>
<td>1310.3</td>
<td>1725.3</td>
<td>1457.0</td>
</tr>
<tr>
<td>Rd $\chi^2$</td>
<td>344.3</td>
<td>264.9</td>
<td>173.1</td>
<td>309.7</td>
<td>261.6</td>
</tr>
<tr>
<td>R-Factor</td>
<td>0.090</td>
<td>0.050</td>
<td>0.024</td>
<td>0.023</td>
<td>0.018</td>
</tr>
<tr>
<td>$v$ R-factor</td>
<td>0.300</td>
<td>0.224</td>
<td>0.155</td>
<td>0.152</td>
<td>0.134</td>
</tr>
<tr>
<td>Amp</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>O</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>P1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>P2</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ca</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha$</td>
<td>0.955</td>
<td>0.985</td>
<td>0.149</td>
<td>0.663</td>
<td>0.663</td>
</tr>
</tbody>
</table>

Table 7.3. F-test for U(VI)-ISA incubation experiment with Fe(III) as an electron acceptor at 1 mM U. Fit No. 362 was chosen as best fit.
<table>
<thead>
<tr>
<th>Fit No.</th>
<th>24</th>
<th>26</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_{\text{min}})</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>(k_{\text{max}})</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>(R_{\text{min}})</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(R_{\text{max}})</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>NIDP</td>
<td>8.60</td>
<td>8.60</td>
</tr>
<tr>
<td>Variables</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>(\chi^2)</td>
<td>2864.1</td>
<td>1702.8</td>
</tr>
<tr>
<td>(R_d\ \chi^2)</td>
<td>511.4</td>
<td>473.0</td>
</tr>
<tr>
<td>(R)-Factor</td>
<td>0.067</td>
<td>0.024</td>
</tr>
<tr>
<td>(\sqrt{R})-factor</td>
<td>0.259</td>
<td>0.155</td>
</tr>
<tr>
<td>Amp</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>O</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>P</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>C biden</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OO rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OO fthr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OO nonflin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\alpha)</td>
<td></td>
<td>0.944</td>
</tr>
</tbody>
</table>

Table 7.4 F-test for U(VI)-ISA incubation experiment with Fe(III) as an electron acceptor at 0.1 mM U. Fit No. 26 was chosen as best fit.
8. Conclusions and Future directions

8.1. Conclusions
The long-term management of the nuclear legacy wastes in the UK focuses on deep geological disposal for which a site has yet to be found and a facility to be built. Until then, there are many challenges to overcome. An important aspect will be to understand the mechanisms of retention of radionuclides within the GDF, including scenarios when incoming groundwater will lead to leaching and degradation processes and extrapolating these data to time periods of a million years. A major problem addressed in this study is the in situ production of ligands, of which ISA is one of the most problematic due to its high solubility and complexing behaviour with radionuclides. The geochemical environment of a GDF and its host microbial population has been discussed in terms of the microbial degradation of ISA. Following results from a study that looked at microorganisms able to degrade ISA at high pH, representative for the “near field” environment, it was considered that ISA removal might remain incomplete due to the limiting energy yields under alkaline conditions, especially when sulfate-reducing conditions dominate (Bassil et al., 2015b). Therefore, the present study focused on ISA biodegradation under circumneutral pH conditions, representative for the “far field” environment of a GDF.

In the far field surrounding a GDF, deep groundwaters from the undisturbed geosphere will meet an alkaline plume emerging from the chemically disturbed zone, resulting in steep biogeochemical gradients. Since this system was not studied before for ISA biodegradation, it was important to sufficiently understand such processes and make predictions on the biogeochemical fate of ISA under geodisposal conditions. To achieve this goal, research was carried out assessing the activity of microbial communities that are expected to be present in the subsurface geosphere of a GDF and may be capable of ISA biodegradation. A thorough lab study was performed using sediment bacteria retrieved from a GDF analogue site contaminated by lime workings, which is characterised by elevated pH and Ca²⁺ concentrations, and known to contain ISA. Initial incubation experiments with inocula from this site were set up with a range of biogeochemical conditions, including aerobic conditions, and anaerobic conditions with nitrate,
Fe(III) or sulfate as alternative electron acceptors, and resulted in complete ISA removal (Chapter 4). In this Chapter methane production from ISA biodegradation under anaerobic conditions was found to be reduced, when an electron acceptor was provided; a very important research outcome when considering pressurisation of the underground facility from the production of gas. The microbial community under anaerobic conditions was complex, but clearly dominated by fermenting organisms. Fe(III) oxyhydroxides are expected to be an abundant source of terminal electron acceptor in the GDF environment capable of supporting ISA biodegradation. The fate of such Fe(III) minerals was studied in detail in Chapter 5. This study showed that chelation of Fe(III) by ISA led to a higher bioavailability of Fe(III) for microbial reduction, resulting in fully reduced Fe(II) forms, vivianite and siderite, rather than a mixed valence Fe(II)/Fe(III) state, such as magnetite. This outcome highlights the shaping effect that ISA biodegradation may have on the mineralogy in the surrounding geosphere of a GDF.

It should be noted that Fe(III) oxyhydroxides have the potential to undergo transformation to more recalcitrant phases, such as goethite or hematite, which are less bioavailable to microbial reduction or might be reduced by other processes before ISA escapes the GDF. In this case, other electron acceptors will become important. For GDF environments in the UK, sulfate might be an important alternative electron acceptor, as it is abundant in UK groundwaters. Thus, the impact of sulfate reduction on the fate of ISA and associated microbial community and mineralogy was studied subsequently under a range of pH conditions, and was found to be constrained to an upper pH range of approximately 9 under the time scales tested thus far (Chapter 9.1).

Following this research on ISA biodegradation under a range of biogeochemical conditions, there was a knowledge gap regarding the fate of radionuclides when complexed to ISA during biodegradation. Therefore, the second part of this study focused on the fate of metals and radionuclides complexed to ISA. The first investigation explored the biodegradability of divalent Ni-ISA complexes under Fe(III)-reducing conditions (Chapter 6), which removed nickel from solution when ISA was biodegraded, but remained in solution in microbially inactive controls (sterile), highlighting the importance of microbial processes in these systems. Small amounts of sulfate were reduced alongside Fe(III) reduction, indicating the activity of sulfate-reducing bacteria. Sulfide, produced from sulfate reduction, precipitated nickel as (Fe,Ni)-sulfide from
solution. Following this study, in a subsequent project hexavalent uranium was added to ISA biodegradation experiments maintained under Fe(III)-reducing and fermenting conditions (Chapter 7). Despite buffering the medium with bicarbonate, the strong complexation of U(VI) with phosphate resulted in uranium initially precipitating from solution. Upon ISA fermentation, small quantities of uranium were remobilised, which were subsequently precipitated as autunite (U(VI)-phosphate). In contrast under Fe(III)-reducing conditions, even though some uranium remained in solution (9% or 33 ppm), the bulk of the U(VI) was reduced to U(IV) and precipitated as a ningyoite (U(IV)-phosphate). The uranium phosphate minerals precipitated in the fermenting and Fe(III)-reducing experiments, are considered recalcitrant and insoluble, and therefore represent a stable sink for uranium; particularly when present as reduced U(IV)-phosphate minerals. A preliminary follow-up study investigating the fate of Ni(II) and U(VI) added to ISA biodegradation experiments under sulfate-reducing conditions, has shown removal of both, nickel and uranium from solution. In the biotic incubation that contained U(VI), reduction to U(IV) was also noted (Chapter 9.2).

In conclusion, the results presented in this thesis significantly extend our knowledge of the microbial cycling of ISA under a wide range of biogeochemical conditions and, for the first time, the biodegradability of radionuclide-ISA complexes with subsequent radionuclide immobilisation was shown. The results demonstrate that an in situ soil microbiome is likely able to remove ISA and associated degradation products from solution as a self-attenuated process. Moreover, our current understanding of a multi-barrier GDF system, comprising chemical and physical barriers, should be extended, to include a newly recognised third barrier, suggested as the ‘bio barrier’. This bio-barrier will additionally retard the migration of radionuclides into the environment. Finally, it is hoped that the results presented here can help underpin the UK Governments’ plan to create underground repositories, which are considered as the safest available long-term solution for the disposal of radionuclides. The outcome of this study will help remove some of the pessimistic concerns raised about the role of microorganisms in the deep geological disposal of nuclear waste. Previously, potentially negative impacts of microbial metabolism have been the focus of concern, for example the enhancement of radionuclide transport via biosorption to planktonic cells, and the beneficial impact of microbial metabolism on a GDF safety case have been largely ignored.
8.2. Future directions

This study has established a deeper understanding of the fate of ISA in the area surrounding a GDF under a wide range of biogeochemical conditions and in presence of different radionuclides. With regard to carbon budgets, the impact of ISA biodegradation on mineralogy and gas production was assessed, which are important aspects for inclusion in a GDF safety case assessment. A major area of interest includes the mechanism of ISA biodegradation, which appeared complex and could not be revealed by the study of enrichment cultures. Another aspect of this thesis comprised the identification of the associated microbial communities (genetic profiling, visual cell investigations), which are key to understanding microbial processes. However, it was challenging to link ISA biodegradation pathways to individual species in this study. Not surprisingly, the microbial community analysis presented here, highlights gaps in our knowledge. However, some of these gaps might be closed in the foreseeable future by the application of other advanced techniques, including the application of genomics, molecular genetics, and new analytical methods that should provide answers to these questions.

Several attempts have been undertaken to isolate a microbial strain on agar plates capable of ISA degradation under sulfate-reducing conditions, however when distinct colonies were obtained on solid agar, and growth on ISA monitored, a mixed microbial community was identified by 16S rRNA gene analysis (Chapter 9.1). Thus, it is possible that the ISA-degrading microorganisms work within syntrophic consortia and it will be a significant future goal to resolve the degradation mechanism in association within such syntrophic microbial interactions.

To shed light on the individual role of certain microorganisms in the degradation chain of ISA, heavy $^{13}$C-labelled carbon can help identify these key microorganisms and associated carbon pools without the requirement of axenic culture techniques. This stable isotope probing (SIP) technique is based on the principle that microorganisms assimilate the specific isotopic signature of the carbon they metabolise and is powerful, because it allows the study of uncultured microorganisms. SIP can be used to study $^{13}$C-labelled DNA and poly lipid fatty acids (PLFA) during a time-course based experiment, thus revealing the order and magnitude of an incorporated label. PLFAs are building blocks of cell membranes and are regarded as
biomarkers, because each individual organism has its characteristic PLFA signature. For this purpose, after incubation a lipid extract will be analysed with the $^{13}$C-labelled ISA using a gas chromatograph coupled to an isotope ratio mass spectrometer (GC-IRMS), from which $^{13}$C assimilation can be inferred. Since lipid biomarkers do not give phylogenetic information at the species level, it is useful to combine this analysis with a genetic study, using for example rRNA genes, to directly assign identified lipid patterns to a specific microorganism. For this purpose, RNA is extracted and amplified after enrichment of a microbial community on a $^{13}$C-labelled compound. This product will be ultracentrifuged to separate heavier ($^{13}$C-labelled) from lighter fragments, of which the $^{13}$C-labelled will be sequenced subsequently (Dumont and Murrell, 2005). To trace the uptake of $^{13}$C from a labelled ISA molecule into bacteria involved in its degradation, $^{13}$C-labelled ISA molecule is required, which cannot be purchased by any chemical supplier. Therefore, in the course of this thesis, it was attempted to synthesise $^{13}$C-labelled ISA by using fully $^{13}$C-labelled α-lactose monohydrate (Chapter 9.3). However, due to difficulties in the process, coupled with time constraints, this project could not be finished and tackling the $^{13}$C-ISA synthesis remains an interesting future project.

Another key aspect for future considerations is that this thesis focused entirely on a lab based approach, which was important to help understand the biochemical cycling of ISA under well-defined conditions. In the future it will be crucial to implement field scale experiments that study the geochemical fate of ISA in natural environments in the surrounding geosphere of a GDF. For this purpose, underground labs offer an opportunity to perform long-term studies that allow analyses of the fate and the stability of end products. These labs would also allow focusing on the perturbation of the wider geosphere upon ISA biodegradation, which can produce soluble degradation products (VFAs) and gases that may migrate further away from a GDF.

Alongside the aforementioned research gaps, there are some open research questions to answer, where analyses were not possible in the time period available for this project. These research goals are outlined below and the status of follow-on work is given:

- Chapter 6 “The biogeochemical fate of nickel during microbial ISA degradation; implications for nuclear waste disposal (research article)”: Complementary EXAFS data have been acquired in the weeks before submission to verify the coordination environment of the (Fe,Ni)-sulfides and are awaiting analysis.
Chapter 9.1 “Biodegradation of isosaccharinic acid (ISA) by sulfate-reducing bacteria (unpublished work)”: Microbial culturing on plates to separate microbial strains degrading ISA, resulted in a highly enriched microbial consortium. This work indicated symbiotic activity of different groups of bacteria, which are currently being characterised. The research is in preparation for submission to PLoS One. However, if $^{13}$C-labelled ISA can be generated in the future, amendment to this microbial consortium will present an interesting project that can reveal individual microbial functions within this consortium (Chapter 9.3).

Chapter 9.2 “The fate of the priority radionuclides U(VI) and Ni(II) in ISA-degrading, sulfate-reducing enrichment cultures (unpublished work)”: The data have been collected, but require further analysis. In particular, fitting of the EXAFS spectra will be focus of future work. Initial fitting indicates precipitation of nickel as a Ni-sulfide, whereas uranium was reduced and appears to be removed as U(IV)-phosphate.

Questions that open up from this project and that could lead future research into new directions include analysis of the options to reduce the amount of cellulosic materials that is co-disposed with ILW. Waste separation before disposal or pre-treatment of the wastes could be attempted to help answer this question. As an example, anaerobic microbial digestion of the waste before disposal has been considered in the US to reduce the amount of disposed cellulose (Lee and Donaldson, 1985). If it is not possible to reduce the amount of cellulose disposed of, the question emerges for a mechanism that can prevent ISA formation in first place. With this regard, a study looked at alkaline treatment in presence of anthraquinone, which would stabilise the cellulose molecule and make it less accessible for alkaline hydrolysis (Helmy and Abd El-Motagali, 1993).

In conclusion, the results presented in this thesis extend our knowledge of the potential fate of ISA under a wide range of biogeochemical conditions and includes, for the first time, analyses of the fate of radionuclides in such systems. These results have satisfactorily answered the objectives formulated at the beginning of this thesis and have opened up follow up research questions, which need to be answered to help support efforts to safely dispose of nuclear waste, while meeting the needs of future generations. The outcome of this thesis has helped add the concept of a ‘bio-barrier’ as a new barrier to the UK Governments’ proposed plan of creating underground repositories. In the future, research needs to be implemented in situ to
explore how this bio-barrier functions in combination with physical and chemical barrier that surround a real ILW repository and the effectiveness of this extended multi-barrier system needs to be assessed in mitigating the migration of radionuclides into the environment.

8.3. References


9. Appendices

This Chapter contains details of research results in preparation for manuscripts or that provided training and insights into the systems being studied. Chapter 9.1 presents a study of the biogeochemical fate of ISA under sulfate-reducing conditions thereby focusing on steep pH gradients that may develop across the near field to the far field surrounding a GDF. Chapter 9.2 is a complementary study to this research focusing on the fate of Ni(II) and U(VI) in such a system. Chapter 9.3 summarises results from a project aimed at synthesising $^{13}$C-labelled ISA, which is planned to be added as carbon source and electron donor to a soil microbiome which facilitates assigning microbial strains in situ to ISA biodegradation and to quantify associated carbon flows.

9.1. Biodegradation of isosaccharinic acid (ISA) by sulfate-reducing bacteria (unpublished work)

9.1.1. Abstract

Geological disposal of Intermediate Level nuclear Waste (ILW), involves the disposal of a range of organic compounds, such as cellulosic items, including cloth, tissue, filters, paper and wood. Upon groundwater resaturation of the cementitious waste repository and development of alkaline and reducing conditions, these items will undergo abiotic alkaline hydrolysis to form mainly isosaccharinic acid (ISA). ISA is a ligand that shows high water solubility and forms strong complexes with radionuclides, which may therefore increase their mobility in the environment. Thus, there exists interest in exploring the fate of ISA in the bio- and geosphere surrounding a GDF. Biodegradation of ISA by soil microorganisms has been found to remove ISA under a range of biogeochemical conditions, however degradation remained incomplete at alkaline pH (=10) when coupled to sulfate as an electron acceptor (Bassil et al., 2015b). Given the importance of biogenic sulfide in precipitating a range of metals and radionuclides, there is significant interest in exploring the upper pH range under which sulfate-reducing bacteria (SRB) can effectively degrade ISA. We therefore studied ISA removal by soil microbial communities obtained from an alkaline legacy lime workings site near Buxton in the UK, a model system representative of the geosphere surrounding a GDF. To do this, enrichment cultures were poised under sulfate-reducing conditions at a pH range of 7 to 10. ISA was removed up to pH 9
and analysis of bioprecipitates by XRD, TEM and ESEM revealed mackinawite [FeS] and vivianite \([\text{Fe}_3(\text{PO}_4)_{2}\cdot8\text{H}_2\text{O}]\) as the dominant biominerals formed. The microbial community in the enrichment cultures was characterised using 16S rRNA gene sequencing, and was dominated by *Elusimicrobia* and *Desulfovibrio* species, which were indicated to live syntrophically as a microbial consortium.

### 9.1.2. Introduction

Plans of the UK Government and other nuclear nations are to manage long-lived higher activity radioactive waste in the long-term through deep geological disposal. The disposal will be implemented in an engineered underground geological disposal facility (GDF) (DECC, 2014). When a suitable site is located, the radioactive waste will be immobilised within a cementitious grout in stainless steel containers placed into the host rock that are again surrounded by a cementitious backfill (DECC, 2014). The waste contents destined for disposal in a GDF include high level waste (HLW), intermediate level waste (ILW) and small portions of low level waste (LLW) not suitable for shallow disposal. The scope of this project encompasses processes in ILWs, which are very heterogeneous, including a range of inorganic and organic materials, the latter comprising cellulosic materials. It is anticipated that in post-closure times of the GDF, hyperalkaline and reducing conditions will develop due to the dissolution of the cementitious backfill and corrosion of steel components. Given the abundance of sulfate in many deep subsurface groundwaters in the UK (Metcalfe et al., 2007) and elsewhere, sulfidation reactions may dominate in the geosphere surrounding a GDF. Under the high pH conditions expected *in situ*, many of the radioactive content will be immobilised by sorption onto the cement, however the cellulosic compounds will undergo alkaline degradation leading to the formation of water-soluble organic acids (Whistler and BeMiller, 1958; Haas et al., 1967). During this hydrolysis of cellulose, glucose units are eliminated from the cellulose chain that will, in the presence of high Ca concentrations, lead to the formation of mainly isosaccharinic acid (ISA) (Glaus et al., 1999a; Vercammen et al., 1999a; Askarieh et al., 2000; Pavasars et al., 2003). ISA forms chemically strong complexes with a range of radionuclides (Baston et al., 1994b; Vercammen et al., 2001; Rai et al., 2003; Warwick et al., 2003, 2004; Rao et al., 2004; Tits et al., 2005), which might cause desorption from the engineered barrier system, thereby mobilising them into the groundwater (Bradbury and Sarott, 1995; van Loon et al., 1997).
Concern about ISA-radionuclide complexes leaching from the GDF into the bio- and geosphere has evoked a significant amount of research focusing at the (bio)geochemical fate of ISA in the vicinity of a GDF at high pH conditions (Bassil et al., 2015b; Rout et al., 2015b) and in the surrounding “far-field” geosphere at neutral pH (Charles et al., 2015; Kuipers et al., 2015). These studies showed that soil microorganisms could use ISA as the sole electron donor and carbon source, and thereby deplete it from solution. Results demonstrated full ISA biodegradation under a range of biogeochemical conditions, including when NO$_3^-$ or Fe(III) was provided as alternative electron acceptors. When SO$_4^{2-}$ was present as an electron acceptor ISA biodegradation was facilitated at neutral pH (Kuipers et al., 2015), but remained incomplete at high pH (≈10) (Bassil et al., 2015b). For this reason, research is warranted exploring the upper pH limit that will lead to ISA biodegradation under sulfate-reducing conditions. We therefore report in this study the fate of ISA in microbial soil enrichments poised under anaerobic sulfate-reducing conditions, at a pH range from 7-10. From these enrichments a stable enrichment culture was created at pH 7, from which a microbial consortium was isolated to identify key microorganisms involved in ISA degradation under the conditions imposed. These results will help modelling the fate of ISA along steep biogeochemical horizons that characterise the geosphere surrounding a GDF, and acknowledge the potential beneficial impacts of microbial metabolism on the GDF safety case.

9.1.3. Materials & Methods

**Materials.** Shallow subsurface sediments (20 cm depth) were collected from a marginal area at a location contaminated by legacy lime workings at Harpur Hill, Buxton in Derbyshire, UK. The samples were stored in the dark at 4°C until use. Ca(ISA)$_2$ was prepared from α-lactose monohydrate and Ca(OH)$_2$ following published protocols (Vercammen et al., 1999a).

**ISA degradation experiments**

*Sulfate-reducing primary enrichment cultures at pH 7 to 10.* Enrichment cultures were set up to obtain a stable sulfate-reducing microbial community able to use ISA as the electron donor. Triplicate incubations were prepared by inoculating 30 mL volumes of defined medium in 100 mL serum bottles, using an inoculum (1% vol/vol) obtained from Harpur Hill, near Buxton. A modified freshwater minimal medium (FWM) (Lovley et al., 1984) was used, containing 30 mM NaHCO$_3$, 4.7 mM NH$_4$Cl, 4.4 mM NaH$_2$PO$_4$·H$_2$O, 1.3 mM KCl, and 0.3 mL of mineral
and vitamin stock solutions (Lovley et al., 1984). Ca(ISA)₂ was added as the sole electron donor to a final concentration of 4 mM ISA and Na₂SO₄ was added as the sole electron acceptor to 12 mM. Incubations were set up, sealed with butyl stoppers and de-aerated with a N₂/CO₂ (80:20) gas mixture for 20 min. A range of pH conditions (pH 7, 8, 9 or 10) was established by adjusting the pH with small amounts of concentrated NaOH. Sterile controls (autoclaved) were also prepared as appropriate. All enrichment cultures were incubated in the dark at 20°C with periodic sampling. After measuring the redox potential (Eh; Denver Instrument Accumet digital meter with a Mettler Toledo Inlab Redox Micro ORP) and pH (Mettler Toledo FEP20 digital meter with a Fisherbrand FB68801 electrode), the samples were frozen at -20°C until further analysis. Geochemical analyses and X-ray diffraction measurements were carried out on these cultures.

**Stable enrichment incubation at pH 7.** From the primary sediment incubation at pH 7, a stable enrichment culture was created over several transfers by using consecutive inocula (1% vol/vol) added to a modified FWM medium (Lovley et al., 1984) containing 30 mM NaHCO₃, 18.7 mM NH₄Cl, 4.4 mM NaH₂PO₄·H₂O, 1.3 mM KCl, 3.7 mM KH₂PO₄, 8.1 mM MgSO₄·7H₂O, 1.8 mM FeSO₄·7H₂O, 12 mM Na₂SO₄, 10 mL L⁻¹ of mineral and vitamin stock solutions and 2 mM Ca(ISA)₂. Controls were prepared as appropriate.

**Isolation of microorganisms.** Isolation of bacterial strains was performed using the same medium as in the stable enrichment culture with a final concentration of 4 mM ISA, but before autoclaving 1% of agar was added. Agar plates were prepared from the medium in petri dishes and left for 2 weeks in an anoxic cabinet to remove residual oxygen. Isolation of cells was done in an anaerobic chamber by dropping 20 µL of sample aliquot from the stable enrichment experiment onto a plate and spreading the inoculum with a 1 µL disposable loop. Inoculated plates were sealed with parafilm and incubated upside-down in a GasPak, equipped with an oxygen indicator paper and a GasPakTM EZ anaerobe container system, at 20°C in the dark. Visible colonies formed after a few days and were sub-cultured by picking and streaking on a new plate, which was incubated again. After several sub-cultures, liquid media, as above, were inoculated with clearly visible, isolated colonies and tested after a few weeks for ISA removal by IC measurement. One isolate showed ISA removal in liquid medium, and was transferred twice.
to fresh medium, as used for the stable enrichment culture, before 16S rRNA gene sequencing was performed.

**Mineralogical characterisation.** Powder X-ray diffraction (XRD) crystallography, Transmission Electron Microscopy (TEM) and Environmental Scanning Electron Microscopy (ESEM) imaging were used to characterize biominerals from ISA biodegradation under sulfate-reducing conditions. For analytical details the reader is referred to Chapter 5.4 and 6.5.

**16S rRNA gene sequencing.** 16S rRNA gene sequencing was performed with the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a Roche ‘Fast Start High Fidelity PCR System’ (Roche Diagnostics Ltd, Burgess Hill, UK). For analytical details the reader is referred to Chapter 6.5.

9.1.4. **Results and discussion**

ISA is predicted to be mobile in groundwater and due to its complexing abilities it might eventually transport radionuclides in the groundwater. In the UK, many groundwaters are sulfate-rich (Metcalfe et al., 2007), which may promote the activity of sulfate-reducing bacteria (SRB). Focusing on SRB, previous soil enrichment cultures using ISA as the sole carbon source and electron donor have demonstrated complete removal of ISA at neutral pH (Kuippers et al., 2015), while at alkaline pH (=10) no ISA removal was observed over the time period tested (Bassil et al., 2015b). For this reason, in this study the upper pH boundaries were tested that may lead to ISA biodegradation with sulfate as the electron acceptor in the geosphere surrounding a GDF.

**Sulfate-reducing primary enrichment cultures; pH 7 to 10**

Soil enrichment cultures were set up to explore the fate of ISA under sulfate-reducing conditions at various pH values, ranging from 7 to 10, representing the pH gradient that will extend from the alkali-disturbed zone adjacent to an ILW GDF ('near field'), to the wider geosphere ('far field'). The inocula used were obtained from a high pH lime-kiln impacted site.
In the biotic incubations, ISA concentrations dropped shortly after incubation started at pH 7, 8 and 9, whereas no decrease was observed at pH 10. At pH 7, ISA was depleted by day 35, whilst at pH 8 and 9 no ISA was detected after 77 days. ISA removal at pH 7, 8 and 9 was accompanied by development of reducing conditions to a redox potential of -300 mV. In addition, sulfate levels decreased in solution (Figure 9.1 C), concomitantly with ISA removal in the biological active incubations up to pH 9. After an extended incubation time (126 days), the experiment at pH 10 was characterised by a drop of approximately 35% of ISA concentration, together with production of 2 mM acetate and a change to a more reducing conditions. Interestingly, the extent of sulfate reduction was lower in enrichments with higher pH resulting in...
73% (pH 7), 45% (pH 8), 16% (pH 9) to 1% (pH 10) of total sulfate reduction. Similarly, the extent of acetate production in solution was lower at higher pH values (Figure 9.1 D). The accompanying sterile control showed no change in ISA or sulfate concentration, and the redox potential remained stable, consistent with ISA removal being due to microbial activity (126 days; Figure 9.1).

These enrichment incubations have demonstrated that ISA degradation can occur at fast rates at a pH of ≤9 and is concomitant with sulfate reduction, and indications are given that over extended incubation time periods small amounts of ISA are biodegraded in alkaline environments with a pH as high as 10. With increasing pH, a slower rate of ISA biodegradation and lower amounts of sulfate reduction were observed, indicating a cessation in microbial activity, presumably as a result of the decreasing energy yield from sulfate reduction at high pH (Rizoulis et al., 2012).

Isolated ISA biodegrading, sulfate-reducing microbial community

Geochemistry

A stable enrichment incubation was created from the initial soil incubation at pH 7, and considered to be more representative for ‘far field’ conditions. Aliquots were streaked onto agar plates from this culture, to isolate microbial strains actively involved in ISA biodegradation. The same ISA/sulfate amended medium that was used in the liquid cultures was used to prepare the agar plates. Colonies from the plates were re-streaked to help obtain axenic cultures, and then

Figure 9.2. ESEM images of late log phase cells from ISA biodegradation experiment poised under sulfate-reducing conditions. Left image shows cells among fine-grained particles, containing Fe and S, right image shows cells surrounding a plated vivianite [(Fe₃PO₄)₂·8H₂O] crystal identified in EDS spectra (not shown).
single colonies inoculated back into liquid medium. However, these isolates lost the ability to degrade ISA (tested with approximately 40 picked colonies). Therefore a sweep across several colonies was re-inoculated into liquid medium, which resulted in ISA biodegradation accompanied by sulfate reduction which could be maintained over 8 consecutive transfers (thus far). This ‘isolated’ microbial community was studied further.

A mineralogical study was performed on biominerals precipitated by the isolated microbial community using XRD, ESEM and TEM analysis. XRD results revealed two sharp peaks identified as mackinawite [FeS] and vivianite [Fe₃(PO₄)₂·8H₂O] by comparison with the standard library. Mackinawite is a common end product from microbial sulfate reduction that generates HS⁻ as a sink for the precipitation of Fe, and other divalent ions, including Ni, Cu and Zn (Ohfuji and Rickard, 2006; Gramp et al., 2007, 2010b; Veeramani et al., 2013). Mackinawite is considered a precursor to more stable forms, such as pyrite and marcasite (Schoonen and Barnes, 1991; Csákberényi-Malasics et al., 2012). Thus, sulfidogenesis constitutes an important sink in sequestering metals (Lloyd and Lovley, 2001; Gramp et al., 2010b).

**Molecular Ecology**

Cells were also fixed and viewed under ESEM, which revealed dense cell agglomerations among mineral precipitates (Figure 9.2). The cell agglomerations were too dense to allow measurement of individual cells, but they appeared to be of elongated shape. 16S rRNA gene sequencing of this ‘isolated’ microbial community showed a dramatic decrease in microbial diversity from approximately 840 species in the original soil inoculum to <100 species in the ‘isolate’ microbial community. The 16S rRNA gene profile comprised two dominant classes most closely affiliated with *Elusimicrobia* and *Deltaproteobacteria*, representing 87% of the whole microbial community (Figure 9.3). However, strains belonging to 11 different Classes were identified, although these comprised of no more than 3% in total abundance.
Sequences affiliated with *Elusimicrobia* (58.7 % of the total number of sequences), belonging to the Order *Lineage I* related to the “*Endomicrobia*” (100% match), are cellulolytic flagellates that have been reported in the gut of termites in co-existence with bacterial symbionts (Ikeda-Ohtsubo and Brune, 2009). These flagellates are specialised in the degradation of lignin-containing compounds and many are unique to the gut of termites and wood-feeding cockroaches (Ikeda-Ohtsubo and Brune, 2009; Zheng et al., 2015). However, a study of 16S rRNA gene sequences from other habitats has revealed many sequences that are related to the TG-1 phylum, even though only distantly (Herlemann et al., 2007). The study of termite gut microorganisms has been impeded by a general resistance to cultivation, and may be as a result of symbiosis (Huber et al., 2002; Brune and Stingl, 2006; Brune and Ohkuma, 2010). A similar problem was encountered in this study, in the attempt to isolate an axenic culture able to couple ISA oxidation to sulfate reduction. The other dominant Class comprised members affiliated with the *Deltaproteobacteria* (28.2% of total abundance). This Class contained members well known for the reduction of sulfate (Figure 9.4). The most abundant representatives of this group were associated with sulfate reduction and were most closely affiliated with *Desulfovibrio* spp. (55.1% of the *Deltaproteobacteria*; Postgate and Campbell, 1966), followed by another sulfate reducer *Desulfovirga adipica strain TsuA1* (22.7%; Fullerton et al., 2013) and the Fe(III) reducer *Geobacter* spp. (16.4%; Caccavo et al., 1994).

![Figure 9.3. 16S rRNA gene sequencing data from the initial sediment inoculum (T0) and the isolated ISA-degrading, sulfate-reducing consortium (Isolate) showing the microbial community diversity.](image_url)
The isolation of microbial species from the experiments conducted at pH 7, resulted in a steep decrease of microbial diversity, however a single strain could not be isolated with ISA and sulfate. Growth on ISA was only noted in two (from over 40) bottles of liquid medium inoculated with single colonies grown on agar plates containing ISA and sulfate. This could be for two reasons, the species coupling ISA oxidation to the reduction of sulfate either did not grow on solid plates, or the biodegradation proceeded via a mixed consortium of bacteria. In this experiment, the dominance of sequences affiliated with the *Elusimicrobia*, known symbiotic microorganisms in cellulose-fermenting habitats, and the *Deltaproteobacteria*, known sulfate-reducing bacteria, suggests a symbiotic relationship between the two Classes involved in the degradation of ISA.

**9.1.5. Summary and future work**

Steep biogeochemical gradients are expected to develop between the ‘near field’ adjacent to a GDF and the ‘far field’ geosphere surrounding a GDF. The biological fate of ISA leached from a GDF is of major interest for the GDF safety assessment. Sulfate-reduction may eventually dominate in and around a GDF, as sulfate in cements and groundwaters can be present at around 9 mM in brackish saline waters, and up to 50 mM in other UK groundwaters (Metcalf *et al.*, 2007).

Figure 9.4. The class of *Deltaproteobacteria* in the ISA-degrading, sulfate-reducing consortium (Isolate) analysed by 16S rRNA gene sequencing.
This study has demonstrated that a stable microbial enrichment culture, obtained from an alkaline site with elevated Ca\(^2+\) concentrations, can use ISA as the sole electron donor with sulfate as the electron acceptor. Consistent with a previous study under similar conditions, where microbial ISA oxidation under sulfate reducing conditions was not feasible at alkaline pH (\(\approx 10\)) (Bassil et al., 2015b), sulfate reduction proceeded in this study up to pH 9. Microbial metabolism resulted in the formation of poorly soluble mackinawite [FeS] and also crystalline vivianite \([\text{Fe}_3(\text{PO}_4)_2\cdot 8\text{H}_2\text{O}]\). The formation of sulfides, such as mackinawite, is advantageous for the disposal of nuclear waste, as they may immobilise a broad range of metals and radionuclides by precipitation. Sulfate-reducing bacteria have also been shown can exert control on the solubility of a range of radionuclides via reduction, including Tc(VII) (Lloyd et al., 1998) and U(VI) (Lovley and Phillips, 1992). Therefore microbial attenuation of ISA by SRB could be a significant process in the geosphere surrounding a GDF that can retard the transport of ISA into the bio- and geosphere and might ultimately exert significant control on the mobility of priority radionuclides. The impact of ISA biodegradation by SRB-containing consortia and in the presence of radionuclides is clearly an area where further research would be valuable. For this reason, further research on the fate of Ni(II) and U(VI) during ISA biodegradation by an enrichment culture of SRB is presented in Chapter 9.2.
9.2. The fate of the priority radionuclides U(VI) and Ni(II) in ISA-degrading, sulfate-reducing enrichment cultures (unpublished work)

9.2.1. Abstract

The UK’s geological disposal concept employed for the long-term management of intermediate level radioactive waste (ILW) aims at immobilising the ILW within a cementitious grout filled into steel drums and placed in a GDF. Eventual resaturation with groundwater will lead to dissolution of the cements and corrosion of metal items, thereby creating a hyperalkaline and reducing environment, which will immobilise most of the radioactive content. However, the co-disposal of organic materials, including cellulosic items, can significantly affect waste stability by abiotic alkaline degradation that forms ligands, of which isosaccharinic acid (ISA) is the most significant due to its ability to form strong complex with radionuclides. Anaerobic biodegradation of ISA has been considered effective in removing ISA from solution, however studies investigating the fate of metals and radionuclides complexed by ISA are lacking. In particular, high sulfate concentrations in UK groundwaters can be important in stimulating sulfate-reducing bacteria (SRB). Therefore, in this study enrichment cultures were set up with ISA as the sole carbon source and electron donor for sulfate reduction, and these cultures were used to assess the potential for immobilisation of Ni(II), and U(VI), in sulfidic systems. 16S rRNA gene sequencing of the associated microbial community identified known SRB affiliated with the Deltaproteobacteria, but the Ni incubation was dominated by sequences affiliated with Elusimicrobia, while cultures with Ni and U were dominated by Firmicutes. The fate of Ni(II) and U(VI) was studied, using ICP, TEM, ESEM and XAS techniques. Ni(II) was shown to be removed and associated with sulfide minerals, whilst U(VI) was reduced to U(IV) phosphate minerals, which both occurred only in biologically active microcosms.
9.2.2. Introduction

As introduced in Chapter 9.1, isosaccharinic acid (ISA) is predicted to dominate as main degradation product from the disposal of intermediate level radioactive wastes (ILW) (Machell and Richards, 1960; Glaus et al., 1999a; Vercammen et al., 1999a; Askarieh et al., 2000; Pahasars et al., 2003). ISA is a stable ligand with known complexing ability for metals and radionuclides, including Ni(II) (Warwick et al., 2003; Almond et al., 2016), and U(IV) (Warwick et al., 2004) and U(VI) (Baston et al., 1994b; Rao et al., 2004) under alkaline conditions. For this reason, concerns have been raised about a decreased sorption of these metals and radionuclides, which could lead to mobilization and facilitate the transport in groundwater into the geological surrounding of a GDF (van Loon and Glaus, 1998a).

There has been a great deal of research focusing on the (bio)geochemical fate of ISA in a GDF and the geological surrounding (e.g. Bassil et al., 2015b; Kuipers et al., 2015; Rout et al., 2015a). This research has mainly focused on microbial metabolism using ISA as a carbon source and electron under high pH cementitious conditions (Bassil et al., 2015b; Rout et al., 2015b) and at neutral pH conditions more representative for the geological surrounding of a GDF (Charles et al., 2015; Kuipers et al., 2015). Less work has been done on the biodegradation of ISA-radionuclide complexes. Under the reducing conditions in the deep terrestrial subsurface surrounding a cementitious GDF, sulfate may become an important electron acceptor for microbial metabolism, as sulfate concentrations can be high (up to 50 mM) in UK groundwaters (Metcalfe et al., 2007). The stimulation of a sulfate-reducing microbial community by ISA that leaches from a GDF into the geological surrounding may impact on its biogeochemical fate and also the geochemistry of the geosphere surrounding such a GDF. Thus, in a previous study conducted at high pH (=10) the diminishing energy yield from sulfate reduction at high pH inhibited ISA biodegradation (Bassil et al., 2015b), whilst under neutral pH conditions, representative for the geological surrounding of a GDF, ISA was degraded completely (Kuipers et al., 2015). A third study formed the link between these two experiments by investigating microbial ISA biodegradation in sulfate-reducing enrichment cultures at a range of pH values (7 to 10), whereby no ISA biodegradation was observed beyond pH 9 (Chapter 9.1).
Given the importance of sulfate in UK groundwaters and the demonstrated ISA biodegradation under a range of pH values in the geological surrounding of a GDF, this work addresses the paucity of data in the biogeochemical cycling of ISA in the presence of metals and radionuclides under sulfate-reducing conditions. Ni(II) and U(VI) were chosen as the metals for study. Ni presents a key radionuclide for nuclear waste disposal, which is used mainly in steel alloys used for nuclear reactor steel components (NDA, 2010f, 2014) to reduce their corrosion (Platt et al., 1997). Ni can also be irradiated thereby forming the activation products Ni-63 (half-life 9.9 x 10^5 years) and Ni-59 (half-life 7.6 x 10^4 years). In addition, sulfate reduction is a very well-known sink to precipitate Ni(II) from solution (Hammack and Edenborn, 1992; Gramp et al., 2007). The radionuclide U(VI) was also investigated, as it will be the dominant radionuclide by mass and shows a complex redox geochemistry (RWM and NDA, 2015), being reduced to its less soluble oxidation state (U(IV)) by SRB (Fletcher et al., 2010; Stylo et al., 2015). Sediments from an alkaline legacy lime workings site in Buxton, U.K., served as model system for this study, to characterise the impact of microbial ISA degradation on the biogeochemistry Ni(II) and U(VI) and identify associated biomineral phases. The overall aim of this study is to help determine the impact of SRB in moderating the transport of ISA and associated metals and radionuclides in sulfate-rich groundwaters in the geosphere surrounding a cementitious GDF.

9.2.3. Materials & Methods

Sediment acquisition. Surface sediment samples were collected from a depth of approximately 20 cm with a pH of 7, at a site contaminated by legacy lime workings at Harpur Hill, Buxton in Derbyshire, UK. The samples were stored in the dark at 4°C until use.

Ca(ISA)_2 preparation. α-lactose monohydrate and Ca(OH)_2 was used for preparation of Ca(ISA)_2 following the protocol of Vercammen et al. (1999a).

Pre-grown enrichment cultures. Enrichment cultures were set up to create stable enrichment cultures of a sulfate-reducing microbial community able to use ISA as the electron donor at pH 7. Triplicate incubations were prepared by adding a sediment inoculum (1% vol/vol) to a modified minimal medium (Lovley et al., 1984), containing 30 mM NaHCO_3, 18.7 mM NH_4Cl, 4.4 mM Na_2HPO_4·H_2O, 1.3 mM KCl, 3.7 mM KH_2PO_4, 8.1 mM MgSO_4·7H_2O, 1.8 mM FeSO_4·7H_2O, 12 mM Na_2SO_4 and 10 mL L\(^{-2}\) of mineral and vitamin stock solutions. The medium was de-aerated with a N_2/CO_2 (80:20) gas mixture for 20 min thereby adjusting the pH
to 7. Ca(ISA)$_2$ was added as the electron donor to a final concentration of 4 mM ISA. The bottles were incubated in the dark at 20°C with periodic sampling to check for biological activity (pH, Eh, ISA concentration).

**ISA biodegradation/Ni(II) immobilisation experiment.** Stock solutions of 0.1 M and 0.01 M NiCl$_2$·6H$_2$O ($n = 237.7$ g/mol) were prepared with deionised water and filter-sterilized prior to use (0.22 µm, diam. 33 mm, Millex-GP, Sigma-Aldrich). Enrichment cultures were prepared, using a 1% vol/vol inoculum from the pre-grown enrichment cultures in a modified FW containing sulfate as above, to which Ca(ISA)$_2$ was added to a final concentration of 4 mM ISA as sole carbon source and electron donor. Respective amounts of nickel stock solution were added to create (i) no added Ni(II); (ii) 0.1 mM Ni(II); and (iii) 1 mM Ni(II) enrichment cultures. Controls were prepared as appropriate, comprising (iv) a no electron donor control at 1 mM Ni(II) (without ISA) and (v) a sterile control (autoclaved) at 1 mM Ni(II) concentration. The bottles were de-aerated with a N$_2$/CO$_2$ (80:20) to create anaerobic conditions and to adjust the pH at 7. The bottles were incubated in the dark at 20°C. Following periodic sampling, redox potential (Eh; Denver Instrument Accumet digital meter with a Mettler Toledo Inlab Redox Micro ORP) and pH (Mettler Toledo FEP20 digital meter with a Fisherbrand FB68801 electrode) were measured of the aliquots, before the samples were frozen at -20°C until further analysis. For this experiment, the solution geochemistry, mineralogy and microbial community composition were studied.

**ISA biodegradation/U(VI) immobilisation experiment.** Enrichment cultures were prepared in triplicate using the modified FW containing sulfate as above, to which Ca(ISA)$_2$ was added to a final concentration of 4 mM ISA as sole carbon source and electron donor. Uranium was added, as uranyl chloride (UO$_2$Cl$_2$), to create (i) 0 mM U; (ii) 0.1 mM U; and (iii) 1 mM U enrichment cultures. Controls were prepared as appropriate, comprising (iv) a no electron donor control at 1 mM U (without ISA) and (v) a sterile control (autoclaved) at 1 mM U concentration. The bottles were de-aerated with a N$_2$/CO$_2$ (80:20) to create anaerobic conditions, a 1% vol/vol inoculum and the enrichment cultures were incubated in the dark at 20°C. Sampling was done as for the Ni-ISA complex biodegradation experiment.

**Solution geochemistry.** ISA and organic acids from the incubations with Ni and U were analysed using ion exchange high performance liquid chromatography (IE-HPLC), as described
Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) was used to analyse total Ni in solution. Samples were prepared centrifuged and diluted to below <10 ppm Ni in 2% nitric acid. Samples were analysed together with standards (from 10 ppm stocks obtained from VWR) on a Perkin-Elmer Optima 5300 DV. For quantification of U(VI) in solution spectrophotometric analysis with 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol was performed at 578 nm (Johnson and Florence, 1971).

**Mineralogical characterisation.** Powder X-ray diffraction (XRD) crystallography, Transmission Electron Microscopy (TEM) and Environmental Scanning Electron Microscopy (ESEM) imaging were used to characterise biominerals from ISA biodegradation under sulfate-reducing conditions. For analytical details the reader is referred to Chapter 5.4 and 6.5.

**16S rRNA gene sequencing.** 16S rRNA gene sequencing was performed with the Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a Roche ‘Fast Start High Fidelity PCR System’ (Roche Diagnostics Ltd, Burgess Hill, UK). For analytical details the reader is referred to Chapter 6.5.

**Speciation and coordination analysis.** To determine speciation and coordination of uranium and nickel at the end of the biodegradation experiments, spectra of the Extended X-ray Absorption Fine Structure (EXAFS) were collected of the L_{III}-edge of U using the B18 XAS beamline at the Diamond Light Source, Harwell, UK. For analytical details the reader is pointed to Chapter 7.3.

### 9.2.4. Results

Microbial metabolism of ISA has been studied under a range of biogeochemical conditions, including sulfate reduction, (Bassil et al., 2015b; Kuipers et al., 2015, n.d., Rout et al., 2015a; b). However, at high pH, representative for the “near field” environment, ISA degradation was prevented under sulfate-reducing conditions over the time periods tested (Bassil et al., 2015b). For this reason, this study looked at the fate of metals and radionuclides in ISA-degrading cultures supplied with sulfate as an electron acceptor under neutral pH conditions, relevant for the “far field” geosphere surrounding a GDF.
The first experiment investigated the fate of a Ni in an ISA biodegradation experiment under sulfate-reducing conditions at a range of concentrations comprising no added Ni(II), 0.1 mM and 1 mM Ni(II) (Figure 9.5). The ISA concentration decreased in the incubations with a microbial inoculum and no added Ni or 0.1 mM Ni after an initial lag phase of 7 days, and was fully depleted after 42 days of incubation. This decrease was accompanied by the formation of approximately 4 mM acetate while sulfate decreased from 22 mM to 16 mM. In the incubation at
1 mM Ni, no changes in the geochemistry were observed, similar to a control incubation which contained a sterile inoculum (heat-killed). Despite Ni being shown to be completely soluble in this medium in the presence of ISA (Chapter 6), monitoring of the soluble Ni concentration in this experiment showed Ni had a lower solubility at that start of the incubation. In the incubations containing 1 mM Ni amendments about 24-40% of the Ni remained in solution, whereas at 0.1 mM Ni 60% was soluble. When ISA biodegradation commenced at 0.1 mM Ni, a concomitant decrease in soluble Ni was observed, which dropped to 9%. This suggests that approximately 40% of the Ni precipitated, probably due to sorption before ISA biodegradation started, and 51% precipitated mediated by the biodegradation of ISA. A drop of the Ni concentration was observed in the cultures with 1 mM Ni added and in the sterile control, but

![Figure 9.6. ESEM images of biominerals from Ni-ISA biodegradation experiment at 0.1 mM Ni concentration showing vivianite [(Fe₃PO₄)₂·8H₂O] crystal (top image) and sulfide aggregates containing Fe and Ni (middle and bottom image).](image)
20% remained in solution. Similarly, the Ni concentration in the no electron donor control remained stable. These results suggest that microbially-mediated ISA degradation enhanced the removal of Ni from solution.

Following geochemical analyses, the Ni-containing precipitates in the 0.1 mM Ni amended cultures were subjected to mineralogical analyses, including XRD, TEM and ESEM. Consistent with results from Chapter 9.1, XRD analysis identified mackinawite [FeS] and vivianite [Fe₃(PO₄)₂·8H₂O] as biominerals (Figure 9.6). ESEM images revealed a crystalline phase that formed layers of minerals, comprising P, Fe, Mg, and O, consistent with vivianite, and second phase of unordered aggregates containing S, Fe and Ni, consistent with mackinawite (Figure 9.6). TEM results (not shown here) indicated that in these experiments Ni was associated with sulfide minerals, which are known scavengers for metals and present stable sinks (Gramp et al., 2007; Paulo et al., 2015).

**ISA biodegradation/U(VI) immobilisation experiment.**

The ISA removal was noted in all of microbially active cultures of the ISA biodegradation/U(VI) immobilization experiment, regardless of the amended concentration of U(VI) (no U(VI), 0.1 mM and 1 mM). After a lag phase a decrease of ISA was observed in all incubations containing an active inoculum (Figure 9.5). ISA was removed by day 63 at 0.1 mM U and with no U, whereas ISA removal took slightly longer with 1 mM U (77 days). ISA removal was accompanied by acetate production, reaching between 3-4 mM acetate and concomitant with the removal of approximately 20% sulfate. Abiotic control incubations (heat-killed or without an electron donor) showed no significant change in the ISA and sulfate levels. Despite using a medium buffered with 30 mM bicarbonate aimed to keep U(VI) in solution (Lovley et al., 1991b, 1993b), the

![Figure 9.7. ESEM image of biovivianite from U-ISA biodegradation experiment at 1 mM U concentration.](image-url)
uranium added to this experiment appeared fairly insoluble and precipitated from solution by day 7 to below the detection limit by ICP-MS. Since ISA removal was noted from day 14 and no other electron donor was present, and the same trend was observed in abiotic controls, the U removal was suggested to be as a result of abiotic processes. In this experiment, ISA was removed at all U concentrations, which was in contrast to the biodegradation experiment with Ni, and suggests lower chemical toxicity of U to microorganisms.

Precipitates in these microcosms were studied before and after incubation by XRD analysis. The only crystalline phase identified with XRD at incubation start was a mixture of metaankoleite and metanatroautunite (data not shown), uranyl-phosphate minerals, whereas after biodegradation of ISA vivianite was identified as the only crystalline biomineral. The presence of vivianite was confirmed with EDS analysis of ESEM images (Figure 9.7). In addition, TEM and ESEM images indicated the presence of irregular agglomerations of crystals in spherical shape (Figure 9.8 A, B). EDS analysis revealed high amounts of U together with P, S, Ca, and Fe. These minerals appear to be Ca-phosphates similar to whitlockite or apatite, and
the high contribution of Fe and S might be contributed by mixture of mackinawite. Indication for mackinawite was found in images with less ordered crystals, which were dominated by peaks of Fe and S with a small contribution of U (Figure 9.8 D, F) and had diffraction rings that matched mackinawite (Figure 9.8 E). Uranium L$_{III}$-edge XANES analysis of the precipitates after biodegradation, showed reduction of U(VI) to U(IV) (Figure 9.9). Thus, the U(VI)-phosphate minerals that were present initially, disappeared after biodegradation of ISA and thus microbial ISA metabolism seems to have affected the fate of U(VI) in this system. U(VI) from the precipitates was indicated to be reduced to U(IV) and was associated with mainly Ca and P in EDS analyses. Due to a lack of a defined U(VI) mineral peak in the XRD analysis, this U(IV) phase seems to be poorly crystalline. Further analysis of the EXAFS data collected in the weeks before submission, are expected to reveal the nature of the biomineral.

![Uranium L$_{III}$-edge XANES](image)

Figure 9.9. Uranium L$_{III}$-edge XANES spectra for U(VI)-ISA experiment at 1 mM U concentration after biodegradation collected at beamline B18, Diamond Lightsource, Harwell.

**9.2.5. Future work**

This study demonstrated the ability of SRB enrichment cultures to degrade ISA in the presence of 0.1 mM Ni and up to at least 1 mM U under conditions representative of the geosphere surrounding a GDF. Sulfidisation had an impact in enrichments with Ni, where Ni was immobilised in (Fe,Ni)-sulfides. In the U(VI)-ISA biodegradation experiment, U(VI) was insoluble, however microbial ISA metabolism affected the speciation of U(VI) which was reduced to U(IV), and seemed to be associated with phosphate. Complementary XAS data and 16S rRNA gene profiles could not be completed in the time of this thesis. However, initial fitting of EXAFS spectra indicate a Ni-sulfide phase and a U(IV)-phosphate phase in the biodegradation experiments at 0.1 mM Ni and 1 mM U, respectively.
9.3. $^{13}$C-labelled ISA biomarker studies

One of the biggest challenges in microbial ecology is the identification of microorganisms carrying out specific metabolic processes in the natural environment, which is traditionally carried out by culturing and using the isolates to draw conclusions on their metabolic role in situ. However, most soil organisms are difficult to characterise by conventional cultivation techniques and it is estimated that between 80-99% of all microorganisms remain uncultured (Amann et al., 1995). This indicates that investigations based on bacterial isolates (e.g. by growth on agar plates) only account for a minor part of the total microbial population. Due to the large number of microorganisms that cannot be cultured to date, there is strong emphasis on the use of culture-independent approaches (Torsvik et al., 1998). Currently, using biomarker approaches presents a valid alternative to overcome the problem of selective culturing. Therefore, the implementation of a stable isotope biomarker assay will be discussed in the context of geological nuclear waste disposal that may help linking biomarkers obtained from microorganisms actively involved in ISA biodegradation.

9.3.1. Stable Isotope Probing

Stable-isotope probing (SIP) is a powerful culture-independent approach that links the genes of bacteria to their metabolic activity. SIP has been used to determine the assimilation of specific substrates into uncultivated organisms in various environments (Radajewski et al., 2000; Dumont and Murrell, 2005). This technique identifies the active population of bacteria utilising the $^{13}$C-labelled carbon among the total bacterial population. Most commonly SIP employs $^{13}$C-labelled organic or inorganic carbon compounds, but other compounds with a stable isotope can be studied too, including deuterated water (Wegener et al., 2012) or $^{15}$N-amino acids (Charteris et al., 2016). The labelled compound will be introduced into a microbial community and will be tracked after a certain incubation time in microbial cell components, such as membrane lipids, DNA or RNA, that provide phylogenetic information and are thus representative as biomarkers. SIP elucidates the active population among a whole population of bacteria and has been used to determine the growth capabilities of uncultivated organisms in various environments (Radajewski et al., 2000; Dumont and Murrell, 2005). SIP also distinguishes between active and dormant cells, because only living cells are able to incorporate the label into their biomass.
Most studies to date are designed with a single fully labelled $^{13}$C-substrate or a combination of $^{13}$C-enriched growth substrates that have been added to an environmental sample or to the field under specified incubation conditions. Typically after a distinct incubation time, $^{13}$C-labelled DNA or lipid membranes are analysed as biomarkers. There are several approaches to link microbial community function with specific phylogenetic groups using DNA, RNA, fluorescence in situ hybridisation and secondary ion mass spectrometry (FISH-SIMS), small-subunit rRNA or polar lipid derived fatty acids (PLFA). Two of these techniques will be highlighted in the following, as these are relevant to the intended project investigating the fate of $^{13}$C-ISA in a biodegradation experiment that makes use of a combination of lipid and DNA biomarkers:

### 9.3.2. SIP using PLFAs

Although the term SIP was first used with DNA biomarkers (Radajewski et al., 2000), the first published example of SIP used polar lipid-derived fatty acids (PLFA; Boschker, 1998) which were isotopically labelled. Two years later, SIP was used in combination with nucleic acids as biomarker molecules (Radajewski et al., 2000).

For the analysis of $^{13}$C-labelled lipids, a lipid extraction is required from which extracts can be separated if lipids of a target organism are already known (e.g. the lipids from archaea vs. bacteria or living vs dead bacteria). The separation of the lipid extract is performed using a gas chromatograph coupled to high performance liquid chromatograph (GC-HPLC). The separated extract is injected to a combustion furnace (GC-C) equipped with an isotope ratio mass spectrometer (IRMS), in order to determine $^{13}$C/$^{12}$C isotope ratios of individual lipid compounds.

This technique comes with two draw-backs. First, with a few exceptions of biomarkers, lipids are not highly phylogenetically specific molecules. This is because many of the same fatty acids are produced by a broad range of organisms, and fatty acid profiles can change with environmental conditions. The obtained fatty acid extract thus tends to represent a group of different organisms. A second disadvantage is that lipid profiles of organisms which have not yet been cultured are unknown.

### 9.3.3. SIP using DNA or RNA

DNA and RNA are the most informative taxonomic biomarkers and the 16S rRNA data bases are robust offering strong capabilities to identify phylogenetic relatives. DNA- and RNA-SIP are
usually aimed by PCR amplification at discovering 16S ribosomal RNA gene sequences to identify enriched microbial strains (Figure 9.10).

After exposure of several microbial generations to a labelled substrate (usually $^{13}$C), DNA or RNA of the organisms capable of growth on the labelled substrate can be purified from unlabelled nucleic acid following isopycnic centrifugation (Kreuzer-Martin, 2007). The genetic content is typically amplified by the polymerase chain reaction (PCR) or reverse transcription (RT) PCR using primers complementary to 16S rRNA or other functional genes. Since the nucleic acids of organisms that have incorporated the $^{13}$C have a higher buoyant density, it can be subjected to either buoyant denaturing gradient gel electrophoresis (DGGE), be cloned directly, or subjected to terminal restriction fragment length polymorphism analysis for phylogenetic characterisation. Typically only the densest DNA or RNA, containing the isotopically most enriched genes, have been subjected to analysis (e.g. Morris et al., 2002b; Hutchens et al., 2004; Singleton et al., 2005). This technique does not require measurement of isotope ratios, however sometimes adjunct measurements to the experiments are made, for example for respired CO$_2$.

The first published study of DNA-SIP produced pure cultures amended with $^{13}$C-methanol and $^{13}$C-methane (Radajewski et al., 2000). These cultures clearly demonstrated an increase in density of DNA by isopycnic density centrifugation in CsCl compared to cultures fed with $^{12}$C-substrates. This approach is quite effective when enough DNA can be collected that delivers robust taxonomic information. At the same time, replication, which is required for $^{13}$C-assimilation into DNA, is its major disadvantage. This may require longer incubation times of typically more than 20 days (Morris et al., 2002a; Wellington et al., 2003). Shorter incubation times resulted in difficulties isolating labelled DNA, whereas longer incubation periods resulted in difficulties from cross feeding whereby $^{13}$C substrate is taken up by organisms other than the primary user resulting in less specific labelling (Manefield et al., 2002; Gallagher et al., 2005). Due to these complexities, there are not many studies using DNA as a biomarker in SIP thus far (Friedrich, 2006; Uhlík et al., 2009). One advantage of RNA compared to DNA is its high copy rate while it uses the same genomic information. Since RNA is labelled faster, RNA-SIP studies have generally used shorter incubation (Manefield et al., 2002, 2004).
In summary, SIP provides an important new tool for investigating members of microbial communities that are directly involved in biodegradation of polymeric substrates. However, SIP studies need to be implemented carefully, in order to avoid misleading growth conditions that trigger the increase of ecologically irrelevant populations (Madsen, 2006). When environmental

Figure 9.10. A) A label, likely $^{13}$C, is introduced into a microcosm with an environmental culture and then incubated until analyses. B) After the incubation time some of the $^{13}$C label was incorporated and samples will be subjected to analysis, such as PCR and gene cloning, to reveal phylogenetic information about the microorganisms (modified after Dumont and Murrell, 2005).

In summary, SIP provides an important new tool for investigating members of microbial communities that are directly involved in biodegradation of polymeric substrates. However, SIP studies need to be implemented carefully, in order to avoid misleading growth conditions that trigger the increase of ecologically irrelevant populations (Madsen, 2006). When environmental
conditions are modelled adequately, this technique poses a vast achievement in molecular microbiology in contrast to established molecular analyses which do not distinguish between active and dormant organisms. All delineated approaches have their advantages but lack other features. Therefore, a combination of different tools is gaining more attention. For example, a group studied the functional diversity of sulfate-reducing bacteria in marine sediments by using DNA and PLFA biomarkers (Webster et al., 2006). In this case, a lipid analysis that helps to identify a whole active population even with small labelling amounts and after a short incubation time, combined with a genetic analysis that gives detailed phylogenetic information, was a strong tool to describe uncultured microbial activities in a complex environment. A similar approach using this promising biomarker combination is also developed for the experiments in this project.

9.3.4. Applications for SIP relevant to contamination/bioremediation

Since the development of SIP, there has been an increasing number of studies using the novel molecular technique for bioremediation approaches, owing to its suitability as tool to study the biodegradation of compounds in the environment via the metabolism of microorganisms. In particular, in complex environments with a broad variety of different organisms involved in elemental cycling that conceal biogeochemical pathways of compounds, this approach provides an enormous potential to identify microorganisms involved in degradation of natural and anthropogenic xenobiotic compounds.

SIP is an excellent technology to trace the fate of contaminants, such as the mobility of arsenic in aquifers. Arsenic is a problematic groundwater contaminant, of which microbial reduction from As(V) to As(III) under anoxic conditions leads to its mobilisation. To reveal active members of a microbial community involved in As reduction, several studies have added $^{13}$C-labelled organic compounds, such as acetate, to incubation experiments (e.g. Héry et al., 2015). Further examples include [methyl-$^{13}$C]-toluene (Pelz et al., 2001), deuterium-labelled styrene (Alexandrin et al., 2001) or a carefully planned field study employing $^{13}$C-labelled naphthalene (Jeon et al., 2003). To this day, there have been fewer bioremediation studies involving DNA-SIP compared to studies using RNA as biomarker because a higher labelling is required for successful application (Manefield et al., 2004). One example is a study that investigated by amendment of $^{15}$C-labelled ethanol as an electron donor U(VI) immobilisation by heterotrophic
microbial reduction coupled to Fe(III) reduction (Akob et al., 2011). However, owing to the high turnover rate of RNA, offering the same sequence as DNA, it is hypothesised to be a stronger biomarker, which has been demonstrated in a $^{13}$C-phenol biodegradation study tracing its fate in a bioreactor (Manefield et al., 2002). Madsen and colleagues (2006) summarised in their review different SIP assays involving the biodegradation of organic pollutants in connection with DNA or RNA as biomarkers.

To my best knowledge there exists no literature on SIP experiments studying the fate of ISA, which is not surprising given that the microbial degradation of ISA has only been recognised recently (e.g. Bassil et al., 2015b; Kuippers et al., 2015). In contrast, many SIP studies have previously been performed using $^{13}$C-labelled glucose and cellulose. Amongst these studies, the degradation of cellulose in situ has been studied extensively to identify bacteria in undisturbed soil environments that are actively involved in cellulose degradation (e.g. Haichar et al., 2007; Eichorst and Kuske, 2012; Chapleur et al., 2014). Haichar and colleagues (2007) used $^{13}$C-labelled cellulose to identify for the first time active cellulose degrading bacteria, including a range of known cellulose degraders and uncultured microorganisms, by analysing their DNA by density-gradient centrifugation. Another recently published study by Chapleur et al. (2014) came up with a clever approach in order to improve cellulose degradation in municipal solid wastes. For this purpose, they prepared batch-incubations co-inoculated with municipal solid waste digester sludge and ruminal content which may contain specified organisms able to degrade cellulose. At the same time $^{13}$C-labelled cellulose was introduced. From this, they identified several active strains of bacteria and archaea degrading cellulose while producing $^{13}$C-labelled gases and VFAs. However, the introduced microorganisms could not outcompete their native competition and thus did not settle permanently.

There are many more studies to be mentioned, as SIP is being adopted widely in combination with bioremediation issues, albeit there is still a huge potential to further exploit this technique. Especially regarding field studies, there is a lack of literature because of the sometimes unknown environmental effects. However, a better understanding of the strengths of the different SIP tools can help to augment the environmental clean-up and the study of the fate of contaminants. In order to choose an appropriate SIP approach for bioremediation applications
Manefield and colleagues (2004) composed a review paper introducing briefly the different biomarkers, such as PLFA, DNA, RNA, FISH-SIMS or small subunit rRNA, that can be studied.

9.3.5. Status of the art and future work

After the successful isolation of a microbial strain capable of ISA degradation under defined laboratory reducing and alkaline (pH 10) conditions (Bassil et al., 2015b), the target of this study is to identify ISA-biodegrading microorganisms at neutral pH (pH = 7) from natural soils without using culturing techniques. The planned experiment will use $^{13}$C-labelled ISA of which the $^{13}$C-atoms will be assimilated into the DNA and membrane lipids of bacterial cells upon ISA degradation. Analyses of the genes and biomarker lipids using 16S gene rRNA sequencing together with GC IRMS, respectively, will help to identify the microorganisms that have incorporated the label into their RNA and cell membranes. By identifying the intensity of the signal, it can be further distinguished between primary feeders and cross-feeders. The identification of ISA-biodegraders from autochthonous soils using $^{13}$C-labelling will be more representative for the description of natural environmental conditions and will in addition allow describing associated carbon flows.

Since $^{13}$C-labelled ISA is not commercially available, it is intended to use fully $^{13}$C-labelled α-lactose monohydrate to prepare $^{13}$C-labelled ISA. The use of a fully labelled carbon molecule provides more reliable results of label incorporation. Fully $^{13}$C-labelled α-lactose monohydrate costs approximately $6,000 (Omicron Biochemicals, 2016). To keep the costs for this experiment reasonable, a purchase was intended at 1 g. However, this would require carrying out the synthesis of Ca(ISA)$_2$ at a 50 times smaller scale compared to the regular procedure done in our labs. The regular procedure yields 8 to 10% product, which would equal a maximum of 0.1 g $^{13}$C-labelled Ca(ISA)$_2$. The synthesis at the small scale was attempted three times without using labelled material, to test the feasibility. The many boiling and evaporating steps required for the synthesis (3.1.3) and the small amounts to handle rendered difficult. In the first two attempts, the ISA precipitate was lost completely in the final reduction step, when the solution was concentrated to about 1 mL on a heating plate. In this step, due to the small liquid volume with high loading of particles, the solution suddenly burst. In the latter attempt, the volume reduction was stopped at about 5 mL, which resulted in a final product of approximately 0.8 g. However, the product was dark brown and still contaminated by degradation by-products,
which were not sufficiently removed. In conclusion, the problematic synthesis of $^{13}$C-labelled Ca(ISA)$_2$ needs to be resolved in the future by improving the final boiling step. From the experiences made, it is suggested to use a heating mantle instead of a heating plate or increase the starting material, which would however increase the costs considerably.

In summary, the identification of ISA-biodegrading microbial strains can deliver crucial information about microbial function within a community. Regarding the management of radioactive waste disposal, the application of $^{13}$C-labelling experiments can be an interesting area of future research to model and predict the fate of ISA and to estimate time scales required for the biodegradation of this compound. Better understanding of the biodegradation pathway of ISA is hoped to help underpin the UK Governments’ plan to implement underground GDFs.

9.3.6. References


9.4. The author

After completion of her B.Sc. degree in September 2010 in “Management of Georesources”, at the RWTH Aachen, the author did a M.Sc. at the University of Bremen in Earth Sciences (completion March 2013). After working a short-term at the MARUM, a marine Centre for Environmental Research, the author took up her Ph.D. at the University of Manchester in October 2013. Since then she has been working on the content of this thesis in the group for Geomicrobiology under supervision of Professor Jon Lloyd and Dr Nicholas Bryan.

Awards

2015 Postgraduate Research Conference of the School of Earth, Atmospheric and Environmental Sciences: 1st prize oral presentations

Society memberships

Since 2013 Royal Society for Chemistry
Since 2013 Mineralogical Society
Since 2014 Society for Microbiology

Collaborations & supervision


- PhD collaboration with colleagues from the Institute of Resource Ecology from HZDR, Dresden-Rossendorf, Germany, namely Dr. Henry Moll and Hannes Brinkmann, was established focusing on ISA research. First exchange trip: Hannes came to Manchester to learn how to synthesise ISA and set up microcosm experiments. Second exchange trip: Author to HXDR, Dresden-Rossendorf to do Time-Resolved Laser Fluorescence Spectroscopy (TRLFS) analyses on U samples after ISA biodegradation (2016).

- Trip with a researcher delegation to a bilateral meeting between the Institute of Resource Ecology from HZDR, Dresden-Rossendorf, Germany, and several groups from the University of Manchester (including the Williamson Research Centre, the Research Centre for Radwaste Disposal and the Centre for Radiochemistry Research) at the HZDR with the aim to build a stronger network between both research groups;
Work undertaken: participation in network meetings and oral presentation about my work (September, 2016).

**Demonstrating**

- Organic Geochemistry and Earth Resources *University of Manchester*, Manchester (1\textsuperscript{st} semester, 2014-2017)
- Earth Resources *University of Manchester*, Manchester (2\textsuperscript{nd} semester, 2014-2016)
- Environmental Investigative Methods *University of Manchester*, Manchester (2\textsuperscript{nd} semester, 2016-2017)

**Research Conference contributions**

*Oral presentations*

2016  
Kuipers, G., Morris, K., Bryan, N., Lloyd, J.R.: *Immobilization of nickel via the biodegradation of isosaccharinic acid - relevant to nuclear waste disposal,* Geomicro Network meeting, Bangor, UK.

2015  

2014  
Kuipers, G., Bassil, N.M., Bryan, N., Lloyd, J.R.: *The biodegradation of Isosaccharinic acid in a geological disposal facility (GDF) and the far field,* ISEB, Cancun, Mexio.
Poster presentations


Kuipers, G., Bassil, N.M., Bryan, N., Lloyd, J.R.: Investigation of the biodegradation of isosaccharinic acid and its degradation products in the far field of geological disposal, Goldschmidt Conference, Prague, Czech Republic.

Kuipers, G., Bassil, N.M., Bryan, N., Lloyd, J.R.: Microbial degradation of isosaccharinic acid under repository conditions & in the far field, PSRS, Manchester, UK.


Kuipers, G., Bassil, N.M., Bryan, N., Lloyd, J.R.: Microbial degradation of isosaccharinic acid under repository conditions & in the far field, PGRC 2014, Manchester, UK.
