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Originally published:

March 2021

Environmental Science & Technology 55(2021)8, 4597-4606

DOI: https://doi.org/10.1021/acs.est.0c03594

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

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- 15 Key words: Isosaccharinic acid, biodegradation, ningyoite, uranyl-phosphate, autunite, nuclear waste 16 management

17 Abstract

Geological disposal is the preferred long-term solution 18 for higher activity radioactive wastes (HAW) 19 20 including Intermediate Level Waste (ILW). In a 21 cementitious disposal system, cellulosic waste items present in ILW could undergo alkaline hydrolysis, 22 23 producing significant quantities of isosaccharinic acid 24 (ISA), a chelating agent for radionuclides. Although 25 microbial degradation of ISA has been demonstrated, 26 its impact upon the fate of radionuclides in a GDF is a



topic of ongoing research. This study investigates the fate of U(VI) in pH neutral, anoxic, microbial enrichment cultures, approaching conditions similar to the far field of a GDF, containing ISA as the sole carbon source, and elevated phosphate concentrations, incubated both (i) under fermentation and (ii) Fe(III)-reducing conditions. In the fermentation experiment, U(VI) was precipitated as insoluble U(VI)-phosphates, whereas under Fe(III)reducing conditions, the majority of the uranium was precipitated as reduced U(IV)-phosphates, potentially via enzymatic reduction (mediated by metal-reducing bacteria including *Geobacter* species detected by 16S rRNA gene sequencing). Overall, this suggests the potential for the establishment of a microbially-mediated "biobarrier" extending into the far field geosphere surrounding a GDF which has the potential to evolve in response to aspects of a GDF and can have a controlling impact on the fate of radionuclides.

36 Introduction

37 In most nuclear countries, the disposal of longer-lived, intermediate level radioactive waste (ILW), a component 38 of Higher Activity Waste (HAW) in the radioactive waste inventory, will be to a deep geological disposal 39 facility (GDF). A GDF will employ a multiple engineered barrier system (EBS), intended to isolate and contain 40 the waste for sufficient time to allow the majority of the radioactivity to decay (DEFRA et al., 2008; Morris et al., 2011; RWM, 2016a). Most EBS concepts include encapsulation of the wastes in cement within steel drums, 41 which are then placed into an excavated vault. Upon re-saturation with groundwater, chemical conditioning will 42 43 be achieved by a cementitious backfill of the vaults to provide a dominantly anoxic and high pH environment 44 that enhances sorption and reduces radionuclide mobility (Holopainen, 1985; Berner, 1992; Crossland and 45 Vines, 2001; RWM, 2015; Duro et al., 2020).

46 In ILW, uranium (U) will typically be the dominant radionuclide by mass (RWM and NDA, 2015). Due to the long half-life of uranium (²³⁸U 4.468 x10⁹ y) and of its resultant decay chain elements, it is important to 47 48 understand the environmental behavior of U to support implementation of a GDF. Redox cycling of U exerts a 49 major control on its mobility. Under oxic conditions uranium, as U(VI), can form mobile carbonate complexes 50 (Clark et al., 1995); although transport is typically limited by sorption. The formation of stable U(VI) colloids at 51 elevated pH has also been demonstrated (Bots et al., 2014; Smith et al., 2015). In contrast, following the onset 52 of anoxic conditions post-closure of a GDF, sparingly soluble U(IV), often precipitated as U(IV) oxide phases, 53 is expected to dominate (Lloyd *et al.*, 2005). In addition, the hyperalkaline conditions (pH > 12.5) in a GDF can 54 reduce the mobility of uranium by formation of uranyl silicates and uranate phases which may sorb to 55 cementitious phases (Wellman et al., 2007; Bots et al., 2014), although stable U(IV) silicate colloids have also 56 recently been reported under alkaline conditions (Neill et al., 2018). A mix of both U(VI) and U(IV) species 57 may co-exist in the EBS; an improved understanding of their different immobilization pathways is important for post-closure modelling. Both, U(IV) and U(VI), can form strong, insoluble complexes with phosphates (Rui et 58 59 al., 2013; Mehta et al., 2015), which may be present, e.g. from the nuclear clean-up process (Thomas and 60 Macaskie, 1996; Chambers et al., 2004; RWM, 2016b) or the surrounding host rock (Porder and Ramachandran, 61 2013). There has been documentation of U(V) species in environmental studies, but it typically

disproportionates (Renshaw et al., 2005; Vettese et al., 2020). The transport behavior of uranium may be further 62 63 complicated by the presence of organic ligands, such as the decontamination agents ethylenediaminetetraacetic 64 acid (EDTA) or nitriloacetic acid (NTA) that may be disposed of with the wastes (Francis, 1998; Hummel et al., 2005; Suzuki and Suko, 2006). Of particular interest, with respect to the disposal of ILW, are cellulosic items 65 which are present at around 1% by mass of the total ILW in some wastes (IAEA, 2003; NDA and DEFRA, 66 67 2014). Under alkaline GDF conditions, cellulosic wastes are expected to undergo alkaline hydrolysis (Machell 68 and Richards, 1957; Greenfield et al., 1992; Glaus et al., 1999; Knill and Kennedy, 2003), resulting in the 69 production of a range of organic degradation products, of which isosaccharinic acid (ISA), a polyhydroxy ligand, is predicted to dominate (Whistler and BeMiller, 1958; van Loon and Glaus, 1998; Knill and Kennedy, 70 71 2003; Pavasars et al., 2003; Glaus and van Loon, 2008). ISA sorbs weakly to surfaces within the cementitious 72 wastes (Bradbury and Sarott, 1995; van Loon et al., 1997) and is known to form stable complexes with 73 radionuclides, such as uranium, which could in turn increase their mobility (Baston et al., 1994; Rao et al., 74 2004; Warwick et al., 2004). ISA has the potential to be degraded, e.g. by microbial activity, and thus understanding the biogeochemical influence of microbial ISA degradation on uranium speciation, and its impact 75 76 upon transport behavior under GDF conditions, is important to help develop an understanding of how waste-77 derived uranium could be affected by processes occurring in the GDF on the long term.

78 Previous biogeochemical studies have focused on the removal of U(VI)(aq) from solution in the absence of strong 79 chelating agents in the context of treatment of contaminated land. These studies used electron donor additions to 80 stimulate microbial U(VI) reduction that promoted the formation of insoluble U(IV) (Lovley et al., 1991; 81 Newsome et al., 2015a). Since ISA may be a potentially significant organic substrate in ILW that could control 82 the fate of U(VI) in and around the GDF, its role as an electron donor and carbon source stimulating microbial 83 metabolism needs to be understood. Indeed, anaerobic microbial ISA degradation has been investigated under a 84 range of biogeochemical conditions and pH values. Under the oxygen-depleted conditions typically expected 85 after closure of a GDF, the oxidation of ISA can be coupled to the reduction of a range of alternative electron acceptors present in the wastes (Bassil et al., 2015b; a; Kuippers et al., 2015; Rout et al., 2015), which may be 86 87 incomplete at high pH (=10) due to diminishing energy yield when coupled to low redox potential electron 88 acceptors, such as sulfate (Bassil et al., 2015b; a; Rout et al., 2015). Complete degradation was observed under 89 a range of conditions with Fe(III) supplied as an electron acceptor, which may occur naturally in the geosphere 90 and might also be produced in situ from the corrosion of steel present in ILW and engineering materials used in 91 GDF construction (Lovley and Phillips, 1986; Konhauser, 1997; Duro et al., 2014). Alternatively, some metal92 reducing bacteria directly utilize U(VI) as an electron acceptor, potentially leading to its reductive precipitation
93 as U(IV). To date, there have been no studies examining the role of U(VI) during microbial degradation of ISA,
94 both as a potential complexant and/or electron donor, under conditions relevant to the near and far field of a
95 GDF.

96 In this study, enrichment cultures growing on ISA were used to represent potential biogeochemical conditions in 97 the pH neutral far field environment surrounding a GDF. The focus was to investigate the fate of $U(VI)_{(aq)}$, 98 when ISA was used as fermentation substrate, and to compare these results to data from an ISA-degrading, 99 Fe(III)-reducing microbial enrichment. A microbial consortium that is expected to be adapted to such 100 biogeochemical conditions, was retrieved from soil samples from an alkaline legacy lime workings site at 101 Harpur Hill, Buxton, UK, a well characterized system and potential analogue for an evolved GDF environment 102 (Williamson et al., 2013). Uranium was removed from solution under both conditions, although the fate of the 103 radionuclide depended on the culturing conditions. Collectively, these data highlight the potential importance of microbial processes in influencing radionuclide mobility in the far field surrounding a GDF (alongside other 104 105 relevant processes).

106 Materials and methods

Sediments. Shallow subsurface sediment samples (approximately 20 cm depth and pH 7.5) were collected at the margins of an alkaline legacy lime workings site at Harpur Hill, Buxton, U.K., a potential analogue site representative of the alkali-disturbed zone surrounding a GDF that is known to contain ISA-degrading bacteria (Williamson *et al.*, 2013; Bassil *et al.*, 2015b; Kuippers *et al.*, 2015). The sediment was kept in the dark at 4°C until use.

112 *Ca*(*ISA*)₂ *preparation.* Ca(ISA)₂ was prepared from α-lactose monohydrate and Ca(OH)₂ following the protocol 113 of Vercammen et al. (Vercammen *et al.*, 1999).

114 Fe(III) oxyhydroxide preparation. Fe(III) oxyhydroxide was produced according to the method of 115 (Schwertmann and Cornell, 2000), briefly, 0.6 M FeCl₃ was hydrolyzed at pH 7, with six washing steps with 116 18 Ω de-ionized water and the Fe(III) suspension was standardized using ICP analysis.

117 *ISA-degrading, Fe(III)-reducing and fermenting enrichments.* Stable enrichment cultures were obtained using 118 a 1% (v/v) sediment inoculum and minimal medium (pH 7), approaching far field conditions of a GDF. The 119 media contained 30 mM NaHCO₃, 4.7 mM NH₄Cl, 4.4 mM NaH₂PO₄·H₂O, 1.3 mM KCl, and 10 mL L⁻¹ of 120 mineral and vitamin stock solutions (Lovley *et al.*, 1984). Ca(ISA)₂ was added as the sole added carbon source and electron donor to a final concentration of 3.5 mM. To create an ISA-degrading and Fe(III)-reducing enrichment culture, approximately 30 mmol L⁻¹ Fe(III) oxyhydroxide was added to one set of enrichment cultures, as the sole added electron acceptor, whilst a second set of ISA-fermenting enrichment cultures was grown without added Fe(III) oxyhydroxide. Microorganisms able to degrade ISA were selected via periodic subculturing (1% v/v inoculum) in fresh medium, typically every four weeks. Stable enrichment cultures were obtained after 12 consecutive transfers for inoculation into U(VI)-containing media.

127 **Preparation of experimental cultures with ISA and U(VI).** Enrichment cultures, approaching potential far field 128 GDF conditions, were prepared with 30 mL anoxic medium at pH 7 containing 3.5 mM ISA. Fe(III) oxyhydroxide (30 mmoles L-1) was added to the "U(VI)-ISA, Fe(III)-reducing" cultures, and these were also 129 supplemented with 1 mM U(VI) (added as a spike of UO_2^{2+} in 0.001 M HCl) after autoclaving. A parallel set of 130 "U(VI)-ISA fermentation" cultures without added Fe(III), and with 1 mM U(VI) as the sole electron acceptor 131 132 were prepared. Finally, an inoculum (1% v/v) was added from the two stable enrichment cultures prepared with 133 and without Fe(III), respectively, to initiate experiments. Controls were also prepared, containing the same 134 media as above with an autoclaved (sterile) inoculum, no inoculum, or no U(VI). All serum bottles were incubated in the dark at room temperature. Periodically, samples were withdrawn and analyzed using a range of 135 136 geochemical and spectroscopic techniques.

137 *Exploring the mechanism of U(VI) reduction.* Here, we explored the fate of U(VI) in Fe(III)-reducing 138 incubations that had reached peak Fe(II) levels and were then either sterilized (autoclaved) or left microbially 139 active, prior to the addition of 1 mM UO_2^{2+} (in 0.001 M HCl). After further incubation for 30 days, the solids 140 were centrifuged anoxically and analyzed by U L_{III}-edge X-ray absorption spectroscopy (XAS) on beamline 141 B18 at Diamond Light Source.

142 Geochemical characterization. Periodically, samples were withdrawn using anaerobic, aseptic techniques and 143 pH, E_h , Fe(II)/Fe(III), U_{total} and U(VI) were measured before preserving the samples at -80°C for further 144 analysis. Microbial Fe(III) reduction was measured using the ferrozine assay (Lovley and Phillips, 1987). Total 145 uranium in solution was quantified with inductively-coupled plasma mass spectrometry (ICP-MS) and on select 146 samples U(VI) in solution was quantified spectrophotometrically with 2-(5-bromo-2-pyridylazo)-5-147 diethylaminophenol at 578 nm (Johnson and Florence, 1971). ISA, organic acids, sulfate and phosphate were 148 analyzed by ion exclusion high performance liquid ion chromatography (IE-HPLC), using a Dionex ICS5000 149 (SI section S1 for methodological details).

150 16S rRNA gene sequencing. 16S rRNA gene sequencing was performed with the Illumina MiSeq platform 151 (Illumina, San Diego, CA, USA) using a Roche 'Fast Start High Fidelity PCR System' (Kuippers et al., 2018). 152 The raw sequencing data were deposited at the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra/) with the accession numbers SRR7769831 - SRR7769835. 153

Mineralogical characterization. All preparation and analyses were performed under anaerobic conditions.
Mineral phase identification was carried out using powder X-ray diffraction (XRD) crystallography on a Bruker
D8 Advance using an anaerobic cell. Environmental Scanning Electron Microscopy (FEG ESEM, FEI Quanta
650) and Transmission Electron Microscopy (FEG TEM, Philips CM200) with selected area electron diffraction
(SAED) were also used to characterize samples.

159 U(VI) speciation and coordination analysis in solids. X-ray Absorption Spectroscopy (XAS) was used to 160 determine speciation and coordination of uranium in solids sampled at incubation end points. XAS data were collected for the L_{III}-edge and M_{IV}-edge of uranium. For studies of the U M_{IV}-edge High-Energy Resolution 161 Fluorescence Detection X-Ray absorption near edge structure (HERFD XANES; Kvashnina et al., 2013, 2014), 162 163 end-point samples were centrifuged and prepared in an anaerobic holder. Spectra were collected on the ID26 164 beamline at the European Synchrotron Radiation Facility (ESRF) in Grenoble (Gauthier et al., 1999), using a 165 Si(111) monochromator and an X-ray emission spectrometer (Glatzel et al., 2013). The U M_{IV}-edge HERFD 166 XANES spectra were normalized according to their maxima, before analysis using Athena linear combination 167 fitting to further quantify the likely proportion of uranyl(IV), uranyl(V) and uranyl(VI) in the samples (Ravel 168 and Newville, 2005).

For U L_{III} -edge XAS, samples were centrifuged and diluted with cellulose to a final concentration of approximately 1% U w/w to form a pressed pellet. All samples were prepared under strictly anaerobic conditions and stored under argon atmosphere at -80 °C until analysis in a cryostat. Samples and standards (schoepite UO₃ and uraninite UO₂) were collected in fluorescence or transmission mode. Background subtraction was performed using Athena and Demeter software (Ravel and Newville, 2005). Shells and multiple scatterers (MS) were only included in the final fits, when they statistically improved the model as assessed by the F-test (Downward *et al.*, 2007).

176 Results and Discussion

Microbial ISA degradation has the potential to impact on the fate and speciation of uranium in the geosphere surrounding a GDF. To test this, anoxic, stable enrichment cultures were set up at pH 7 containing ISA as the sole electron donor and carbon source, and aqueous U(VI). The enrichment cultures were prepared from sediments collected at Harpur Hill, Buxton in media selective for ISA-fermenting and ISA-degrading, Fe(III)reducing conditions with U(VI).



Figure 1. Solution chemistry of U(VI)-ISA fermentation (left panel) and U(VI)-Fe(III)-reducing cultures (right panel), both with 1 mM U(VI). Panel A & C: IC results for ISA (red line), acetate (blue line), butyrate (green line) and ISA in sterile control (grey dashed line); B & D: ICP MS results for total uranium in solution (yellow line) and sterile control (grey dashed line); E: Ferrozine results for Fe(II) in solution for microbially active Fe(III)-reducing culture (red line) and sterile control (grey dashed line); E: Ferrozine results for Fe(II) in solution for microbially active Fe(III)-reducing culture (red line) and sterile control (grey dashed line). Note: results for U(VI)_(aq) analyses using Bromo PADAP are presented in SI Section S1, Figure S6).

188 Solution chemistry. There was a lag of two days in both microbial enrichment experiments (with and without 189 added Fe(III)) before a decrease in the ISA concentrations was observed. ISA was fully depleted by 14 days 190 (Figure 1 A, C). In contrast, no decrease in soluble ISA concentration was observed in the heat-sterilized control 191 incubations (Figure 1 A, C; SI Figure S1). The decrease in ISA concentration in the microbial enrichments was 192 accompanied by a drop in redox potential to -110 mV in ISA-fermenting treatments and to -200 mV in 193 treatments amended with Fe(III) (SI Figure S2). In the ISA-fermenting experiment, the pH remained broadly 194 constant and in the Fe(III)-reducing experiment, modest generation of alkalinity was evident as the pH increased 195 from 7.1 to 7.4 (SI Figure S3). No changes in E_h or pH were observed in abiotic controls (SI Figure S2 & S3). In 196 microbially active incubations, an increase in concentrations of volatile fatty acids (VFAs; dominated by acetate

197 and lower concentrations of butyrate) was noted during ISA degradation (Figure 1 A, C). VFA production 198 plateaued in both treatments at day 14, when ISA was depleted, and the amount of carbon converted to VFAs 199 was slightly higher in the fermentation experiment at 53% $\pm 1.5\%$ compared to 45% $\pm 0.5\%$ in the Fe(III)-200 reducing experiment. Fe(II) ingrowth to solids (0.5 N HCl-extractable) in the Fe(III)-reducing incubations was 201 only detected in biotic experiments. Here, Fe(II) production started after a lag phase of 7 days and continued to increase until day 14 when the concentration plateaued at approximately 56% \pm 3% (16 mmol L⁻¹) of the 0.5 N 202 HCl-extractable Fe(II) fraction (Figure 1E and SI Figure S4). In addition, the total 0.5 N HCl-extractable iron 203 204 fraction (Fe(II) + Fe(III)) decreased from a peak of 30 mmol L^{-1} at the start of the experiment by approximately 25% ±5% over the course of the incubation time (SI Figure S5), indicating formation of recalcitrant 205 206 Fe(II)/Fe(III)-bearing solids. XRD analysis at the end of the incubation confirmed formation of siderite (FeCO₃) 207 and vivianite ((Fe₃PO₄)₂·8H₂O) in the incubations (SI Figure S11). Overall, ISA degradation in the Fe(III)-208 reducing experiment was considered to proceed via a mixed microbial community of fermenting and Fe(III)-209 reducing bacteria. However, the Fe(III)-reducing experiments showed a 10% decrease in the amount of carbon 210 converted from ISA to VFAs compared to the U(VI)-ISA fermentation experiment, and following depletion of 211 ISA the amount of VFAs decreased further, both indicators for continued oxidation of fermentation products to 212 CO₂ coupled to Fe(III) reduction.

213 The concentration of total uranium (added as 1 mM U(VI)) in culture supernatants was monitored using ICP-214 MS (Figure 1 B, D), and spectrophotometric analysis of U(VI) was consistent with these results (SI Figure S6). 215 At the start of incubation, U(VI) was removed rapidly from solution, before ISA degradation was detected. This 216 rapid removal of uranium was also noted in sterile control incubations, indicating oversaturation of the system 217 was occurring (Figure 1 B, D & SI Figure S6). XRD analysis of the uranium precipitate revealed formation of a 218 uranyl phosphate in sterile systems (SI Figure S11), potentially due to complexation with phosphate (1 mM) 219 from the medium (Langmuir, 1978). However, U(VI) adsorption to ferrihydrite in the Fe(III)-amended 220 experiment could not be excluded (Waite et al., 1994). When ISA levels started to decrease in the biotic 221 experiments, a small proportion was concomitantly remobilized in both the ISA-fermenting and Fe(III)-reducing 222 experiments; 2% and 13% U(VI), respectively. In the ISA-fermenting experiment, this U(VI) release was 223 transient and removal was essentially complete by 35 days. By contrast, in the Fe(III)-reducing system, a small 224 proportion (approximately 4% uranium) remained in solution throughout the remainder of the experiment. The 225 spectrophotometric assay for U(VI) suggested the re-solubilized uranium was dominated by U(VI). Finally,

comparison of the U(VI)-amended biotic experiments with controls that did not contain uranium (SI Figure S7),
showed that uranium did not impede the rates of ISA biodegradation.

Solid phase characterization. Biominerals that formed were characterized using XRD, TEM and ESEM. In addition, XAS was used to define the average U(VI) oxidation state and coordination environment in key samples, including the collection of U M_{IV} -edge (SI Figure S8) and U L_{III} -edge (SI Figure S9) spectra. ICP MS data showed that in all microcosms the majority of uranium precipitated at the start of the incubation. XRD analysis on the sterile controls indicated this was potentially a uranyl phosphate phase similar to autunite (SI Figure S10 and S11).

234 ISA-fermenting experiment with U(VI)

235 In the ISA-fermenting enrichment culture, brief remobilization of approximately 2% of uranium between day 7 236 and 21 was observed, with complete removal of uranium from solution by the end of the incubation period. 237 Using XRD, the precipitate was identified as a uranyl phosphate (either K- or Na-autunite), a similar structure to 238 the precipitate in the sterile controls (SI Figure S11). Further characterization by ESEM showed well-defined 239 spherules with an average size of 800 nm \pm 14 nm that appeared to consist of smaller plates (SI Figure S13A). 240 TEM images confirmed a typical autunite-like morphology (Gudavalli et al., 2013), and revealed individual 241 plates ranging in size from 15 to 40 nm that aggregated into spherules (Figure 2A). EDS analysis of the 242 corresponding TEM images identified O, P, U and trace Na (Figure 2B), consistent with metanatroautunite. U 243 M_{IV}-edge spectroscopy confirmed U(VI) was dominant (SI Figure S8), with linear combination fitting (LCF) of 244 the U M_{IV}-edge HERFD XANES showing approximately 98% U(VI) in the solid phase by the end of the 245 incubation period (SI Figure S10; Table S1). EXAFS analyses were informed by the relevant U(VI)-phosphate 246 literature (Locock and Burns, 2003) and the best fit contained 2 axial O backscatterers at 1.78 Å, 4 equatorial O backscatterers at 2.43 Å, and 3 P backscatterers at 3.62 Å (Table 1). This model suggests a U(VI) uranyl species 247 248 coordinated by 3 phosphate ions around the equatorial plane, consistent with the autunite-like U(VI)-phosphate 249 identified from XRD analysis (Singh et al., 2012; Mehta et al., 2016).

Interestingly, although uranium was not reduced in the U(VI)-ISA fermentation experiment, TEM EDS analysis showed significant accumulation of U- and P-containing precipitates on the cells (SI Figure S14). These uranium precipitates could have complexed with ligands in the microbial cell surface, such as carboxyl, amine, hydroxyl, phosphate and sulfhydryl groups (Beveridge and Murray, 1980; Lloyd and Macaskie, 2000). Thus, 254 uranium in the U(VI)-ISA fermentation experiment was primarily mineralized as an U(VI)-phosphate mineral



which seemed partially associated with the microbial cell surface.

Figure 2. Mineralogical analysis of ISA-fermenting U(VI) experiment (A-D) and ISA degradation, Fe(III)reducing U(VI) experiment (E-H) both with 1 mM U(VI). In detail: A) & E) TEM images with SAED; B) & F) corresponding EDS of TEM images; C) & G) non-phase shift corrected U L_{III}-edge EXAFS data and D) & H) corresponding k³ weighted Fourier transform of EXAFS data. EXAFS data are represented by blue lines and corresponding fits by red dotted lines.

10

| Sample | Scattering path | Coordination number | Atomic distance (Å) | Debye-Waller factor σ^2 (Å ²) | R-factor | ΔΕο |
|--------------|-------------------------|------------------------|------------------------|--------------------------------------------------|----------|---------|
| U(VI)-ISA | | | | | | |
| fermentation | O _{ax} | 2 | 1.76(1) | 0.002(1) | 0.0018 | 2.0(21) |
| | O_{eq} | 4.5 | 2.26(1) | 0.004(1) | | |
| | Pmonodentate | 3 | 3.62(3) | 0.005(3) | | |
| | O _{ax-rattle} | 2 | 3.52(1) | 0.008(1) | | |
| | Oax-non-forward | 2 | 3.52(1) | 0.004(1) | | |
| | Oax-forward | 2 | 3.52(1) | 0.004(1) | | |
| U(VI)-ISA + | | | | | | |
| Fe(III) | O_{eq} | 3 | 2.27(2) | 0.006(3) | 0.0013 | 5.2(14) |
| | O _{eq} | 5 | 2.43(2) | 0.005(2) | | |
| | P _{bidentante} | 1.2 | 3.14(2) | 0.005(2) | | |
| | Pmonodentante | 1.8 | 3.71(3) | 0.005(2)* | | |

Table 1. EXAFS fit parameters for U(VI)-ISA degradation experiments with Fe(III) and without Fe(III) added both with 1 mM U(VI). ΔE_0 is energy shift from calculated Fermi level in eV. The amplitude factor (S0²) was fixed as 1.0 for the U(VI)-ISA + Fe(III) sample and 0.9 for the U(VI)-ISA fermentation sample. Indices are: ax for axial atoms and eq for equatorial atoms. * indicates linked to parameter above.

265 U(VI)-Fe(III)-reducing, ISA biodegradation experiment

266 At the start of the Fe(III)-reducing experiment, uranium was precipitated as a uranyl phosphate, similar to the

267 ISA-fermenting experiment. After the incubation period, no crystalline uranium phase was detected by XRD in

the microbially active incubations, whereas in the sterile, Fe(III)-reducing control a uranyl precipitate was 268 269 identified (SI Figure S11). Instead, in the biotic experiment, siderite (FeCO₃) and vivianite ((Fe₃PO₄)₂·8H₂O) 270 had formed. U M_{IV}-edge HERFD XANES data for the experiment end point confirmed the uranium was present 271 predominantly as U(IV) (LCF showed 97% U(IV); SI Figure S10; SI Table S1). In ESEM (SI Figure S13B) and 272 TEM images (Figure 2E; SI Figure S15) these U(IV) particles appeared as thin sheets agglomerated into clusters 273 with a clearly different morphology to the end point uranium precipitate in the ISA fermentation experiment. 274 Corresponding EDS analysis from several different areas of these clusters showed O, Fe, P, U and Ca (Figure 275 2F).

276 Again, EXAFS fitting for the sample was informed by relevant published literature and a good fit was achieved with a split shell of three equatorial O backscatterers at 2.29 Å, five equatorial O backscatterers at 2.42 Å, and 277 two P backscatterer shells containing 1.2 atoms at 3.01 Å and 1.8 atoms at 3.75 Å (Figure 2G, H; Table 1). This 278 279 model is consistent with a range of previously described U(IV)-phosphate structures, including a nanocrystalline 280 ningyoite-like structure (Dusausoy et al., 1996; Newsome et al., 2015a) and phosphate coordinated monomeric 281 U(IV) (Boyanov et al., 2011; Bargar et al., 2013; Alessi et al., 2014). Due to similar bond distances and coordination numbers, delineating between these phases to identify the exact U(IV)-phosphate phase is not 282 possible using solely EXAFS and so from hereon, the U(IV)-phosphate phase present in this study will be 283 284 referred to as 'ningyoite-like'. In a sterile control prepared alongside with Fe(III) and U(VI), U(VI) reduction 285 was absent, indicating U(VI) reduction to be a microbially-mediated process in the U(VI)-Fe(III)-reducing experiment. This is in contrast to the biotic U(VI)-ISA fermentation experiment, where U(VI) reduction was 286 287 circumvented. Microbially-mediated mechanisms for U(VI) reduction include direct enzymatic reduction where 288 U(VI) serves as an alternative electron acceptor (Lovley et al., 1991; Gorby and Lovley, 1992; Fredrickson et 289 al., 2000), or indirectly microbially-mediated reduction of an electron acceptor, such as via reduction of Fe(III), 290 which generates reducing capacity via Fe(II) for abiotic U(VI) reduction (Jeon et al., 2005; O'Loughlin et al., 291 2010; Veeramani et al., 2011). To further explore the mechanism of reduction, an additional set of Fe(III)-292 reducing microcosms was set up and U(VI) was added to these microcosms after Fe(III) reduction was 293 complete. One set of these controls was autoclaved before U(VI) was added to study abiotic U(VI) reduction 294 mediated by biogenic Fe(II), whilst U(VI) was added to the other control in a microbially active state. After 295 incubation for a month, U L_{III}-edge XANES indicated that U(VI) was reduced to U(IV) only with a viable 296 microbial inoculum (SI Figure S12), suggesting that direct microbial processes catalyzed U(VI) reduction in the 297 presence of Fe(II)-bearing mineral phases.

Microbial community analysis. 16S rRNA gene profiles were obtained by Illumina sequencing for all 298 299 treatments at the end of incubation (Figure 3), to identify the microorganisms that may have been involved in 300 controlling the biogeochemical fate of uranium. Sequence analyses of the original, pH 7.5 soil samples, 301 retrieved from the potential analogue site, Harpur Hill, indicated a complex background microbial community 302 dominated by sequences most closely affiliated with the classes Betaproteobacteria (22.3% of total sequence 303 abundance) and Bacteroidia (16.6% of total sequence abundance; Figure 3). Alpha-rarefaction curves showed a 304 significant decrease in species diversity after enrichment from over 1,000 observed operational taxonomic units 305 (OTUs) in the original sediments, to approximately 135 OTUs in the ISA-fermenting experiment and 150 OTUs 306 in the Fe(III)-reducing experiment (SI Figure S16). At the end of the experiments, both treatments were 307 dominated by a class most closely affiliated with members of Clostridia (over 40% of total sequences), but close 308 examination of the 16S rRNA gene sequences revealed a marked difference between the treatments at the family 309 level.



Figure 3. Microbial community fingerprinting showing the most important phylogenetic classes within sediments before incubation, alongside bioreduced ISA-fermenting and ISA, Fe(III)-reducing cultures with and without added U(VI) after 14 days of incubation performed by using 16S rRNA gene sequencing.

Focusing on the ISA-fermenting enrichment with U(VI), this experiment was characterized, in addition to the strong enrichment of Clostridia (46.8% of genes detected), by a high relative abundance of sequences associated with members from the class Betaproteobacteria (18.7% of genes detected) and an enrichment of sequences affiliated with the class Negativicutes (of 12.8% of genes detected). The Negativicutes were comprised of sequences from the family *Veillonellaceae* (98% match). Another significant class comprised the Bacteroidia (12.5% of genes detected), of which most sequences were associated with a member from the *vadinBC27* wastewater sludge group (approximately 73% of this class) from the family *Rikenellaceae*. The microbial
 profile of the ISA-fermenting culture without added U(VI) comprised the same classes and species.

321 By contrast, in the U(VI)-amended Fe(III)-reducing enrichment, the second most abundant class (after the 322 Clostridia) comprised of members belonging to the Deltaproteobacteria (14.7% of total sequence abundance). 323 Two families were enriched within this class, with the majority related to the Fe(III)-reducing family 324 Geobacteraceae (11.8% of total sequence abundance, 97% match) and a smaller proportion related to species 325 from the sulfate-reducing (and Fe(III)-reducing) Desulfovibrionaceae (3% of total sequence abundance, 98% 326 match). Geobacter sp. are dissimilatory metal-reducing bacteria that can couple oxidation of organic carbon to 327 the reduction of a wide range of terminal electron acceptors, including Fe(III) and U(VI) (Lovley et al., 1991). 328 These species were not enriched in the -ISA-fermenting cultures which were established without an alternative 329 electron acceptor. As discussed, the controls prepared to study the mechanism of U(VI) reduction indicated that 330 enzymatic U(VI) reduction occurred in these microcosms, and indeed several potential candidates, known to be 331 capable of enzymatic U(VI) reduction, were enriched, comprising Geobacter spp. (Lovley et al., 1993; Vali et 332 al., 2004; Shelobolina et al., 2008), Clostridia spp. (Francis et al., 1994; Boonchayaanant et al., 2009) and members of the family Veillonellaceae from the Negativicutes (Woolfolk and Whiteley, 1962; Gihring et al., 333 334 2011). However, *Clostridia* spp. and *Veillonellaceae* spp. do not appear to be associated with the reduction of 335 U(VI), as these species were also strongly enriched in the U(V)-ISA-fermenting experiment, where U(VI)336 reduction was absent. This implies species affiliated with Geobacteraceae potentially played an important role 337 in the reduction of U(VI) to U(IV) in these systems.

338 Overall, anaerobic microbial enrichment cultures fueled by ISA degradation, were able to couple the oxidation 339 of ISA or its degradation products to the reduction of U(VI)-phosphate, when enriched on Fe(III) as an electron 340 acceptor, whilst no U(VI) reduction was observed in the cultures incubated under ISA-fermenting conditions 341 alone. In addition, 16S rRNA gene profiling identified Geobacter species as potential candidates involved in 342 enzymatic U(VI) reduction, alongside other metal-reducing bacteria including species from the 343 Desulfovibrionaceae and Veillonellaceae. These findings shed light on how the subsurface microbial 344 community in and around a GDF may adapt to changing geochemical environments, and highlight the 345 availability of terminal electron acceptors as a controlling factor in these systems. The microbial community 346 structure in turn can have a profound impact on the oxidation state of uranium (and potentially other 347 radionuclides) and therefore the speciation of end-member precipitates. Although early work on microbial 348 U(VI) reduction noted uraninite (UO₂) as the dominant end point mineral, U(IV)-phosphates have also been

observed more recently as the products of microbial U(VI) reduction, especially in the presence of aqueous 349 350 phosphate (Bernier-Latmani et al., 2010; Lee et al., 2010; Newsome et al., 2015a) or after reduction of a U(VI) 351 phosphate mineral phase (Khijniak et al., 2005; Rui et al., 2013). Although the autunite-like mineral that was 352 precipitated under ISA-fermenting conditions is a stable end product and a sink for U(VI) that can persist over 353 geological timescales (Langmuir, 1978; Sato et al., 1997; Jerden and Sinha, 2003), the ningyoite-like precipitate 354 from U(VI)-Fe(III)-reducing conditions is considered more recalcitrant towards oxidation and thus remobilization (Finch and Murakami, 1999; Jerden and Sinha, 2003; Pinto et al., 2012; Newsome et al., 2015b; 355 356 a). Additional reducing capacity, associated with the Fe(II)-bearing minerals vivianite and siderite, was also 357 identified, and both minerals have been shown to mediate the reduction of priority radionuclides, e.g. Tc(VII) 358 and Np(V), and leading to their reductive immobilization (Lloyd et al., 2000; Law et al., 2010; McBeth et al., 359 2011; Thorpe et al., 2014).

Our work suggests that ningyoite is an ideal end member of U(VI) bioreduction associated with anoxic environments in radioactive waste disposal, and that biogeochemical factors (e.g. bioavailable Fe(III) and phosphate concentrations) can promote its formation within a 'bio-barrier', enhancing the strength-in-depth approach afforded by the multiple barriers of the EBS and geosphere.

364 Acknowledgements

365 This work was supported by Radioactive Waste Management Limited (RWM). The authors would like to thank 366 NERC for additional funding under the OPTIUM consortium (NE/R011230/1). JRL also acknowledges the financial support of the Royal Society and the EU MIND programme under "Euratom2014-2015" and the call 367 368 "NFRP-06-2014: Supporting the implementation of the first of-a-kind geological repositories". We thank both Diamond Light Source (SP12767, SP13599) and European Synchrotron Radiation Facility (EV/192) for 369 beamtime and EnvRadNet for travel and subsistence to ESRF. We would like to thank Dr Z Azlam of the 370 371 University of Leeds LENNF facilities for TEM support. Further thank goes to Paul Lythgoe, Alastair Bewsher 372 and John Waters for analytical support and Kurt Smith for assistance with data analysis.

373 **Competing financial interests**

The authors declare no competing financial interest.

375 Author contributions

376 GK – primary investigator, preparation and monitoring of experiments, data acquisition, analysis and
 377 interpretation, manuscript drafting; LT, PB, KM, KK and – support with XANES & EXAFS data collection,

- analysis and interpretation; CB sample analysis for microbial ecology (sequencing); KM, LT manuscript
- 379 review; JRL Planning experiments with GK, data interpretation, review and editing of manuscript.

380 Associated Content

381 Supporting Information

- 382 Methodological details, pH and E_h data, Fe(II) + Fe(III) data, XRD data, additional TEM figures, α -rarefaction
- 383 curves, additional XANES and EXAFS fitting results and parameters used for fitting.

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