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

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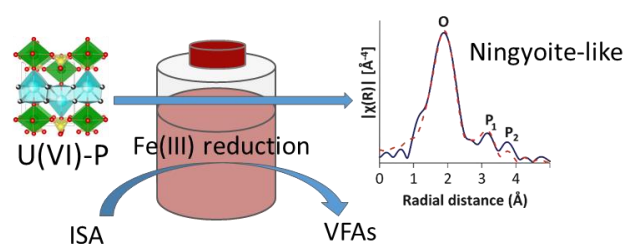
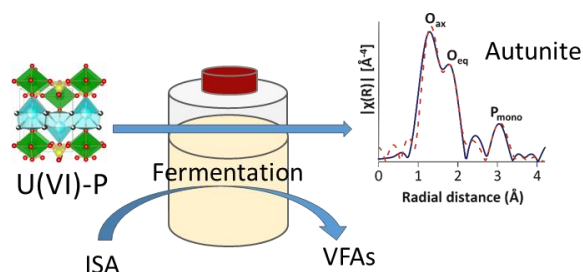
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Abstract

Geological disposal is the preferred long-term solution for higher activity radioactive wastes (HAW) including Intermediate Level Waste (ILW). In a cementitious disposal system, cellulosic waste items present in ILW could undergo alkaline hydrolysis, producing significant quantities of isosaccharinic acid (ISA), a chelating agent for radionuclides. Although microbial degradation of ISA has been demonstrated, 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

32 enzymatic reduction (mediated by metal-reducing bacteria including *Geobacter* species detected by 16S rRNA
33 gene sequencing). Overall, this suggests the potential for the establishment of a microbially-mediated “bio-
34 barrier” extending into the far field geosphere surrounding a GDF which has the potential to evolve in response
35 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*
41 *al.*, 2011; RWM, 2016a). Most EBS concepts include encapsulation of the wastes in cement within steel drums,
42 which are then placed into an excavated vault. Upon re-saturation with groundwater, chemical conditioning will
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
47 long half-life of uranium (^{238}U 4.468 x10⁹ y) and of its resultant decay chain elements, it is important to
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
58 post-closure modelling. Both, U(IV) and U(VI), can form strong, insoluble complexes with phosphates (Rui *et*
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

62 disproportionates (Renshaw *et al.*, 2005; Vettese *et al.*, 2020). The transport behavior of uranium may be further
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.*,
65 2005; Suzuki and Suko, 2006). Of particular interest, with respect to the disposal of ILW, are cellulosic items
66 which are present at around 1% by mass of the total ILW in some wastes (IAEA, 2003; NDA and DEFRA,
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
70 ligand, is predicted to dominate (Whistler and BeMiller, 1958; van Loon and Glaus, 1998; Knill and Kennedy,
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
75 understanding the biogeochemical influence of microbial ISA degradation on uranium speciation, and its impact
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
86 acceptors present in the wastes (Bassil *et al.*, 2015b; a; Kuippers *et al.*, 2015; Rout *et al.*, 2015), which may be
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 metal-

92 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
104 microbial processes in influencing radionuclide mobility in the far field surrounding a GDF (alongside other
105 relevant processes).

106 **Materials and methods**

107 **Sediments.** Shallow subsurface sediment samples (approximately 20 cm depth and pH 7.5) were collected at the
108 margins of an alkaline legacy lime workings site at Harpur Hill, Buxton, U.K., a potential analogue site
109 representative of the alkali-disturbed zone surrounding a GDF that is known to contain ISA-degrading bacteria
110 (Williamson *et al.*, 2013; Bassil *et al.*, 2015b; Kuippers *et al.*, 2015). The sediment was kept in the dark at 4°C
111 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

121 and electron donor to a final concentration of 3.5 mM. To create an ISA-degrading and Fe(III)-reducing
122 enrichment culture, approximately 30 mmol L⁻¹ Fe(III) oxyhydroxide was added to one set of enrichment
123 cultures, as the sole added electron acceptor, whilst a second set of ISA-fermenting enrichment cultures was
124 grown without added Fe(III) oxyhydroxide. Microorganisms able to degrade ISA were selected via periodic sub-
125 culturing (1% v/v inoculum) in fresh medium, typically every four weeks. Stable enrichment cultures were
126 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)
129 oxyhydroxide (30 mmol L⁻¹) was added to the “U(VI)-ISA, Fe(III)-reducing” cultures, and these were also
130 supplemented with 1 mM U(VI) (added as a spike of UO₂²⁺ in 0.001 M HCl) after autoclaving. A parallel set of
131 “U(VI)-ISA fermentation” cultures without added Fe(III), and with 1 mM U(VI) as the sole electron acceptor
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
135 incubated in the dark at room temperature. Periodically, samples were withdrawn and analyzed using a range of
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 UO₂²⁺ (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' (Kuipers *et al.*, 2018).
152 The raw sequencing data were deposited at the NCBI Sequence Read Archive
153 (<http://www.ncbi.nlm.nih.gov/sra/>) with the accession numbers SRR7769831 – SRR7769835.

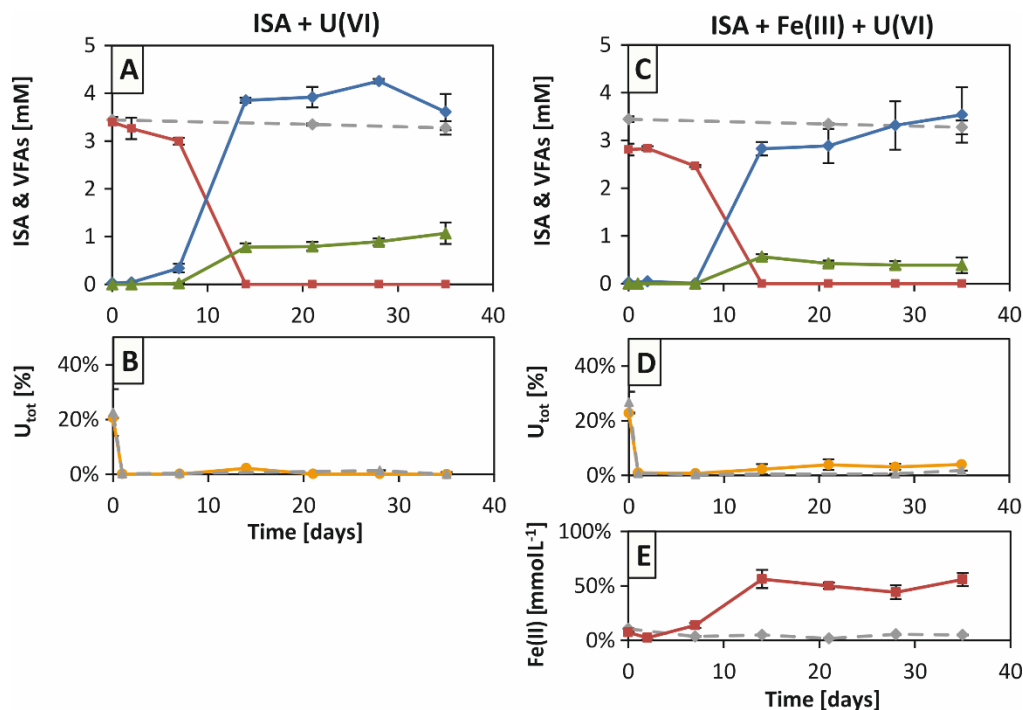
154 **Mineralogical characterization.** All preparation and analyses were performed under anaerobic conditions.
155 Mineral phase identification was carried out using powder X-ray diffraction (XRD) crystallography on a Bruker
156 D8 Advance using an anaerobic cell. Environmental Scanning Electron Microscopy (FEG ESEM, FEI Quanta
157 650) and Transmission Electron Microscopy (FEG TEM, Philips CM200) with selected area electron diffraction
158 (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
161 collected for the L_{III}-edge and M_{IV}-edge of uranium. For studies of the U M_{IV}-edge High-Energy Resolution
162 Fluorescence Detection X-Ray absorption near edge structure (HERFD XANES; Kvashnina *et al.*, 2013, 2014),
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).

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

176 **Results and Discussion**

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



182 Figure 1. Solution chemistry of U(VI)-ISA fermentation (left panel) and U(VI)-Fe(III)-reducing cultures (right
 183 panel), both with 1 mM U(VI). Panel A & C: IC results for ISA (red line), acetate (blue line), butyrate (green
 184 line) and ISA in sterile control (grey dashed line); B & D: ICP MS results for total uranium in solution (yellow
 185 line) and sterile control (grey dashed line); E: Ferrozine results for Fe(II) in solution for microbially active
 186 Fe(III)-reducing culture (red line) and sterile control (grey dashed line). Note: results for U(VI)_(aq) analyses using
 187 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
202 increase until day 14 when the concentration plateaued at approximately 56% \pm 3% (16 mmol L⁻¹) of the 0.5 N
203 HCl-extractable Fe(II) fraction (Figure 1E and SI Figure S4). In addition, the total 0.5 N HCl-extractable iron
204 fraction (Fe(II) + Fe(III)) decreased from a peak of 30 mmol L⁻¹ at the start of the experiment by approximately
205 25% \pm 5% over the course of the incubation time (SI Figure S5), indicating formation of recalcitrant
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,

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

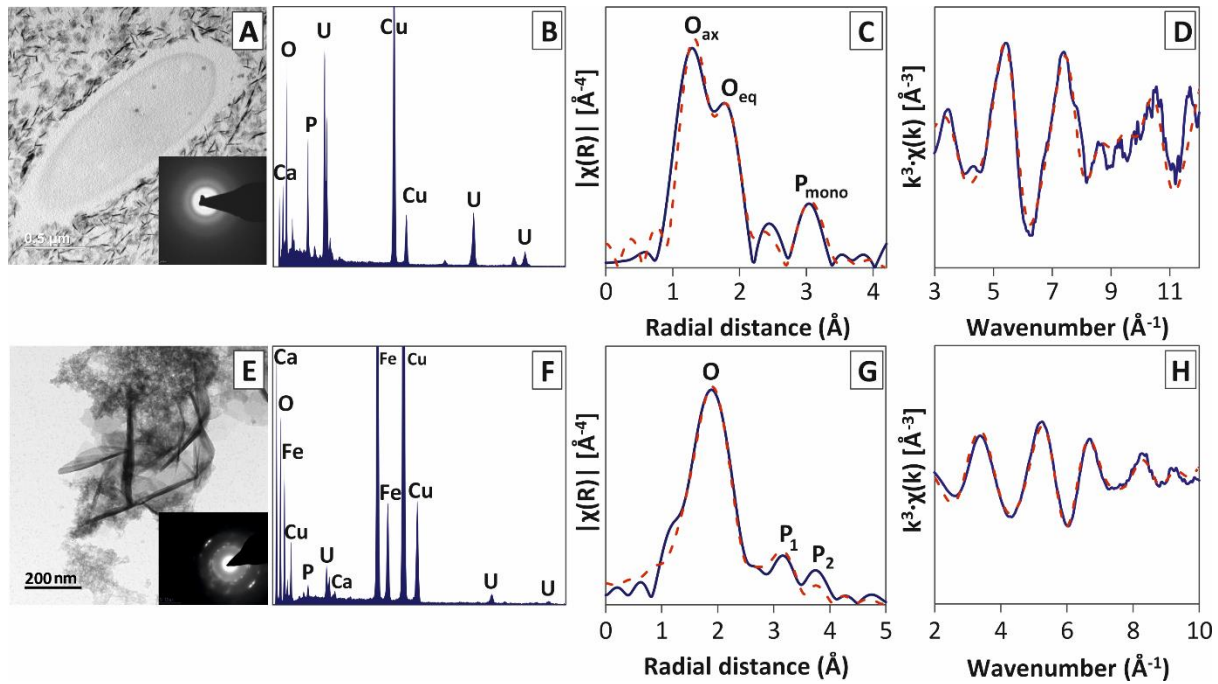
228 **Solid phase characterization.** Biominerals that formed were characterized using XRD, TEM and ESEM. In
229 addition, XAS was used to define the average U(VI) oxidation state and coordination environment in key
230 samples, including the collection of U M_{IV}-edge (SI Figure S8) and U L_{III}-edge (SI Figure S9) spectra. ICP MS
231 data showed that in all microcosms the majority of uranium precipitated at the start of the incubation. XRD
232 analysis on the sterile controls indicated this was potentially a uranyl phosphate phase similar to autunite (SI
233 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 ±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
247 backscatterers at 2.43 Å, and 3 P backscatterers at 3.62 Å (Table 1). This model suggests a U(VI) uranyl species
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).

250 Interestingly, although uranium was not reduced in the U(VI)-ISA fermentation experiment, TEM EDS analysis
251 showed significant accumulation of U- and P-containing precipitates on the cells (SI Figure S14). These
252 uranium precipitates could have complexed with ligands in the microbial cell surface, such as carboxyl, amine,
253 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
 255 which seemed partially associated with the microbial cell surface.



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

Sample	Scattering path	Coordination number	Atomic distance (Å)	Debye-Waller factor σ^2 (Å ²)	R-factor	ΔE_0
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)		
	P _{monodentate}	3	3.62(3)	0.005(3)		
	O _{ax-rattle}	2	3.52(1)	0.008(1)		
	O _{ax-non-forward}	2	3.52(1)	0.004(1)		
	O _{ax-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 _{bidentate}	1.2	3.14(2)	0.005(2)		
	P _{monodentate}	1.8	3.71(3)	0.005(2)*		

261 Table 1. EXAFS fit parameters for U(VI)-ISA degradation experiments with Fe(III) and without Fe(III) added
 262 both with 1 mM U(VI). ΔE_0 is energy shift from calculated Fermi level in eV. The amplitude factor (S_0^2) was
 263 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}
 264 for axial atoms and _{eq} for equatorial atoms. * indicates linked to parameter above.

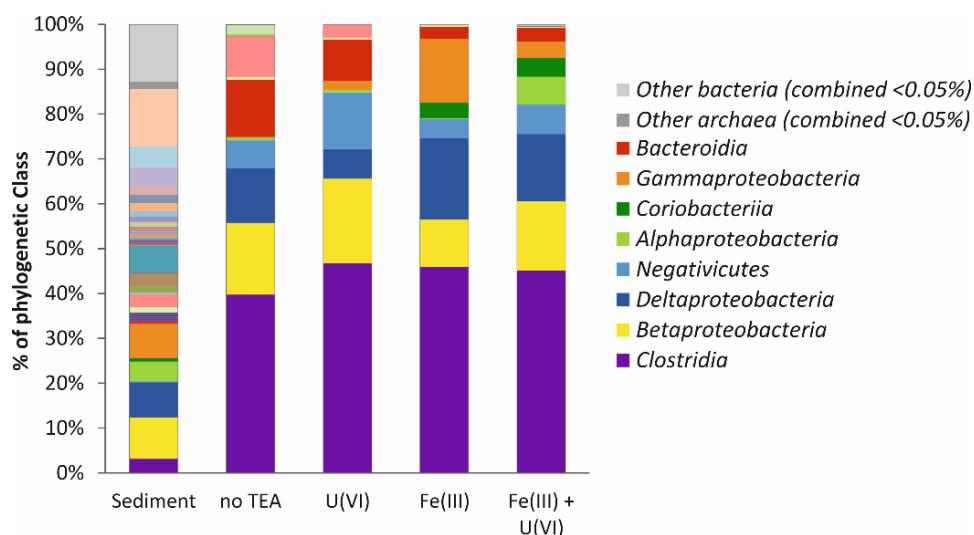
265 U(VI)-Fe(III)-reducing, ISA biodegradation experiment

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

268 the microbially active incubations, whereas in the sterile, Fe(III)-reducing control a uranyl precipitate was
269 identified (SI Figure S11). Instead, in the biotic experiment, siderite (FeCO_3) and vivianite ($(\text{Fe}_3\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}$)
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
277 with a split shell of three equatorial O backscatterers at 2.29 Å, five equatorial O backscatterers at 2.42 Å, and
278 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
279 model is consistent with a range of previously described U(IV)-phosphate structures, including a nanocrystalline
280 ningyosite-like structure (Dusauso *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
282 coordination numbers, delineating between these phases to identify the exact U(IV)-phosphate phase is not
283 possible using solely EXAFS and so from hereon, the U(IV)-phosphate phase present in this study will be
284 referred to as 'ningyosite-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
286 experiment. This is in contrast to the biotic U(VI)-ISA fermentation experiment, where U(VI) reduction was
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 al.*,
289 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.

298 **Microbial community analysis.** 16S rRNA gene profiles were obtained by Illumina sequencing for all
 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.



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

313 Focusing on the ISA-fermenting enrichment with U(VI), this experiment was characterized, in addition to the
 314 strong enrichment of Clostridia (46.8% of genes detected), by a high relative abundance of sequences associated
 315 with members from the class Betaproteobacteria (18.7% of genes detected) and an enrichment of sequences
 316 affiliated with the class Negativicutes (of 12.8% of genes detected). The Negativicutes were comprised of
 317 sequences from the family *Veillonellaceae* (98% match). Another significant class comprised the Bacteroidia
 318 (12.5% of genes detected), of which most sequences were associated with a member from the *vadinBC27*

319 wastewater sludge group (approximately 73% of this class) from the family *Rikenellaceae*. The microbial
320 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
333 members of the family *Veillonellaceae* from the Negativicutes (Woolfolk and Whiteley, 1962; Gihring *et al.*,
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

349 observed more recently as the products of microbial U(VI) reduction, especially in the presence of aqueous
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
355 remobilization (Finch and Murakami, 1999; Jerden and Sinha, 2003; Pinto *et al.*, 2012; Newsome *et al.*, 2015b;
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).

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

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373 **Competing financial interests**

374 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,

378 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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