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Microbial reduction of uranium(VI) in sediments of different lithologies collected from Sellafield

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ABSTRACT

The presence of uranium in groundwater at nuclear sites can be controlled by microbial processes. Here we describe the results from stimulating microbial reduction of U(VI) in sediment samples obtained from a nuclear-licensed site in the UK. A variety of different lithology sediments were selected to represent the heterogeneity of the subsurface at a site underlain by glacial outwash deposits and sandstone. The natural sediment microbial communities were stimulated via the addition of an acetate/lactate electron donor mix and were monitored for changes in geochemistry and molecular ecology. Most sediments facilitated the removal of 12 ppm U(VI) during the onset of Fe(III)-reducing conditions; this was reflected by an increase in the proportion of known Fe(III)- and U(VI)-reducing species. However U(VI) remained in solution in two sediments and Fe(III)-reducing conditions did not develop. Sequential extractions, addition of an Fe(III)-enrichment culture and most probable number enumerations revealed that a lack of bioavailable iron or low cell numbers of Fe(III)-reducing bacteria may be responsible. These results highlight the potential for stimulation of microbial U(VI)-reduction to be used as a bioremediation strategy at UK nuclear sites, and they emphasise the importance of both site-specific and borehole-specific investigations to be completed prior to implementation.

1. Introduction

Contamination of groundwater by aqueous uranium is a common problem at sites where mining, milling and reprocessing of uranium for nuclear fuel has taken place. In the UK, Sellafield is a nuclear reprocessing site in the north west of England (Fig. S1), with the largest contaminated land liability in the UK Nuclear Decommissioning Authority's estate (Cruickshank, 2012). Uranium is known to be present as a contaminant in groundwater underlying the Sellafield site (McKenzie et al., 2011). In oxidising groundwaters uranium is generally present as the mobile uranyl ion (UO_2^{2+}) or uranyl hydroxide complexes below pH 6.5 or as uranyl carbonate complexes at higher pH, whereas under reducing conditions, insoluble U(IV) predominates (Choppin et al., 2002; Newsome et al., 2014). Bioreduction, whereby indigenous soil bacteria are supplied with an electron donor which they oxidise coupled to reduction of aqueous U(VI) to insoluble U(IV), could offer a promising *in situ* remediation strategy to prevent further migration and dispersal of uranium and other radionuclides in groundwater.

Over twenty years of research has identified a diverse range of relatively common soil bacteria that are able to facilitate the enzymatic reduction of U(VI) to U(IV), mainly, but not limited to Fe(III)- and sulphate-reducing bacteria (Lovley and Phillips, 1992; Lovley et al., 1991; Newsome et al., 2014; Williams et al., 2013). Although abiotic reduction of U(VI) by Fe(II) minerals is possible e.g. Latta et al. (2012), Veeramani et al. (2011), most studies show that under environmental conditions the dominant mechanism is direct enzymatic reduction (Bargar et al., 2013; Law et al., 2011; Singer et al., 2012; Williams et al., 2013, 2011). The form of microbially reduced U(IV) is often stated to be uraninite e.g. Suzuki et al. (2002), however, more recently another form, commonly termed monomeric U(IV), has been identified e.g. Bernier-Latmani et al. (2010), Kelly et al. (2008). Field trials stimulating microbial reduction of U(VI) in the subsurface have successfully demonstrated uranium immobilisation over periods of up to a year, although the long-term stability of biogenic U(IV) *in situ* warrants further investigation (Anderson et al., 2003; Bargar et al., 2013; Tang et al., 2013a,b; Williams et al., 2011).

Laboratory microcosm experiments using sediments representative of the Sellafield and Dounraey nuclear facilities, and a sediment sample from the Low Level Waste Repository have

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demonstrated the potential for bioreduction of U(VI) as a remediation technique (Begg et al., 2011; Law et al., 2011; Wilkins et al., 2007). To date, there are no data on U(VI) behaviour in sediments from the Sellafield site. The geology underlying Sellafield is complex, with a varying thickness of made ground underlain by a mixture of Quaternary glacial outwash deposits and till, channel sands and gravels (drift deposits), and Sherwood Sandstone (Smith and Cooper, 2004). The Sherwood Sandstone also outcrops at certain locations on site. It is a major aquifer, with the regional groundwater flow southwest towards the Irish Sea. Groundwater in the Sherwood Sandstone is probably in hydraulic continuity with the overlying drift deposits, which have more variable groundwater flow directions. In certain areas of the site there are localised bodies of perched groundwater (Cruickshank, 2012; McKenzie and Armstrong-Pope, 2010). Contaminant flow pathways vary across the site due to the complex heterogeneity of the subsurface (Hunter, 2004).

The aim of this work was to assess the potential for microbial U(VI) reduction within these different lithological units. Here we present the results from biostimulated U(VI)-reduction experiments using unique on-site sediments obtained from a Sellafield site drilling programme and covering a range of different lithologies underlying the site. In brief, sediments were incubated with an artificial groundwater representative of the site, and containing acetate and lactate as electron donors to stimulate microbial U(VI) reduction. Changes in geochemistry were monitored. Generally, the indigenous microbial community could facilitate the removal of 12 ppm (50 μM) U(VI) from solution through reduction to U(IV). Some variations in the rate and extent of U(VI) reductive precipitation were observed, and the reasons for this are explored further.

2. Materials and methods

2.1. Sediments

A range of sediments from the 2009 to 2010 Sellafield site drilling programme which were either non-active or had very low levels of radionuclides were shipped to The University of Manchester in 2011 and stored in the dark in a cool store room. A fresh sediment sample was obtained from a 2012 site excavation and stored at 4 °C in the dark. Seven sediment samples (Table 1) were selected as representative of key lithologies on sites and were further characterised using X-ray fluorescence (PANalytical Axios), X-ray diffraction (Bruker D8 Advance), for surface area using BET (Gemini 2360 Surface Area Analyser), pH (measured after equilibrating 1 g sediment in 1 ml deionised water for an hour, after ASTM (2006)), total organic carbon (Leco TruSpec) and total bioavailable iron using the ferrozine assay (Lovley and Phillips, 1987).

2.2. Stimulation of microbial U(VI) reduction

Sediments were incubated in triplicate in sterile glass serum bottles with a 1: 10 ratio with sterile anaerobic artificial groundwater representative of the Sellafield area and composed of (g/l) KCl, 0.0066; $MgSO_4{\cdot}7H_2O$, 0.0976; $MgCl_2{\cdot}6H_2O$, 0.081; $CaCO_3$, 0.1672; $NaNO_3$, 0.0275; NaCl, 0.0094; $NaHCO_3$, 0.2424 (Wilkins et al., 2007). Electron donors were also supplied as 5 mM acetate and 5 mM lactate, while control bottles contained no added electron donor. Uranium as U(VI) in 0.001 M HCl was added to the microcosms to give a final concentration of 12 ppm, representative of elevated concentrations of uranium reported in Sellafield site groundwaters (McKenzie et al., 2011). The bottles were crimp sealed with butyl rubber caps, the headspace purged with N_2, and the experiments incubated in the dark at room temperature over three months. In an additional experiment, one replicate set of sediment was stored at 10 °C to represent the average temperature of UK groundwater, in order to investigate the impact of reduced temperature on the rate of microbial U(VI) reduction.

Sediment slurry was extracted at set time points using N_2 flushed syringes and aseptic technique. An aliquot was immediately added to 0.5 N HCl for analysis of Fe(II) as a fraction of total bioavailable iron (Lovley and Phillips, 1987). Porewaters were then separated via centrifugation (14,800*g*) and monitored for U(VI) by the bromo-PADAP assay (Johnson and Florence, 1971), nitrite (Harris and Mortimer, 2002), pH and Eh. Surplus porewaters and sediment pellets were frozen at −80 °C for later analysis of nitrate and sulphate via ion chromatography (Dionex) or microbial community composition via pyrosequencing (see below).

2.3. X-ray absorption spectroscopy (XANES)

Uranium speciation in samples of microbially-reduced clay (RB23) and gravelly sand (RB27) frozen back at day 90 was analysed at the DIAMOND Lightsource, Harwell, UK on Beamline B18. U L_{III}-edge spectra were collected in fluorescence mode using a 9 element Ge detector (Dent et al., 2009) with samples loaded in the cryostat. Data were calibrated, background subtracted and nor-

Table 1
Characteristics of Sellafield sediment samples.

Sediment description, depth and year of excavation	Dark brown SAND and GRAVEL (RB10) 1.5–2.5 m 2009	Red brown clayey SANDY SILT (RB18) 8.0 m 2008	Friable brown gravelly CLAY (RB23) 2.0–2.5 m 2008	Brown fine SAND (RB24) 8.0–8.5 m 2008	Brown GRAVELLY SAND (RB27) Unknown 2012	Red brown SANDSTONE (IS16) 9.5–10 m 2009	Red brown SAND and GRAVEL (IS18) 9.5–10 m 2009
pH	8.38 ± 0.03	8.36 ± 0.03	5.94 ± 0.03	8.55 ± 0.07	8.31 ± 0.03	5.05 ± 0.01	8.56 ± 0.09
TOC (%)	0.35	0.43	2.1 ± 0.045	0.46	0.75 ± 0.11	0.27	0.29
BET (m^2/g)	3.57	5.91	5.43	2.22	5.66	3.51	5.00
Ca (%)	1.23	0.377	0.57	0.38	9.72	0.066	0.878
Mn (%)	0.117	0.08	0.119	0.075	0.079	0.009	0.121
Fe (%)	4.46	2.99	3.42	2.32	2.52	0.666	3.72
P (%)	0.073	0.102	0.244	0.065	0.080	0.044	0.153
U (ppm)	3.5	3.6	3.4	1.2	3.9	1.9	3.9
Bioavailable Fe (%)	0.0392 ± 0.003	0.0307 ± 0.003	0.230 ± 0.010	0.0384 ± 0.009	0.158 ± 0.010	0.0074 ± 0.001	0.138 ± 0.022
Mineralogy (XRD)	Quartz Clinochlore Muscovite Albite Orthoclase	Quartz Clinochlore Muscovite Albite Orthoclase Pyrite	Quartz Clinochlore Muscovite Albite Orthoclase Pyrite	Quartz Clinochlore Muscovite Albite Orthoclase Pyrite	Quartz Clinochlore Muscovite Albite Orthoclase Calcite	Quartz Calcite	Quartz Clinochlore Muscovite Albite

malised for drift to a standardised E_0 position using ATHENA (Ravel and Newville, 2005). Linear combination fitting was performed on XANES spectra compared to data from the Actinide Reference Database for Spectroscopy (Scheinost et al., 2013) for U(IV) as uraninite (Opel et al., 2007) and U(VI) as a uranyl carbonate complex (Rossberg et al., 2009). EXAFS was not performed due to the low, but environmentally-relevant, concentrations of uranium included in the experiment.

2.4. Molecular ecology

To compare changes in the microbial community during development of U(VI) reducing conditions, DNA was extracted from two sediments (clay RB23 and gravelly sand RB27) at the start of the experiment and after approximately 90 days incubation. DNA was extracted from soil/slurry samples (200 μl) using a PowerSoil DNA Isolation Kit (MO BIO Laboratories INC, Carlsbad, CA, USA). The 16S-23S rRNA intergenic spacer region from the bacterial RNA operon was amplified using primers ITSF and ITSReub as described previously (Cardinale et al., 2004). The amplified PCR (polymerase chain reaction) products were separated by electrophoresis in Tris–acetate–EDTA gel. DNA was stained with ethidium bromide and viewed under short-wave UV light. Positive microbial community changes identified by RISA (ribosomal intergenic spacer analysis) justified further investigation by 16S rRNA gene sequencing.

A pyrosequencing methodology was then applied to investigate the microbial diversity within the samples. Isolated DNA from each sample was subjected to PCR amplification of the V1–V2 hypervariable region of the bacterial 16S rRNA gene, using universal bacterial primers 27F (Lane, 1991) and 338R (Hamady et al., 2008). The primers were synthesised by IDTdna (Integrated DNA Technologies, BVBA, Leuven, Belgium) and their design was based on Roche's guidelines for one way amplicon sequencing with the 454 Life Sciences GS Junior system. The fusion forward primer (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNA-GAGTTTGATGMTGGCTCAG-3′) contained 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 (NNNNNNNNNN), and bacterial primer 27F. The reverse fusion primer (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGTGCTGCCTCCCG-TAGGAGT-3′) contained the 454 Life Sciences "Lib-L Primer B", a 4 base "key" sequence (TCAG), and bacterial primer 338R. The PCR amplification was performed in 50 μl volume reactions using 0.4 μl (2.0 units) FastStart High Fidelity DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), 1.8 mM $MgCl_2$, 200 μM of each dNTP, 0.8 μM of each forward and reverse fusion primers, and 2 μl of DNA template (8.6–11.0 ng DNA per reaction). The PCR conditions included an initial denaturing step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and a final elongation step at 72 °C for 5 min. PCR products were then loaded in an agarose gel, and following gel electrophoresis, bands of the correct fragment size (approximately 410 base pairs) were excised, cleaned up using a QIAquick gel extraction kit (QIAGEN, GmBH, Hilden, Germany), and eluted in 30 μl of DNAse free H_2O. The cleaned up PCR products were quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA), and pooled so that the mixture contained equal amounts of DNA from each sample. The emulsion emPCR and the pyrosequencing run were performed at the University of Manchester sequencing facility, using a 454 Life Sciences GS Junior system (Roche).

The 454 pyrosequencing reads were analysed using Qiime 1.6.0 release (Caporaso et al., 2010). Low quality reads (mean quality score less than 25) and short sequences (less than 300 base pairs) were discarded, and both forward and reverse primers were removed from further analysis. Denoising and chimera removal was performed during OTU picking (at 97% sequence similarity) with USEARCH (Edgar, 2010) in Qiime, and a representative sequence for each OTU was identified. Taxonomic classification of all reads was performed in Qiime using the Ribosomal Database Project (RDP) at 80% confidence threshold (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.

2.5. Sequential extractions

Sequential extractions were performed on sediment samples to provide insight into the mineral phases that Fe and microbially-reduced U(IV) were present in. The modified BCR sequential extraction procedure was used (Rauret et al., 1999). Briefly the extraction steps included 0.11 M acetic acid "exchangeable", 0.5 M hydroxylamine hydrochloride "reducible", 8.8 M hydrogen peroxide and 1.0 M ammonium acetate "oxidisable", and aqua regia "residual" fractions. The Fe-extractions were performed on 0.5 g of sediment. For the U-extractions, 0.5 g of microbially-reduced sediment slurry was used, and the first two extractions were performed under anaerobic conditions (Keith-Roach et al., 2003). Extracts were diluted in 2% nitric acid and analysed for uranium via ICP-MS (Agilent 7500CX).

2.6. Enrichment culture

To enrich for Fe(III)-reducing bacteria present in the clay sediment, 1 ml of microbially reduced sediment slurry was inoculated into 100 ml of anaerobic sterile freshwater minimal medium (Lovley et al., 1991; Thorpe et al., 2012) at pH 7, with 5 mM acetate and 5 mM lactate as an electron donor and approximately 15 mmoles per litre ferrihydrite as the electron acceptor (Cornell and Schwertmann, 2006; Schwertmann and Cornell, 2000). The proportion of Fe(II) in the experiment was monitored using the ferrozine assay (Lovley and Phillips, 1987). When the Fe(II) had reached 30% or greater (usually occurring between two and four weeks of incubation), 1% of the enrichment culture was inoculated into the next batch of freshwater minimal medium. This was repeated until the thirteenth enrichment. From each enrichment culture, the DNA was extracted and the microbial diversity assessed using RISA (as above). The microbial diversity of the first, eighth and thirteenth enrichments was investigated by pyrosequencing (as above). The identity of the reduced Fe(II) mineral was characterised using XRD and ESEM.

To investigate whether sediments IS16 (sandstone) and IS18 (sand and gravel) contained sufficient bioavailable Fe to support a robust population of Fe(III)-reducing bacteria, an aliquot of the Fe(III)-reducing enrichment culture (1%) was added to sediment microcosms with no added U(VI). These were then monitored for changes in Fe(II)/Fe(III) over 100 days. Due to the low concentrations of bioavailable Fe(III) present in the sandstone, it was added at a 2:5 sediment to artificial groundwater ratio instead of 1:10. This ensured that measurements were well above the limit of detection and so could be analysed with greater confidence. The acid-digested sediment slurry (Lovley and Phillips, 1987) was filtered before adding to ferrozine solution in order to avoid turbidity affecting colorimetric measurements.

2.7. Most probable number (MPN) enumerations

Most probable number (MPN) enumerations were used to assess the abundance of Fe(III)-reducing bacteria in sediments RB23 (clay), RB27 (gravelly sand), IS16 (sandstone) and IS18 (sand and gravel). Here, 1 g of sediment was added to 10 ml anaerobic

sterile freshwater minimal medium at pH 7 in triplicate, with 3.9 mM nitrilotriacetic acid (as the trisodium salt), 2 mM acetate as the electron donor and approximately 4 mmoles per litre ferrihydrite as the electron acceptor. Serial dilutions were performed by adding 1 ml of the slurry to 9 ml of the medium, until a dilution factor of 10^{-7} was reached. Fe(III) reduction was monitored using the ferrozine assay (Lovley and Phillips, 1987) after 7 and 11 weeks incubation, parallel to reduction times seen in sediment incubations. MPN estimates were then made using published MPN tables (Man, 1983).

3. Results

3.1. Characteristics of Sellafield sediment samples

Prior to assessing the potential for microbial U(VI) reduction, the sediment samples were characterised using a range of techniques. The seven sediment samples comprised mainly silicate minerals, namely quartz, mica, chlorite and feldspar (Table 1). Two sediments contained calcite, and three contained pyrite. Lithology ranged from clay and silt, to sand and gravels, broadly representative of glacial till and the buried channel and fluvioglacial units. One sample (IS16) was of the upper sandstone bedrock. The mean (±1 SD) composition of the seven soils determined by XRF was: Ca 1.89% (3.5), Mn 0.086% (0.04), Fe 2.87% (1.2), P 0.11% (0.07), U 3.06 ppm (1.1), by total organic carbon analysis was 0.84% (0.74), total bioavailable Fe by ferrozine assay was 0.092% (0.08), with a BET derived surface area of 4.47 m^2/g (1.4) and with sediment pH ranging from 5.1 to 8.6 (Table 1). In contrast to the other sediments, after 1 h equilibration with artificial groundwater the clay (RB23) released approximately 1.0 mM nitrate and 0.4 mM sulphate to solution, indicating it contained significant quantities of labile electron acceptors. The clay also contained the highest concentration of organic carbon and bioavailable iron.

3.2. Microbial U(VI) reduction

To determine whether the range of sediments and their extant microbial communities underlying Sellafield were able to support microbial reduction of U(VI), samples of different lithology were incubated with added electron donors for approximately 90 days. In five of the seven incubations, U(VI) was removed from solution, and Fe(II) produced (Fig. 1). Sediments were observed to darken in colour and geochemical indicators demonstrated the development of progressively reducing conditions. An initial peak of nitrite was detected up to around 300 μM, representing reduction of the nitrate present in the artificial groundwater. Ion chromatographic analyses confirmed nitrate removal, and subsequent sulphate removal (Fig. S2). All microcosms remained around circumneutral pH throughout the incubation period. Final Eh measurements ranged from −79 to −153 mV. For the additional experiment incubated at 10 °C (RB27), a slight time lag before U(VI) reduction occurred compared to the 21 °C incubation (Fig. 1), but the overall extent of U(VI) and Fe(III) reduction was the same after 90 days. Control samples with no added electron donor generally did not exhibit U(VI) removal or Fe(III) reduction; nitrate and sulphate remained in solution and final Eh values were between +102 and +188 mV. The exception was the clay (RB23) control, in which after 90 days U(VI), nitrate and sulphate were completely removed from solution, Fe(II) was generated, and the Eh was −116 mV.

In contrast U(VI) remained in solution in two of the electron donor stimulated sediment incubations over the 90 day incubation period; these were the sandstone (IS16) and the sand and gravel (IS18) (Fig. S3). The colour remained orange–brown and the final

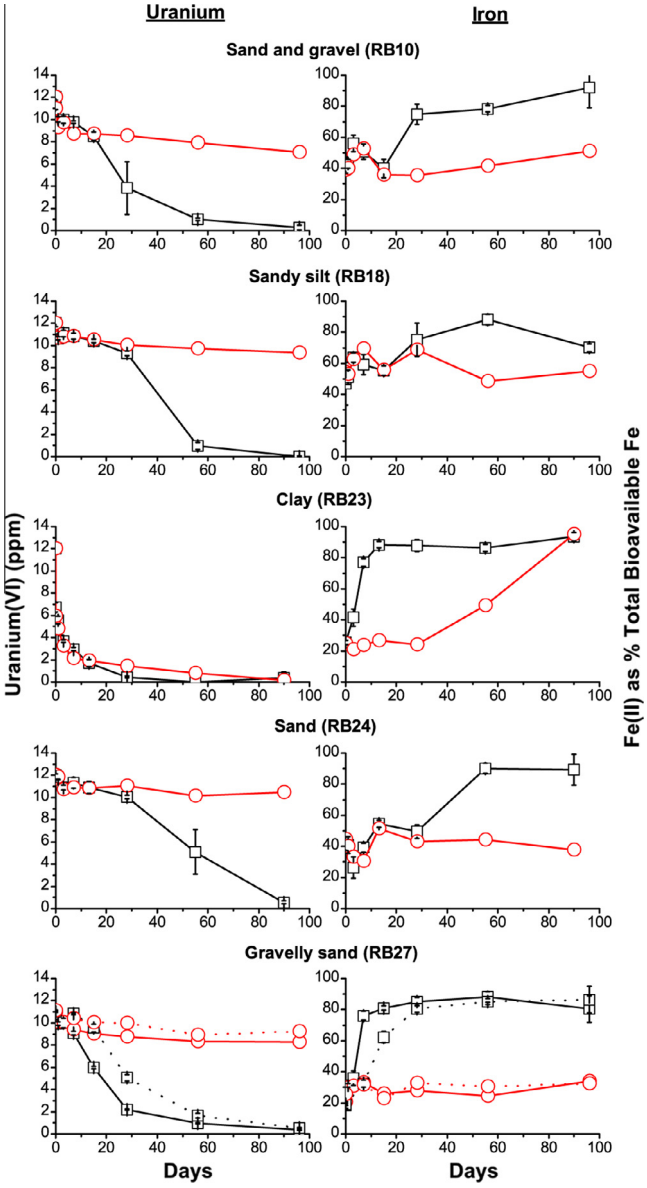

Fig. 1. Results from microbial reduction experiments demonstrating removal of U(VI) and reduction of Fe(III) in sediments of different lithology. Triplicates with electron donor (□), controls without added electron donor (○), error bars represent +/-1 standard deviation. Dashed lines for gravelly sand RB27 represent samples incubated at 10 °C. Results for two sediments which failed to remove U(VI) are presented in Fig. S3.

Eh measurements of +89 and +143 mV indicated that bioreducing conditions did not develop. Possible reasons for this were investigated further using sequential extractions and adding an inoculum of the Fe(III)-reducing Sellafield enrichment culture, to determine if Fe(III) reduction was feasible in these sediments (see below).

XANES spectra were collected for two sediments to identify uranium speciation after 90 days incubation. Both the clay (RB23) and gravelly sand (RB27) samples had a very similar shape to the U(IV) standard, with the same edge position and clearly lacking the post-edge "shoulder" of the U(VI) standard (Fig. 2). Linear combination fitting of these spectra indicated that 100% of the uranium in each sample was present as U(IV).

3.3. Molecular ecology

A rapid molecular profiling approach (gel electrophoresis of the PCR amplified products of the bacterial 16S-23S rRNA intergenic

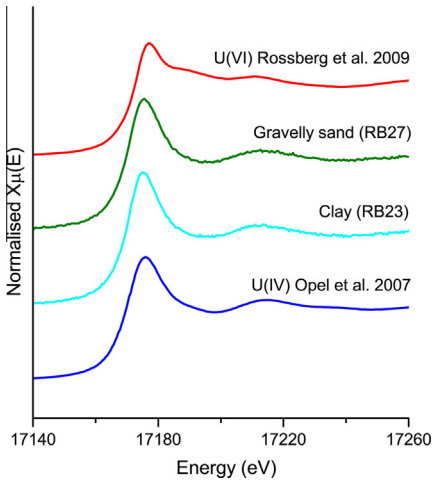

Fig. 2. Uranium LIII-edge XANES spectra for uranium amended sediments and U(IV) uraninite and U(VI) uranyl carbonate reference standards. For both sediments the edge position and shape of the spectra closely resemble the uraninite standard indicating that U is present as U(IV).

spacer region) indicated that there were clear shifts in the structure of bacterial communities in the two sediment samples after 90 days of incubation (Fig. S4). The phylogenetic diversity of the bacterial communities within these samples was investigated further by 16S rRNA gene sequencing, using pyrosequencing (Fig. 3 and Table 2). The results indicated that both sediments were characterised by a diverse range of bacterial phyla prior to incubation (Fig. 3), with most of the sequences affiliated to terrestrial or soil environments (Table 2) and belonging to ubiquitous bacterial orders such as Rhodospirillales, Acidobacteriales, Solibacterales, Xanthomonadales, Rhizobiales, Sphingobacteriales, Hydrogenophilales, Pseudomonadales and Burkholderiales.

In the clay sediment (RB23), after 90 days incubation with acetate and lactate there was a clear shift in the structure of the bacterial community, as sequences affiliated with nitrogen-fixing *Azospirillum* sp., iron-reducing *Rhodoferax ferrireducens,* organisms from agricultural soils or environments associated with U(VI) reduction and organic degradation became enriched (Table 2, Fig. S5). Furthermore, the number of sequences affiliated with the known Fe(III)- and U(VI)-reducing *Geobacter* (2.9%) genus more

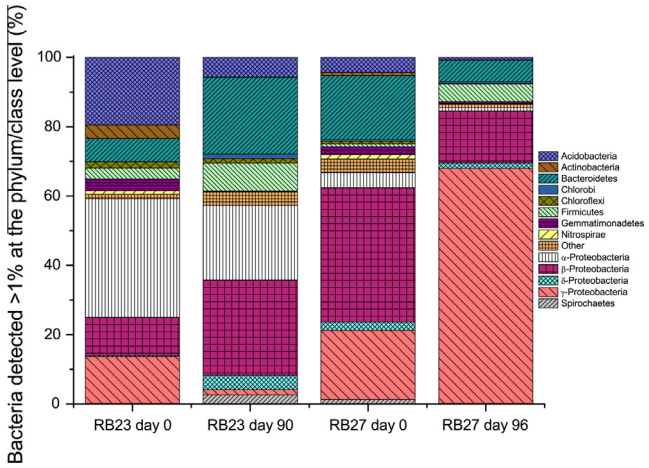

Fig. 3. Bacterial phylogenetic diversity within clay (RB23) and gravelly sand (RB27) sediments before and after development of bioreducing conditions (at the phylum level/class for the Proteobacteria). Phyla/classes detected at greater than 1% of the bacterial community are illustrated.

than doubled, as did sequences of genera belonging to the Bacteroidales, Sphingobacteriales (*Flavisolibacter*), Clostridiales, Rhodospirillales (*Azospirillum*), Burkholderiales (*Limnohabitans, Polaromonas, Rhodoferax*), Hydrogenophilales, Desulfuromonadales (*Geobacter*) and Spirochaetales (*Spirochaeta*) bacterial orders. Of these, Clostridiales, Burkholderiales and Desulfuromonadales contain known U(VI)- or Fe(III)-reducers (Finneran et al., 2003; Gao and Francis, 2008; Lovley et al., 1991).

The gravelly sand sediment (RB27) was characterised by a large increase in sequences belonging to the Pseudomonadales order (from 11.6% to 65.3%), with most of these being closely affiliated to *Pseudomonas peli* strain R-20805 isolated from a nitrifying inoculum (Table 2, Fig. S5). Other bacterial orders containing genera which increased by a factor of two or more were Bacteroidales, Neisseriales (*Vogesella*), Desulfuromonadales (*Geobacter*) and Alteromonadales (*Shewanella*), and within these were sequences affiliated to known U(VI)- and Fe(III)-reducing *Geobacter* (1.0%) and *Shewanella* (1.5%).

3.4. Sequential extractions

As the sandstone (IS16) and sand and gravel (IS18) were unable to generate microbial U(VI) reduction, one possible explanation is that they contained insufficient bioavailable Fe(III) to maintain an active Fe(III)- and consequently U(VI)-reducing microbial community. BCR sequential extractions were performed alongside XRF and 0.5 N hydroxylamine hydrochloride extractions to gain insight into the operationally defined sediment associations of Fe in IS16 and IS18 compared to two sediments which successfully removed U(VI) from solution; the clay (RB23) and gravelly sand (RB27). The proportion of Fe in the four sediments, measured via aqua regia digestion, ranged from 0.1% to 1.8%; similar to but slightly lower than the results obtained by XRF, probably due to refractory Fe present in the mineral lattices of clays not dissolving with aqua regia. The results for total bioavailable iron measured using the ferrozine assay were broadly comparable to the sequential extraction reducible fraction, ranging from 0.07 to 2.3 milligrams per gram of sediment. While the sand and gravel (IS18) contained similar concentrations of total Fe and "bioavailable" Fe to the clay (RB23) and gravelly sand (RB27), the concentration in the sandstone (IS16) were much lower (Fig. 4).

An additional set of sequential extractions were performed on the clay (RB23) and gravelly sand (RB27) to investigate which sediment fraction microbially reduced U(IV) was associated with. In the clay, most uranium was found in the reducible (38% ± 2.0%) and oxidisable (50% ± 7.6%) fractions, while in the gravelly sand, most uranium was in the exchangeable (41% ± 3.7%) and reducible (44% ± 19%) fractions (Fig. 5). This suggests that microbially reduced U(IV) may partition to a more recalcitrant fraction in the clay compared to the gravelly sand.

3.5. Enrichment culture

An Fe(III)-reducing enrichment culture was generated from the clay sediment. This enrichment culture reduced more than 30% of bioavailable Fe(III) in the medium within 2–4 weeks, forming a golden coloured platy mineral. This was identified by XRD as a mixture of the Fe(II) bearing minerals siderite ($FeCO_3$) and vivianite ($Fe_3(PO_4)_2{\cdot}8H_2O$) (Fig. 6).

RISA indicated that after eight subcultures, a relatively diverse microbial community remained present (Fig. S4). This was confirmed with the 16S rRNA gene sequencing results, with 582 sequences present in the eighth subculture (compared to 898 in the initial sample). A relatively stable microbial community had developed by the eighth enrichment subculture which remained broadly similar at the thirteenth subculture (Fig. 7 and Table 3),

Table 2
Closest phylogenetic relatives of the five most abundant OTUs from clay (RB23) and gravelly sand (RB27) sediments before and after the development of bioreducing conditions.

Number of clones	% of clones	Closest phylogenetic relative	Accession number	ID similarity (%)	Environment
RB23 day 0					
155	10.3	Uncultured bacterium clone NC42e10_14934	JQ384246.2	98	Elevated atmospheric CO_2/warmed Antarctic soils/methane oxidisers
103	6.9	Bacterium Ellin643	DQ075307.1	100	Elevated atmospheric CO_2/anaerobic cellulose degraders/rice paddy soil
76	5.1	Uncultured *Rhodospirillaceae* bacterium	EF018478.1	99	Elevated atmospheric CO_2/activated sludge/drinking water treatment
60	4.0	Uncultured bacterium clone WC1_a1	GQ263698.1	98	Cellulosic waste/elevated CO_2 soils/Antarctic soils
55	3.7	Uncultured bacterium clone WC2_31	GQ263951.1	100	Cellulosic waste/elevated CO_2 soils
RB23 day 90					
349	13.8	*Azospirillum* sp. 7C	AF411852.1	97	Nitrogen-fixing bacteria from rhizosphere/fuel contaminated Antarctic soils
181	7.1	Type strain *Pseudorhodoferax caeni* strain SB1	NR_042216.1	97	Activated sludge/anaerobic digester/organics degrader/drinking water treatment
180	7.1	*Flavisolibacter ginsengisoli*	NR_041500.1	97	Ginseng cultivating soil/organics degrader/rhizosphere
115	4.5	Uncultured bacterium clone 5MhU1878D11	JF395212.1	97	Microbial community response to U(VI) bioremediation/organics degrader
63	2.5	Type strain *Rhodoferax ferrireducens* T118 strain DSM 15236	NR_074760.1	99	Acetate amended soils/organics degrader
RB27 day 0					
288	6.3	*Algoriphagus terrigena* strain DS-44	NR_043616.1	99	Korean soil
216	4.7	*Thiobacillus denitrificans* ATCC 25259	NR_074417.1	97	Chemolithoautotrophic, facultatively anaerobic bacterium. Acetate amended soils/thiosulphate or sulphur oxidiser/acid mine draining/PCB contaminated soil
203	4.4	Type strain *Pseudomonas mohnii*	NR_042543.1	100	Degrader of chlorosalicylates or isopimaric acid
195	4.3	Type strain *Polaromonas* sp. JS666	NR_074725.1	99	Microbial community response to U(VI) bioremediation/anaerobic digester/drinking water treatment/snow/glaciers
106	2.3	*Thiobacillus thiophilus* strain D24TN	NR_044555.1	97	Chemolithoautotrophic, thiosulphate-oxidising bacterium. Acetate amended soils/hydrocarbon bioremediation
RB27 day 96					
6963	27.9	Type strain *Pseudomonas peli* strain R-20805*	NR_042451.1	99	Nitrifier/organic degrader/nitrate reducer
5182	20.8	Type strain *Pseudomonas peli* strain R-20805*	NR_042451.1	99	Nitrifier/organic degrader/nitrate reducer
897	3.6	Type strain *Pseudomonas mohnii*	NR_042543.1	100	Degrader of chlorosalicylates or isopimaric acid
497	1.9	Type strain *Pseudomonas peli* strain R-20805*	NR_042451.1	96	Nitrifier/organic degrader/nitrate reducer
435	1.7	*Pseudomonas sagittaria* sp. nov.	JQ277453	100	Siderophore producer/oil or herbicide contaminated soil/phenol degrader/magnetite mine drainage

* These represent bacteria assigned to different operational taxonomic units, but each are most closely related to *Pseudomonas peli* strain R-20805.

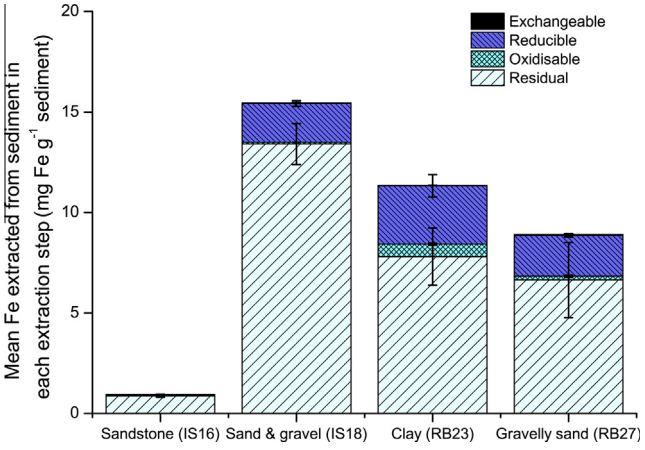

Fig. 4. Profile of Fe in sediments which were able to facilitate U(VI) bioreduction (RB23, RB27) and were not able to (IS16, IS18), as determined by sequential extraction. Bars represent the average of triplicate samples, error bars are +/-1 standard deviation.

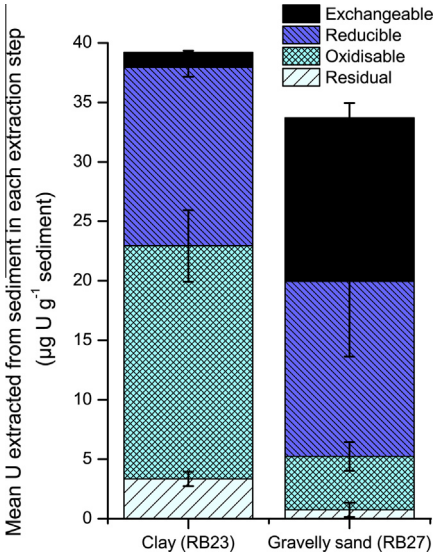

Fig. 5. Profile of bioreduced U in clay and gravelly sand sediments, as determined by sequential extraction. Bars represent the average of triplicate samples, error bars are +/-1 standard deviation.

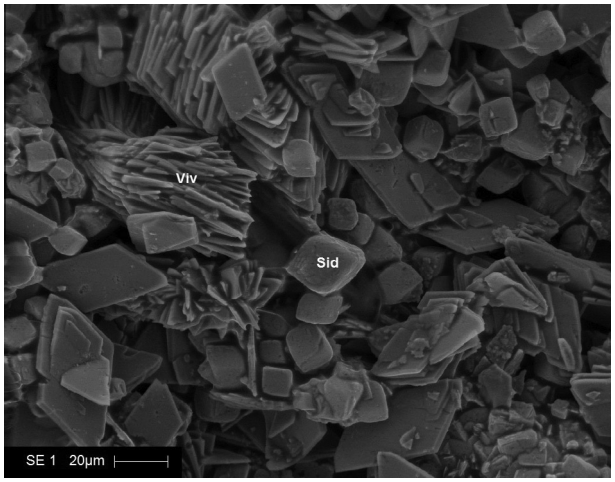

Fig. 6. ESEM image showing cubic siderite "Sid" and acicular/platy vivianite "Viv" minerals. Mineral identification was confirmed using XRD.

with 41–46% of sequences closely related to four species of *Thaurea, Geobacter, Sporotalea* (reassigned as *Pelosinus* (Moe et al., 2012)), *Acidovorax* and *Simplicispira*; genera found in anaerobic environments and typically associated with organic degradation or Fe(III)-reduction (Fig. S6).

An alternative explanation for some sediments not being able to support stimulated microbial reduction of U(VI) is that the extant microbial community did not contain viable Fe(III)-reducing bacteria. Results showed that adding an inoculum of enrichment culture to sediment microcosms of the sandstone (IS16) and sand and gravel (IS18) increased the extent of Fe(III)-reduction compared to controls without the added inoculum and Eh data were consistent with this interpretation (Fig. S7). As the sequential extractions demonstrated that the sand and gravel (IS18) contained comparable amounts of bioavailable iron to the sediments which did stimulate U(VI)-reduction, it is possible that this sediment lacks an active Fe(III)-reducing microbial community. Given the low concentration of bioavailable iron in the sandstone (IS16), it is not surprising that when the proportion of sediment was increased and an enrichment culture added, some Fe(III)-reduction could be measured. This indicates that the lack of bioavailable Fe(III) and Fe(III)-reducing bacteria in this sediment might preclude U(VI)-reduction from taking place, unless manipulated through the addition of electron acceptor (Fe(III) or sulphate) and/or microbial inocula.

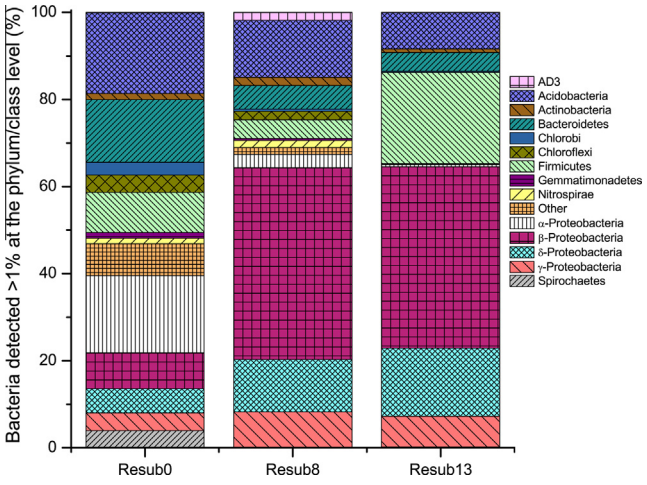

Fig. 7. Bacterial phylogenetic diversity within the enrichment culture (at the phylum level/class for the Proteobacteria). Phyla/classes detected at greater than 1% of the bacterial community are illustrated.

3.6. Most probable number enumerations

After 11 weeks of incubation, approximately 1×10^5 – 1×10^7 cells of Fe(III)-reducing bacteria per gram of sediment were measured in the sediments which had previously been shown to reduce U(VI) (Table 4). As expected from biogeochemical measurements, this exceeded the numbers present in the sediments which could not reduce U(VI), with approximately 1×10^4 cells per gram of sandstone (IS16) and sand and gravel (IS18).

4. Discussion

Sellafield site sediments representing a range of different lithologies underlying the site have been characterised in terms of their potential to develop reducing conditions and consequently remove U(VI) from solution. The indigenous microbial community in most of the sediments was able to reduce U(VI) to U(IV) and remove it from solution over approximately 90 days after stimulation with added electron donor, including at 10 °C representative of UK groundwater temperatures. The clay sediment (RB23) contained sufficient indigenous and labile organic matter to stimulate microbial U(VI) reduction without addition of an electron donor.

As expected, over the incubation period a cascade of anaerobic redox processes was observed in most of the sediments, as the available nitrate was depleted quickly (within 1 week) followed by periods of Fe(III)- and sulphate reduction. In addition, within the identified 16S rRNA sequences, there were sequences closely related to known nitrogen-fixing (*Azospirillum*) nitrifying (*Pseudomonas*), and Fe(III)-/metal-reducing (*Geobacter*, *Shewanella*, *Rhodoferax*) genera as well as a number of bacteria closely related to those known to degrade a range of organic compounds. Thus both geochemical and molecular ecology results indicate clearly that the indigenous microbial population in the majority of these Sellafield sediments have the capacity to utilise a wide range of electron donors and acceptors and carry out diverse biogeochemical processes.

Regarding U(VI) reduction, it is noteworthy that during the incubation period the proportion of sequences related to known U(VI)-reducing bacterial genera more than doubled, including *Geobacter* and *Shewanella* (Lovley et al., 1991), albeit they remained in low abundances (1–3%). It is not clear whether U(VI) reduction was carried out by these microorganisms or by more dominant members of the microbial communities, since many of the sequences of this study were not closely related to cultured organisms with known physiological properties. Moreover, in the gravelly sand sediment there was a significant enrichment of sequences closely related to *P. peli* after incubation (Fig. 3 and Table 2). Although *Pseudomonas* is known predominately as a facultative anaerobic denitrifying genus, sequences related to *Pseudomonas stutzeri* dominated similar U(VI) bioreduction experiments established with soil from the nearby Low Level Waste Repository site (Wilkins et al., 2007). Future anaerobic microcosm studies should explore in more detail the potential of these indigenous microbial communities for the reduction of U(VI) and other radionuclides.

Important differences in sediment composition include the clay content, and the amount of total organic carbon and bioavailable Fe(III) present. Although calcium may inhibit microbial U(VI) reduction through the formation of stable Ca-uranyl-carbonate complexes (Brooks et al., 2003), this effect was not observed in these sediments. That is, while calcium was present in the artificial

Table 3
Closest phylogenetic relatives of the five most abundant OTUs from the initial sediment enrichment and the eight and thirteenth enrichment subcultures.

Number of clones	% of clones	Closest phylogenetic relative	Accession number	ID similarity (%)	Environment
First enrichment culture					
349	3.5	Uncultured Acidobacteria bacterium clone 4OLI_9	GQ342374.1	99	Biostimulated U mining soil/drinking water treatment/hydrocarbon contaminated aquifer/bioremediation of U(VI) or TCE/rice paddy soil
281	2.8	*Geothrix fermentans*	NR_036779.1	97	Fe(III)-reducer/biostimulated U mining soil/rice paddy soil/drinking water treatment
281	2.8	Uncultured bacterium clone B0610D002_F03	AB660901.1	99	Rice paddy soils/acetate amended soils/activated sludge/Arctic sediments
229	2.3	Uncultured bacterium clone ORFRC-FW102–726d-55	FJ451813.1	99	*In situ* uranium bioremediation
189	1.9	*Flavisolibacter ginsengisoli*	NR_041500.1	97	Ginseng cultivating soil/organics degrader/rhizosphere
Eighth enrichment subculture					
1878	17.9	*Thauera* sp. G3DM-88	EU037291.1	99	Landfill sediments contaminated with Cr/anaerobic digester/activated sludge/iron reducer/organic degrader
1036	9.9	*Geobacter bemidjiensis* strain Bem	NR_075007.1	99	Fe(III)-reducer/rice paddy soil/acetate amended soils/wetlands/mining soils
828	7.9	*Acidovorax defluvii* strain BSB411	NR_026506.1	100	Activated sludge/ammonium rich aquifer/wastewater or drinking water treatment
542	5.2	*Simplicispira metamorpha* strain DSM 1837	NR_044941.1	99	Activated sludge/anaerobic digester/water treatment/nitrifying chemostat/ammanox
300	2.9	Type strain *Pseudomonas peli* strain R-20805	NR_042451.1	99	Nitrifier/organic degrader/nitrate reducer
Thirteenth enrichment subculture					
2029	15.9	*Thauera* sp. G3DM-88	EU037291.1	99	Landfill sediments contaminated with Cr/anaerobic digester/activated sludge/iron reducer/organic degrader
1981	15.6	Uncultured bacterium clone B0610D003_J14 (*Geobacter*)	AB660562.1	98	Rice paddy soil
1137	8.9	Type strain *Sporotalea propionica* strain TmPN3*	NR_042513.1	97	U(VI) removal by anaerobic microbial communities
1116	8.8	*Acidovorax defluvii* strain BSB411	NR_026506.1	100	Activated sludge/ammonium rich aquifer/wastewater or drinking water treatment
783	6.2	*Simplicispira metamorpha* strain DSM 1837	NR_044941.1	99	Activated sludge/anaerobic digester/water treatment/nitrifying chemostat/ammanox

* *Sporotalea propionica* has since been reassigned as *Pelosinus propionicus* comb. nov. (Moe et al., 2012).

Table 4
MPN enumerations of Fe(III)-reducing bacteria.

Sediment	7 weeks MPN (cells g^{-1})	11 weeks MPN (cells g^{-1})
Sand (RB10)	7.4×10^3	2.4×10^5
Clay (RB23)	1.5×10^5	7.4×10^6
Gravelly sand (RB27)	1.5×10^4	9.3×10^4
Sandstone (IS16)	2.3×10^3	9.3×10^3
Sand and gravel (IS18)	9.2×10^2	2.4×10^4

groundwater (1.6 mM) and within the sediments (up to 9.7% Ca measured by XRF (Table 1)), complete U(VI) reduction was observed in most sediments after 90 days incubation. Indeed, microbial reduction of U(VI) as Ca-uranyl-carbonate complexes has also been observed *in situ* at the US DOE Rifle site (US Department of Energy, 2011), perhaps suggesting that the influence of calcium on microbial U(VI) reduction is less important in natural soil systems compared to microbial pure cultures.

Sorption effects were observed in the clay (RB23) sediment incubations; after one hour just over half the added U(VI) had been removed from solution. As other geochemical indicators clearly demonstrated development of reducing conditions (e.g. Fe(II) ingrowth, and nitrate and sulphate reduction) it is likely that the sorbed U(VI) was also reduced. Indeed, reduction of U(VI) sorbed to soils has been observed previously (Begg et al., 2011; Law et al., 2011) and XANES data confirmed that uranium in the solid phase was present entirely as U(IV) in the sample with added electron donor (Fig. 2). Furthermore, results from the sequential extractions indicate that microbially reduced U(IV) partitioned to more recalcitrant phases of the clay compared to the gravelly sand. Relatively high concentrations of organic matter in the clay sediment (RB23) allowed for bioreducing conditions to be established even when no additional electron donor was supplied. Together this suggests that this clay could act as a natural attenuant of U(VI), through both sorption and, if the organic fraction is bioavailable, microbial reduction. Although groundwater does not flow through clay strata, many soils contain a clay mineral component which may be able to offer some capacity for uranium retention.

In certain sediments, the paucity of bioavailable Fe(III) might preclude an active Fe(III)-reducing microbial community from developing and consequently being able to reduce U(VI), such as in the sandstone IS16. However, the ability to reduce U(VI) is not restricted to just Fe(III)-reducers, and this does not explain the lack of U(VI) reduction in the sand and gravel (IS18) as this sediment contained comparable amounts of bioavailable Fe(III) to the others. The issue of certain amendments working in some locations but not others, or in the laboratory but not in the field, is common and it is often difficult to determine the reasons why, especially in complex heterogeneous systems (Lovley, 2003). Evidence obtained by adding an active Fe(III)-reducing enrichment culture to the sandstone (IS16) and sand and gravel (IS18) sediment incubations, and also from MPN enumerations suggested that these sediments contain fewer Fe(III)-reducing bacteria than the U(VI)-reducing sediments, which may have contributed to their failure to reduce U(VI) in these experiments.

This work highlights the potential for stimulated microbial U(VI) reduction to be a suitable technique for treating uranium contaminated groundwater *in situ*, both at Sellafield, and given the range of different lithology sediments tested, at other UK nuclear sites. Should remediation of uranium in groundwater

become a priority, further development of this work such as scale-up to columns prior to field deployment would clearly be warranted.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apgeochem.2014.09.008.

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