

RESEARCH ARTICLE

Postbiostimulation microbial community structure changes that control the reoxidation of uranium

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Received 3 December 2009; revised 29 April 2010; accepted 9 June 2010.
Final version published online 10 August 2010.

DOI:10.1111/j.1574-6941.2010.00933.x

Editor: Alfons Stams

Keywords

postbiostimulation; uranium; reoxidation; biomass turnover; iron-oxidizing bacteria; sulfide-oxidizing bacteria.

Abstract

This study evaluated the influence of changes in the microbial community structure on reoxidation of reduced uranium during a postbiostimulation period. Effluent groundwater from acetate-stimulated sediment flow-through columns was analyzed over 60 days after acetate amendment was discontinued. Only a small reoxidation of iron or uranium (17%) occurred in the presence of 1–2 mg L⁻¹ O₂ influent groundwater for the 2-month period. Most uranium reoxidation occurred during the first 2 weeks after biostimulation with acetate was discontinued. Groundwater and sediment microbial community compositions suggested that two processes played important roles immediately after the cessation of acetate addition. The first process was characterized by a predominance of both sediment-bound and planktonic microorganisms most closely related to *Hydrogenophaga* sp., *Thiobacillus* sp., and *Gallionella* sp., which could oxidize a variety of reduced compounds. The second process was characterized by organisms closely related to *Lysobacter* sp. and *Sterolibacterium* sp., with the potential to feed on complex organic compounds from biomass turnover. The presence of these bacteria and the lack of uranium oxidation implied that after acetate addition was stopped, reduced inorganic compounds and dead biomass became electron donors for a microbial community capable of using low ambient oxygen as a terminal electron acceptor, contributing to the preservation, at least temporarily, of biogenic U(IV).

Introduction

Over the past decade, many studies have used a variety of approaches to remediate uranium-contaminated groundwater (i.e. Morrison *et al.*, 2001; Finneran *et al.*, 2002). Biostimulation has been a promising uranium remediation strategy (Anderson *et al.*, 2003; Wu *et al.*, 2006; Cardenas *et al.*, 2008). The current bioremediation strategies for subsurface plumes of uranium stimulate an indigenous microbial community to reduce soluble U(VI) to insoluble U(IV), preventing further mobility of uranium in groundwater. This is achieved through the addition of a carbon source such as acetate, lactate, or ethanol (Abdelouas *et al.*, 1998; Anderson *et al.*, 2003; Istok *et al.*, 2004; Sani *et al.*, 2004). Past studies have focused on the microbially mediated U(VI) reduction to biogenic insoluble U(IV) minerals (Lovley & Phillips, 1992; Abdelouas *et al.*,

1998; Anderson *et al.*, 2003; Wu *et al.*, 2006), their reoxidation in the presence of different oxidants (Finneran *et al.*, 2002; Senko *et al.*, 2002; Gu *et al.*, 2005; Zhou & Gu, 2005; Moon *et al.*, 2007; Komlos *et al.*, 2008a), and the microbial communities associated with uranium biostimulation (Holmes *et al.*, 2002; Chang *et al.*, 2005; Cardenas *et al.*, 2008; Hwang *et al.*, 2009). However, little is known about the changes in the microbial community dynamics and geochemistry during poststimulation (the period after acetate amendment has been discontinued), which could control the stability of bio-reduced uranium when the electron donor addition is discontinued.

It has been suggested that a potential drawback of biostimulation to immobilize uranium in groundwater is the dependence of uranium-sequestering biological processes on the availability of an external source of electron donor. This is because the postbiostimulation stability of biogenic U(IV)

may rely on the maintenance of anoxic conditions that are generated during biostimulation (Komlos *et al.*, 2008a).

It is well known that biogenic U(IV) can be oxidized to U(VI) by oxidants common in groundwater systems such as oxygen (Gu *et al.*, 2005; Zhou & Gu, 2005; Komlos *et al.*, 2008a, c) and nitrate (Finneran *et al.*, 2002; Senko *et al.*, 2002; Moon *et al.*, 2007, 2009). Although nitrate does not directly oxidize U(IV) abiotically, denitrification intermediates (nitrite, nitric oxide, and nitrous oxide) can oxidize U(IV) directly (Senko *et al.*, 2002). Bacteria such as *Geobacter* sp. (Finneran *et al.*, 2002), *Desulfovibrio* sp. (Senko *et al.*, 2005), and *Thiobacillus* sp. (Beller, 2005) have been shown to oxidize U(IV) in the presence of nitrate. The concentration of nitrate at the Rifle IFRC site is very low ($< 3 \mu\text{M}$) such that it has minimal to no effect on the biogeochemical processes affecting uranium reduction and reoxidation. Although Mn oxides (Fredrickson *et al.*, 2002) and Fe(III) minerals have been shown to oxidize biogenic U(IV) under limited geochemical conditions (Sani *et al.*, 2005; Ginder-Vogel *et al.*, 2006), Mn oxides were not detected in these sediments (Komlos *et al.*, 2008c), indicating that U(IV) oxidation by Mn oxides should not be a factor in these experiments.

In contrast, a recent field study at a uranium-contaminated site in Rifle, CO (N'Guessan *et al.*, 2008), has shown that uranium immobilization may continue long (months to years) after biostimulation via acetate injection has ceased. Biosorption was proposed as one of the processes contributing to the long-term sequestration of uranium (N'Guessan *et al.*, 2008). Other researchers (Kelly *et al.*, 2008) reported that after biostimulation, uranium was no longer bound to carbon ligands, but was adsorbed to Fe/Mn minerals, and that reduction of U(VI) to U(IV) continued in sediment samples stored under anaerobic conditions. In addition, Wan *et al.* (2005) reported that microbial reduction of uranium can be transient, even under sustained reducing conditions, as a result of the increase in the bicarbonate concentration due to microbial respiration. Using sediments from the Rifle site and synthetic groundwater, Moon *et al.* (2009) have shown that in the absence of nitrates and in the presence of low dissolved oxygen (DO) levels, precipitated uranium remained stable for extended time periods. Oxygen, as opposed to nitrate, was characterized by a very delayed breakthrough, indicating that oxygen was consumed in the flow-through sediment columns before it could react with the U(IV) phases.

Although the oxygen consumption described above could be mediated biologically, little research on the microbial community changes during postbiostimulation has been performed and little is known of how it might affect geochemical conditions and U(IV) stability. Because the oxidants mentioned above (DO and nitrate) that influence the U(IV) stability are all affected by microbial activity, strong feedbacks are expected between the poststimulation microbial dynamics and uranium stability. Therefore, the

objectives of this study were to investigate the postbiostimulation microbial community dynamics in sediments and groundwater, under the conditions observed at the Rifle site, after acetate addition is stopped, and note how this microbial change is linked to major biogeochemical processes and uranium stability. For this purpose, sediment flow-through columns were operated under acetate biostimulation in the presence of $< 2 \text{ mg L}^{-1}$ DO in the influent, to simulate the low ambient oxygen in the groundwater. After the biostimulation period, acetate injection was discontinued and only groundwater entered the columns at typical groundwater flow rates. Columns were sacrificed periodically during the postbiostimulation period, allowing for the characterization of the microbial community and the geochemistry over time. To our knowledge, this is the first investigation of the poststimulation microbial dynamics of carbon turnover under conditions that closely simulate the field and linking these dynamics to the geochemical changes and U(IV) stability.

Materials and methods

Sediment and groundwater

The sediment and groundwater used in the study were collected from the US Department of Energy's Integrated Field Research Challenge site located in Rifle, CO. Detailed descriptions of the geology and hydrogeology of the study site have been presented elsewhere (US Department of Energy, 1999; Anderson *et al.*, 2003; Vrionis *et al.*, 2005; Yabusaki *et al.*, 2007; Komlos *et al.*, 2008c). Both sediment and groundwater were taken from locations upgradient of field experimental plots. The sediment was collected with a backhoe, placed in mason jars, and stored at 4°C without any further treatment until use. Before loading the columns, the sediment was sieved through a 2-mm sieve to homogenize the sample and remove pebbles. Groundwater was pumped to the surface with a peristaltic pump, directly into 5 gallon plastic carboys, and stored at 4°C until use.

Column setup and operation

Seven glass columns (15 cm long, 2.6 cm in diameter, Kimble Kontes) were wet packed with $150 (\pm 10)$ g of the sediment described above. Groundwater from the Rifle site, which contains *c.* 9 mM sulfate, amended with $20 \mu\text{M}$ U(VI), was pumped in an up-flow mode through the columns at a flow rate of 0.04 mL min^{-1} , resulting in a linear flow velocity of 30 cm day^{-1} . The influent media were continuously purged with a gas mixture of nitrogen, containing 0.1% O_2 , to maintain the low ambient DO concentration (Moon *et al.*, 2009), and 2% CO_2 , to maintain circumneutral pH of influent water. Because oxygen diffuses through the influent and pump tubing, influent DO was measured to range between 1 and 2 mg L^{-1} , which is within the range of what is observed in the groundwater at the Rifle

site. The influent groundwater was pumped into the columns until complete uranium breakthrough was observed. Biostimulation was initiated by pumping acetate via syringe pumps (KD Scientific) into the main influent lines just before the column influent, resulting in a concentration of 3 mM after mixing with the influent media. This acetate concentration was the same as that used in previous column experiments (Komlos *et al.*, 2008c; Moon *et al.*, 2009) as well as at the UMTRA field site during biostimulation (Anderson *et al.*, 2003). The columns were biostimulated for 100 days at 17 °C, after which the acetate addition was discontinued. After the biostimulation period, and without a flow interruption, the same influent media (without acetate) were continuously pumped through the column for an additional 60 days. Effluent samples were collected to analyze the microbial community and effluent chemistry during the postbiostimulation phase. Individual columns were destructively sampled at 10–20-day intervals. Sediment samples were collected in two equal parts (sediments closer to the inflow, or 'influent sediment', and those closer to the outflow of the columns, or 'effluent sediment') in an anaerobic glove box (3% H₂ in N₂) as described (Moon *et al.*, 2009). Each of these sediment samples was then homogenized, to minimize variability, and was analyzed for solid-phase U and Fe, as well as biomass and microbial community structure.

Groundwater and sediment analysis

Column effluent concentrations were monitored for acetate, Fe(II), U(VI), and sulfate during the biostimulation and postbiostimulation periods. Effluent Fe(II) concentrations were measured by adding 0.5 mL of effluent solution to 0.5 mL of 1 N HCl, and after a 1-h extraction, were analyzed using ferrozine (Lovley & Phillips, 1987). Acetate and sulfate were analyzed using a Dionex DX500 ion chromatograph equipped with a CD25 conductivity detector and a Dionex IonPac AS14 4 mm column. Influent and effluent U(VI) concentrations were analyzed using reverse-phase chromatography coupled to post-column derivatization with the dye Arsenazo III (Sigma-Aldrich) as described by Lack *et al.* (2002). The U(VI) detection limit using this procedure is 0.5 µM. All anion and uranium samples were filtered (0.2 µm) and stored at 4 °C until analyzed.

DO was measured using a Corning 317 DO meter fitted to an inline sampling device attached to the effluent of the column. DO was measured right at the influent to the columns as well as at the outflow.

Uranium was extracted from the sediments by adding 2–3 g of sediment to 5 mL of 1 M NaHCO₃ and allowing the extraction to proceed under aerobic conditions for 4 days, after which the supernatant was analyzed as described above.

Fe(II) in sediments was measured in an anaerobic glove box (3% H₂ in N₂) by adding 0.2–0.3 g of sediment to 5 mL 0.5 N

HCl, extracting for 24 h (Kukkadapu *et al.*, 2006; Komlos *et al.*, 2007), and analyzing as described above. The HCl-extractable Fe concentration in sediments was determined in the same manner as the sediment-associated Fe(II) concentration, except that 0.2 mL of 6.25 N hydroxylamine hydrochloride was added to the 0.5 N HCl solution at a final concentration of 0.25 N before the sediment addition (Komlos *et al.*, 2007).

The SDs of the measurements for acetate and sulfate concentrations were 0.04 and 0.05 mM ($n = 4$ samples). The SD of the uranium analyses was 0.33 µM ($n = 3$ samples).

Molecular analysis of groundwater and sediment communities

Culture-independent molecular microbial analyses were performed on sediments and on groundwater samples from the flow-through columns. Sediment samples were collected from sacrificed columns at different postbiostimulation time points. Groundwater samples were collected by filtering 50 mL of effluent with a 0.2-µm inline Sterivex filter. DNA was extracted from sediment samples, and the 16S region of the DNA was amplified and sequenced as described previously (Anderson *et al.*, 2003). Phospholipid lipid fatty acid (PLFA) and quantitative PCR (qPCR) analyses were performed as described previously (Komlos *et al.*, 2008c). DNA was extracted from sediment columns using the Power Soil DNA extraction kit (MO BIO Laboratories) as per the manufacturer's protocol. Bacteria were enumerated by qPCR using the PCR primers BACT1369F-PROK1492R with the TaqMan probe 1389F as described previously (Suzuki *et al.*, 2000). The PCR primer set 361F-685R specific for the 16S rRNA gene of *Deltaproteobacteria* (Stults *et al.*, 2001) and the TaqMan probe 1839F were used as an index of potential sulfate-reducing and iron-reducing bacteria. PCR primer set 561F-825R and TaqMan probe Gbc2 specific for *Geobacter* sp. (Stults *et al.*, 2001) were used to enumerate potential iron-reducing bacteria. The dissimilatory sulfite reductase gene (1F/5R) (Karr *et al.*, 2005) was used to enumerate sulfate-reducing bacteria. All real-time PCR was performed on an ABI Prism 7000 Sequence Detector or an ABI 7300 Real-Time PCR System (Applied Biosystems). All experiments included control reaction mixtures without added DNA and calibration curves with appropriate positive control DNA.

PLFA analysis was performed using the previously reported precautions (White & Ringelberg, 1998). Column sediment samples (5 g) were extracted with a single-phase chloroform-methanol-buffer system of Bligh & Dyer (1954), as modified by White *et al.* (1979). The total lipid extract was fractionated into neutral, glyco, and polar lipids by silicic acid column chromatography (Guckert *et al.*, 1985). The polar lipids were then transesterified to fatty acid methyl esters (FAMES) by a mild alkaline methanolysis (Guckert *et al.*, 1985). The resulting FAMES were analyzed

by capillary GC with flame ionization detection on a Hewlett-Packard 5890 Series 2 chromatograph with a 50 m nonpolar column (0.2 mm I.D., 0.11 μ m film thickness). Preliminary peak identification was performed by a comparison of retention times with known standards. Definitive identification of peaks was accomplished by GC/MS of selected samples using a Hewlett-Packard 6890 series gas chromatograph interfaced to a Hewlett-Packard 5973 mass-selective detector using a 50 m nonpolar column (0.2 mm I.D., 0.11 μ m film thickness).

Results

Geochemical changes associated with biostimulation

Biostimulation was initiated in the flow-through columns by amending the groundwater with acetate (3 mM final influent concentration) (Fig. 1a). Although uranium reduction was somewhat less than that in previous column experiments, the sediment flow-through columns responded to biostimulation

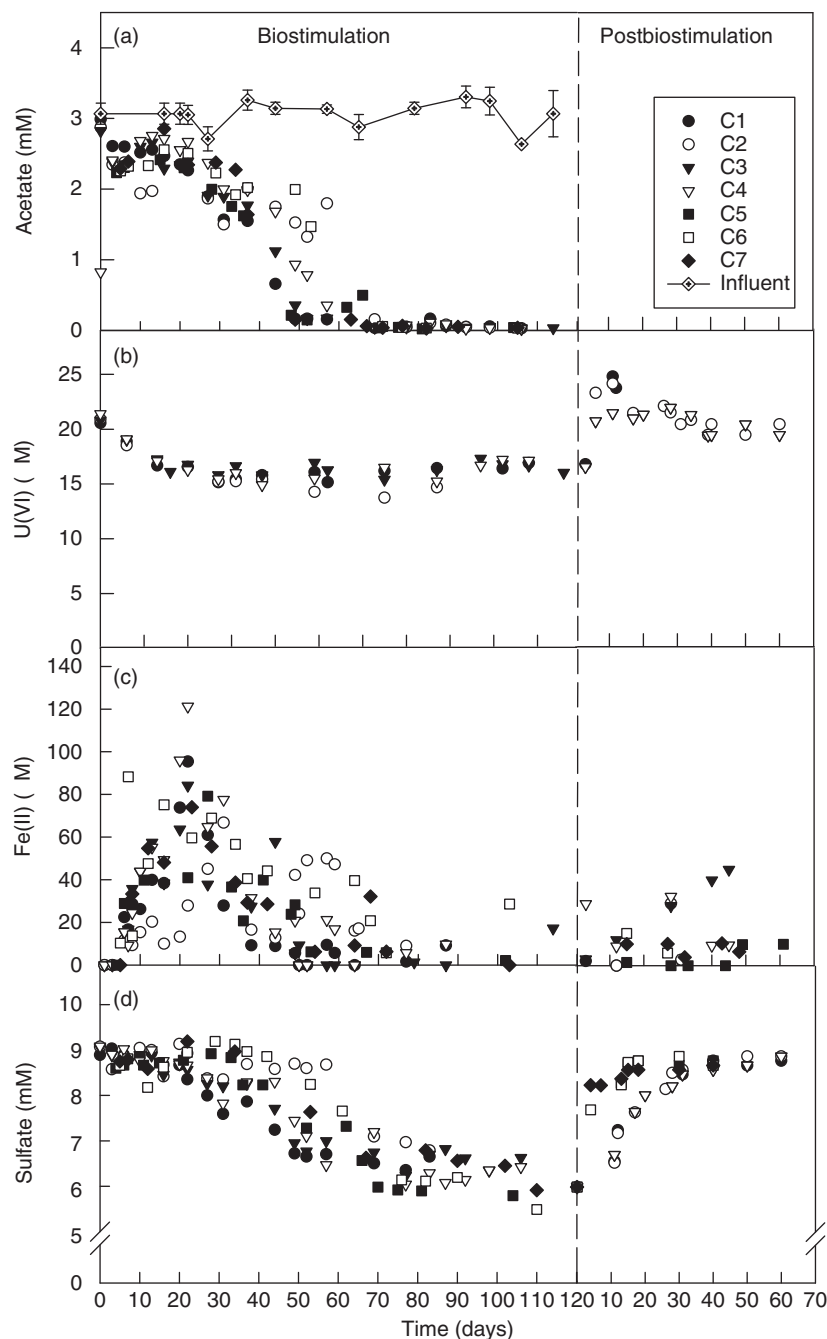


Fig. 1. Geochemical changes in flow-through columns' effluent groundwater. Acetate (a), uranium (b), iron (c), and sulfate (d).

as observed previously (Moon *et al.*, 2007, 2009; Komlos *et al.*, 2008b, c; N'Guessan *et al.*, 2008). The addition of acetate to the columns resulted in the immediate removal of a fraction of the uranium from the groundwater along with the simultaneous release of Fe(II) (Fig. 1b and c). Approximately $5 \mu\text{M}$ U(VI) was continuously removed from the influent groundwater for the duration of the acetate amendment period (i.e. 100 days). A total of $23.8 \mu\text{mol}$ of uranium precipitated during this biostimulation phase. Sulfate reduction became prevalent after 30 days of biostimulation, and equimolar amounts (i.e. $\sim 3 \text{ mM}$) of sulfate and acetate were removed from the groundwater (Fig. 1d). During this sulfate reduction phase, Fe(II) was removed from the groundwater, presumably by reduced sulfur species (Fig. 1c). This same trend was also observed in previous studies (Moon *et al.*, 2007; Komlos *et al.*, 2008c). No DO was detected in the effluent during biostimulation or during the postbiostimulation period (data not shown).

Geochemical changes associated with the postbiostimulation reoxidation

The removal of sulfate and uranium from groundwater stopped once acetate addition was terminated (Fig. 1b and d), although some sulfate was consumed for an additional 30 days after acetate amendment was discontinued. No sulfate rebound over the influent levels was observed. During the first month of the postbiostimulation period, about 17% of the total immobilized uranium ($4 \mu\text{mol}$) was released from the columns (with higher spikes during the first 2 weeks), after which the uranium effluent concentrations became equal to the influent levels until the end of the experimental period, meaning that no detectable loss of immobilized uranium occurred during that period (Fig. 1b).

Low levels of Fe(II) continued to be released from the columns during the 60-day postbiostimulation period (Fig. 1c). Analysis of the HCl-extractable iron in the sediment showed that the Fe(II) to total Fe ratios remained the same in all columns for the entire duration of the postbiostimulation period (Supporting Information, Fig. S1). Furthermore, Fe(II) in the sediments at the influent half of the column decreased during the first 2 weeks of the postbiostimulation period and remained stable thereafter. No changes in Fe(II) in the sediments with time were observed in the effluent half of the columns (Fig. S1).

Based on the extractions and analyses for Fe(II) at the beginning and at the end of the postbiostimulation period, $482 \mu\text{mol}$ of Fe(II) was reoxidized during the 60-day postbiostimulation. During this period, $162 \mu\text{mol}$ of O_2 entered the column (flow = 0.4 mL min^{-1} , $\text{DO} = 1.5 \text{ mg L}^{-1}$). Based on a stoichiometry of $1/4 \text{ mol O}_2 \text{ mol}^{-1} \text{ Fe(II)}$ oxidized, $121 \mu\text{mol}$ of O_2 would have reacted with the Fe(II), leaving about $40 \mu\text{mol}$ of O_2 to react with other reduced species.

PLFA and bacterial abundance changes in groundwater and sediment during the postbiostimulation period

During the 60 days following the end of biostimulation, the microbial cell abundance decreased by two orders of magnitude in the groundwater effluent as well as in the sediment (Fig. 2a and b). Iron-reducing bacteria (GEO and IRB) and sulfate-reducing bacteria (SRB and DSR) were below detection limits in the groundwater, but they represented a more significant fraction of the total bacteria in the sediment (Fig. 2a and b). While there were distinct changes in the bacterial population in the sediment throughout the experiment, the

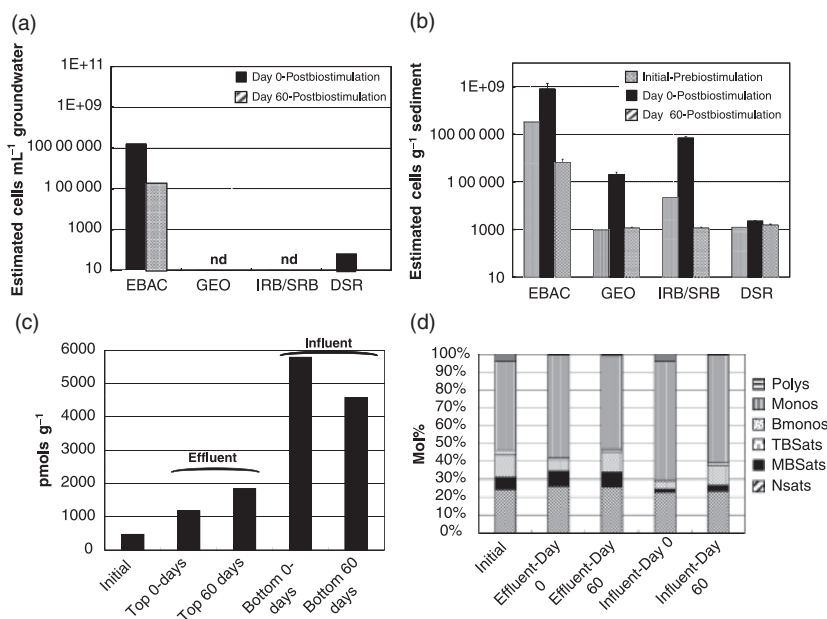


Fig. 2. Postbiostimulation biomass changes in flow-through columns. (a) Groundwater and (b) sediment qPCR with primers specific for bacteria (EBAC), *Geobacteraceae* (GEO), iron- and sulfate-reducing bacteria (IRB/SRB), and dissimilatory sulfite reductase gene (DSR), (c) PLFA biomass changes, and (d) community composition PLFA in sediment, Polys, polyunsaturated PLFA; Monos, monounsaturated PLFA; BMonos, branched-chain monounsaturated PLFA; TBSats, terminally branched saturated PLFA; MBSats, mid-chain branched saturated PLFA; NSats, normal saturated PLFA.

cell abundance for sulfate-reducing bacteria (DSR) remained the same (Fig. 2b). PLFA analysis of the sediment collected from the influent and effluent of the columns showed that there was significantly more biomass at the influent of the columns (i.e. close to the injection point) than at the effluent (Fig. 2c). Additionally, the PLFA profiles, which were dominated by monounsaturated fatty acids, suggested that Gram-negative bacteria remained prevalent throughout the postbiostimulation monitoring period (Fig. 2d). Furthermore, terminally branched fatty acids, indicative of Gram-positive bacteria, were low at the start of the postbiostimulation monitoring period and increased slightly within 60 days postbiostimulation (Fig. 2d). However, these represented only a small fraction of the PLFA profile.

Fitting first-order decay rates to the bacterial numbers during the poststimulation period, shown in Table S1, results in $k = 0.08 \text{ day}^{-1}$ for Eubacteria ($r^2 = 0.39$, $n = 12$); $k = 0.10 \text{ day}^{-1}$ for *Geobacter* ($r^2 = 0.76$, $n = 12$); and $k = 0.15 \text{ day}^{-1}$ for iron- and sulfate-reducing bacteria ($r^2 = 0.70$, $n = 12$). These are averaged over the influent and the effluent sediments.

Postbiostimulation phylogenetic microbial community dynamics

Proteobacteria were predominant in the sediments and in the groundwaters for at least 50 days postbiostimulation (Table 1). These correlated well with the results from the PLFA analysis described earlier (Fig. 2c and d). *Proteobacteria* represented > 50% of the total bacterial community in the sediment both at the influent and at the effluent of the columns. In the groundwater, *Proteobacteria* accounted for no < 96% of the total microbial community postbiostimulation (Table 1). *Verrucomicrobia* were present in the sediment, but not in the groundwater (Table 1). *Firmicutes* were not prevalent in any of the samples from this experiment. Again, this correlated well with the low percentage of terminally branched fatty acids from the community composition PLFA analysis (Fig. 2d).

Groundwater postbiostimulation community dynamics within the phylum *Proteobacteria*

Organisms that belong to the class *Betaproteobacteria* were most prevalent in the groundwater and accounted for 56–98% of the postbiostimulation microbial community (Fig. 3a), including *Hydrogenophaga* sp. (91–99% similarity), representing 98% of the total microbial community in the groundwater early during the postbiostimulation (Fig. 3b). By day 32, organisms closely related to the betaproteobacterium *Sterolibacterium* sp. (90–94% similarity) represented 47.8% of the total microbial community, while the proportion of *Hydrogenophaga*-like bacteria decreased to 43% (Fig. 3b). After day 32 of postbiostimulation monitoring, *Gammaproteobacteria* became increasingly important in the groundwater (Fig. 3a),

Table 1. 16S rRNA gene frequencies in clone libraries from genomic DNA extracted from effluent groundwater as well as effluent and influent sediment from flow-through columns

Groundwater Phylum	% in samples collected on the following days postbiostimulation					% in samples collected on the following days postbiostimulation					% in samples collected on the following days postbiostimulation				
	– 50*	16	32	42	50	– 50*	16	32	42	50	– 50*	16	32	42	50
<i>Acidobacteria</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Actinobacteria</i>	5 (2)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Bacteroidetes</i>	30 (13)	2 (1)	2 (1)	4 (2)	2 (1)	2 (1)	13 (6)	5 (2)	5 (2)	13 (4)	13 (5)	8 (3)	10 (4)	6 (2)	3 (1)
<i>Chlorobi</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Chloroflexi</i>	2 (1)	0	0	0	0	14 (6)	6 (3)	11 (5)	14 (6)	9 (3)	0	3 (1)	2 (1)	3 (1)	8 (3)
<i>Firmicutes</i>	9 (4)	0	0	0	0	5 (2)	4 (2)	2 (1)	5 (2)	0	10 (4)	5 (2)	0	0	5 (2)
<i>Gemmatimonadetes</i>	0	0	0	0	0	0	0	9 (4)	0	0	0	0	0	0	0
<i>Planctomycetes</i>	0	0	0	0	0	0	0	0	2 (1)	0	0	0	2 (1)	0	3 (1)
<i>Proteobacteria</i>	55 (24)	98 (44)	98 (43)	96 (45)	98 (42)	62 (26)	70 (33)	68 (30)	50 (22)	53 (17)	69 (27)	76 (28)	76 (32)	69 (25)	74 (29)
<i>Verrucomicrobia</i>	0.0	0	0	0	0	0	0	0	5 (2)	0	0	5 (2)	7 (3)	0	0
Unknown	0.0	0	0	0	0	14 (6)	0	0	21 (9)	22 (7)	3 (1)	3 (1)	0	17 (6)	8 (3)
Total	100 (44)	100 (45)	100 (44)	100 (47)	100 (43)	100 (42)	100 (47)	100 (44)	100 (44)	100 (32)	100 (39)	100 (37)	100 (42)	100 (36)	100 (39)

Numbers in parentheses represent the number of sequences in a library. * – 50 represents the community structure at 50 days of biostimulation with acetate.

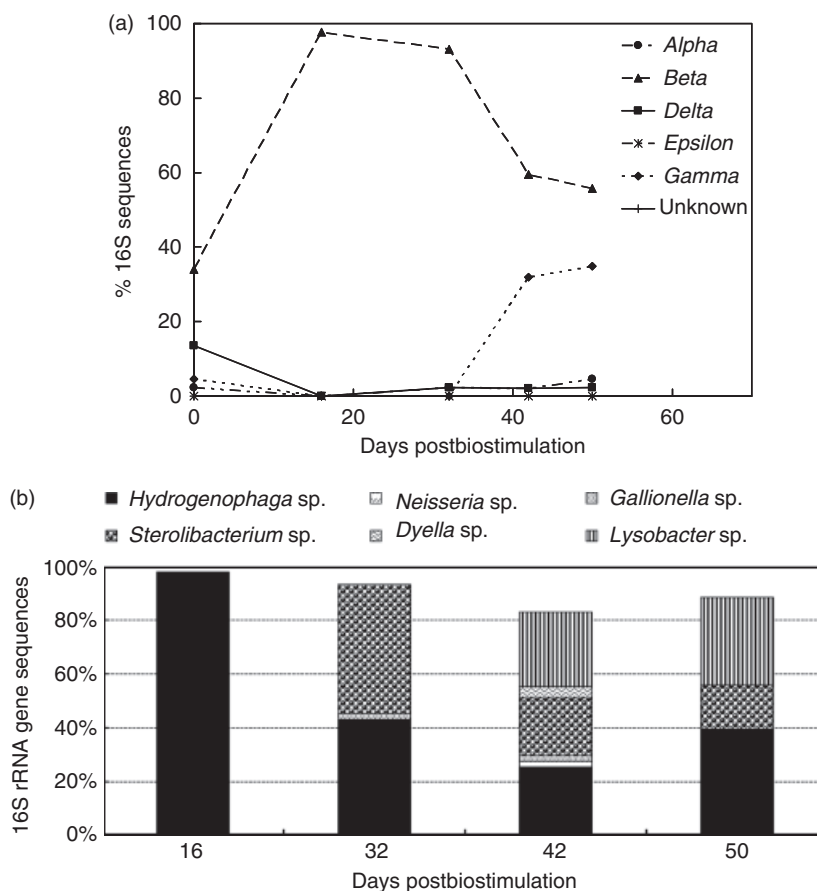


Fig. 3. (a) 16S rRNA gene-based groundwater microbial community dynamics within the phylum *Proteobacteria* and (b) distribution of *Betaproteobacteria* and *Gammaproteobacteria* in the effluent groundwater throughout the postbiostimulation monitoring.

with bacteria closely related to *Lysobacter* sp. (89–98% similarity) representing 33% of the total groundwater microbial community by day 50 (Fig. 3a).

Sediment postbiostimulation community dynamics within the phylum *Proteobacteria*

In contrast to the groundwater, where *Betaproteobacteria* were predominant, the postbiostimulation bacteria community in the sediment was dominated by *Deltaproteobacteria* (Fig. 4). Dissimilatory metal-reducing bacteria (DMRB), belonging to the family *Geobacteraceae*, as well as sulfate-reducing bacteria (SRB) within the *Desulfobacteraceae*, *Desulfobulbaceae*, and *Desulfuromonadaceae* were the most prevalent *Deltaproteobacteria* in sediments (Fig. 5). Yet, there were distinct differences between the distribution of *Deltaproteobacteria* at the columns influents and effluents. At the effluent, DMRB were the predominant organisms, while SRB thrived at the influent (Fig. 5). Additionally, after 16 days postbiostimulation, bacteria within the class *Betaproteobacteria* increased from 8% to 31% of the total 16S sequences from the sediment at the influent of the columns (Figs 4 and 6). On the other hand, *Betaproteobacteria* repre-

sented < 14% of the microbial community at the effluent of the columns (Figs 4 and 6). The *Betaproteobacteria* at the influent were composed of *Hydrogenophilaceae*, such as *Thiobacillus* sp. (92–98% similarity), as well as other bacteria most closely related to *Gallionella* sp. (92% similarity) and *Hydrogenophaga* sp. (94–99% similarity) (Fig. 6).

Discussion

The geochemical changes and microbial dynamics observed in this study suggested that there were two important processes that followed the end of biostimulation in the presence of 1–2 mg O₂ L⁻¹. The first process, 'bio-oxidation', is described in this manuscript as the oxidation of reduced minerals by microorganisms [e.g. Fe(II) by *Gallionella* sp., and reduced sulfur by *Thiobacillus* sp. and *Hydrogenophaga* sp.], and is a process that requires oxygen. The second process, biomass turnover, refers to the oxidation of complex organics from decaying biomass by bacteria. This biomass turnover can take place under aerobic and anaerobic conditions; however, organisms such as *Lysobacter* sp., and *Sterolibacterium* sp. detected in this experiment, can oxidize complex organic materials under aerobic conditions

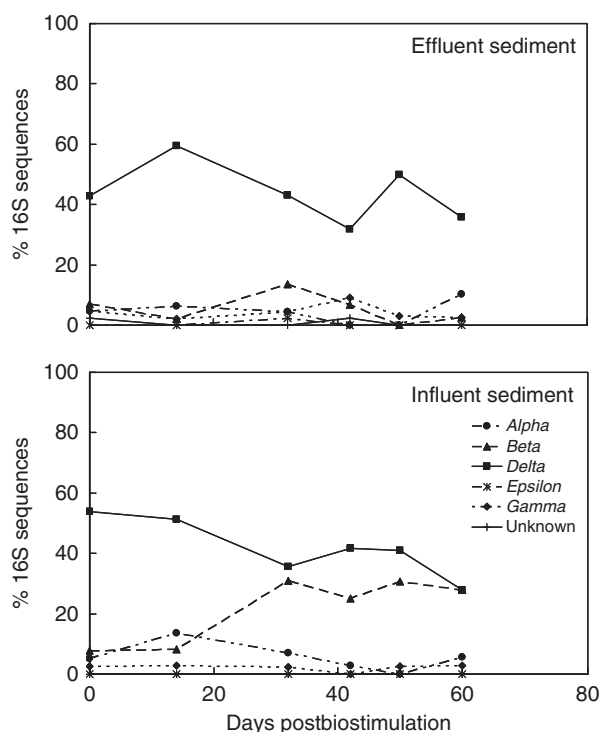


Fig. 4. 16S rRNA gene-based sediment microbial community dynamics within the phylum *Proteobacteria* at the effluents and influents of flow-through columns.

(Christensen & Cook, 1978; Tarlera & Denner, 2003). These two processes, which require oxic or suboxic conditions, may have been mediated by specialized bacteria that became predominant during the postbiostimulation period. Bio-oxidation and biomass turnover could have contributed to the consumption of incoming low-level oxygen, therefore minimizing any reoxidation of Fe(II) or biogenic U(IV) in the sediment. In addition, biomass turnover may have resulted in the production of low levels of simple organic carbon compounds as byproducts that could have been used by the surviving population of *Deltaproteobacteria* (i.e. DMRB and SRB), therefore explaining the persistence of these organisms throughout the 2-month postbiostimulation monitoring period (Fig. 6).

The bio-oxidation stage was characterized by the presence of organisms with the potential to oxidize reduced species. These bacteria, which include *Hydrogenophaga* sp. and *Thiobacillus* sp., are able to use H₂, Fe(II), sulfide, or thiosulfate as their electron donor in the presence of O₂. Organisms from the genus *Hydrogenophaga* are generally motile (Willems *et al.*, 1989; Kampfer *et al.*, 2005), while those from the genus *Thiobacillus* are generally nonmotile (Willems *et al.*, 1989; Wood *et al.*, 2001; Kampfer *et al.*, 2005; Kellermann & Griebler, 2009). This could potentially explain the prevalence of *Hydrogenophaga*-like organisms in the groundwater and the predominance of *Thiobacillus*-like organisms in the sediment.

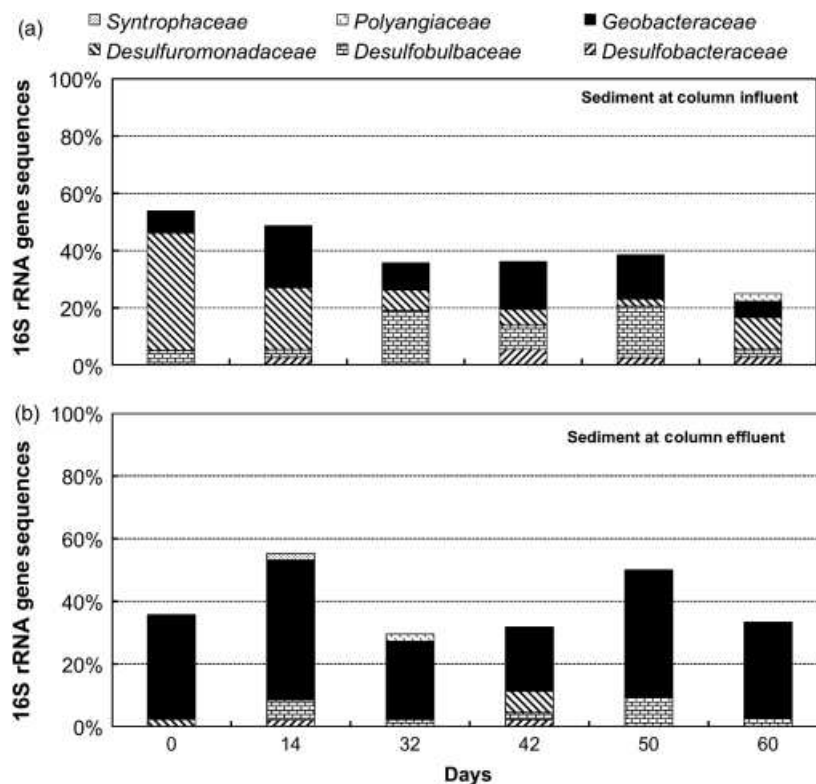


Fig. 5. Distribution of *Deltaproteobacteria* in sediment at the influents and effluents of flow-through columns throughout the postbiostimulation monitoring.

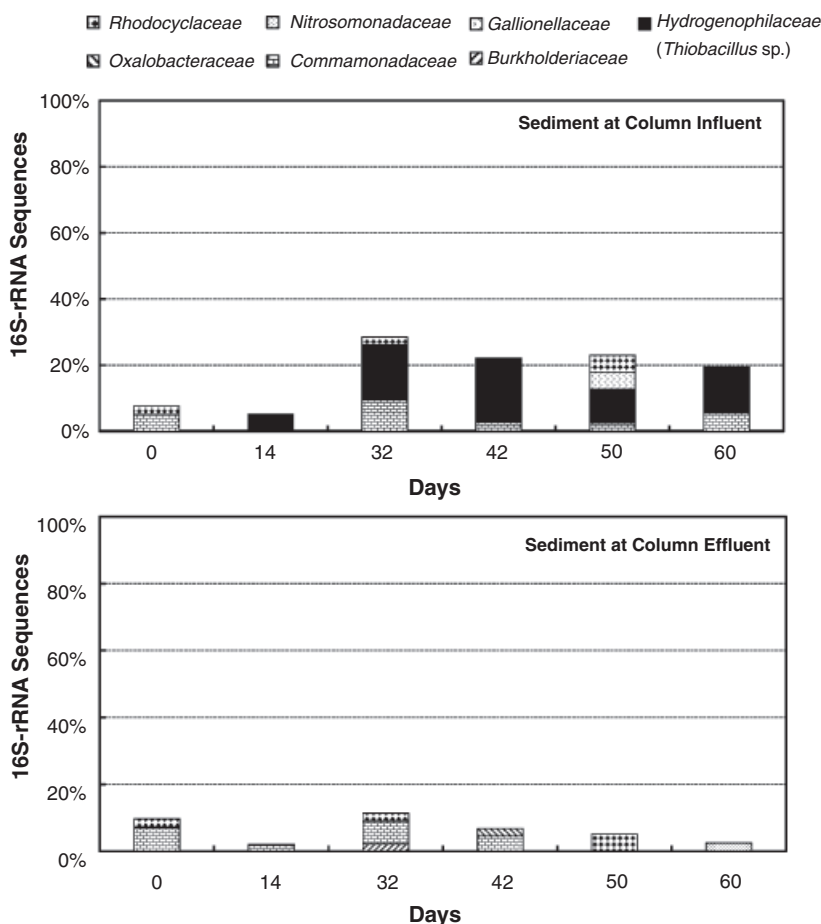


Fig. 6. Distribution of *Betaproteobacteria* in sediment at the influent and effluents of flow-through columns throughout the postbiostimulation monitoring.

Hydrogenophaga sp. are chemoorganotrophic or chemolithoautotrophic bacteria that typically utilize H_2 as an energy source and CO_2 as a carbon source (Willems *et al.*, 1989; Kampfer *et al.*, 2005). Some *Hydrogenophaga* sp. have also been shown to oxidize thiosulfate to sulfate (Cytryn *et al.*, 2005). Some 16S *Hydrogenophaga*-like sequences were also recovered from the sediment (Fig. 3). Phylogenetic comparison of these sequences showed that most *Hydrogenophaga*-like organisms in the groundwater were most closely related to *Hydrogenophaga atypica* (95–98% similarity) and *Hydrogenophaga defluvii* (91–98% similarity), while bacteria most closely related to *Hydrogenophaga taeniospiralis* (94–99% similarity) were only found in the sediment (Fig. S2).

In contrast to *Hydrogenophaga* sp. dominating the groundwater, *Thiobacillus*-like bacteria were only found in the sediment at the influent of the column (Fig. 6). This suggested that the conditions in this location were most favorable for their growth. The presence of *Thiobacillus* sp. in the sediment at the influent of the columns correlated well with the pronounced presence of SRB at this location and therefore the potential for the largest deposit of reduced sulfur species (Fig. 5). In general, bacteria of the genus *Thiobacillus* are able to oxidize reduced sulfur species (Schedel & Truper, 1980;

Beller *et al.*, 2006). *Thiobacillus thiophilus* (92–98% similarity), *Thiobacillus thioparus* (93–96% similarity), and *Thiobacillus denitrificans* (95–96% similarity) were the closest relatives of the *Thiobacillus*-like organisms found in these sediments (Fig. S3). *Thiobacillus* sp. are chemolithoautotrophic bacteria that use thiosulfate or hydrogen sulfide as an electron donor, with oxygen and nitrate acting as electron acceptors (Starkey, 1935; Parker & Prisk, 1953; Sublette & Sylvester, 1987; Kellermann & Griebler, 2009). Other *Thiobacillus* sp. are capable of catalyzing the oxidation of Fe(II) to Fe(III) (Nicolle *et al.*, 2009).

Gallionella sp., which was also detected during the post-stimulation period, is a microaerophilic iron oxidizer, commonly found in groundwater at the anaerobic/aerobic interface (Hallbeck & Karsten, 2005).

Unlike the bio-oxidation process where mineral-oxidizing bacteria were found both in the sediment and in the groundwater, biomass turnover seemed to be most pronounced in the groundwater. The biomass turnover process was characterized by the presence of organisms closely related to *Sterolibacterium* sp. (90–94% similarity) and *Lysobacter* sp. (89–98% similarity). These organisms are bacteria with the potential to use complex organic matter, such as decaying biomass. *Sterolibacterium denitrificans* was the closest match for the 16S

sequences from the effluent groundwater samples (Fig. S4). It is therefore possible that following the end of biostimulation, soluble fractions of decaying biomass could have served as a source of carbon for these *Sterolibacterium*-like organisms. Additionally, *Lysobacter* sp., which produce proteases and chitinases, may have contributed to the carbon turnover from decaying biomass. *Lysobacter daecheongensis* (91–98% similarity) was the closest relative to the *Lysobacter*-like organisms found in the sediment and groundwater (Fig. S5).

The low abundance of *Firmicutes* was atypical, as previous experiments have suggested that the *Firmicutes* play an important role during and after biostimulation (Vrionis *et al.*, 2005; N'Guessan *et al.*, 2008). Field conditions are much more heterogeneous than the column sediments, which could have led to the lower initial numbers of *Firmicutes* in the column sediments than that of the average field sediments. A previous study has suggested that *Firmicutes* play an important role in uranium retention postbiostimulation (N'Guessan *et al.*, 2008). Therefore, the absence of organisms from this phylum may explain the lower U(VI) reduction efficiency observed in this experiment.

Because iron or sulfur oxidizers scavenge the low DO levels ($1\text{--}2\text{ mg O}_2\text{ L}^{-1}$), as evidenced by the nondetectable DO levels in the effluent during the postbiostimulation phase, their activity may have protected the reduced uranium from remobilization (Moon *et al.*, 2009). Uranium was detected in the column effluents during the first month, with the highest levels during the first 2 weeks of biostimulation. During this time, about 17% of the uranium that was immobilized during the biostimulation period was remobilized. As shown in Fig. 6, the *Hydrogenophilaceae* family, which includes *Thiobacillus* sp., was only detected for times longer than 2 weeks of postbiostimulation, when uranium loss from the columns was stabilizing. Therefore, although we cannot rule out that some oxygen reacted abiotically, the results from this study suggest that the activity of oxidizing bacteria contributes towards maintaining the low oxygen levels in the subsurface, hence preventing the reoxidation of U(IV) while these bacteria are actively consuming the DO.

Because uranium remobilization had taken place before the detection of these oxygen-consuming organisms, the results show that abiotic oxygen utilization (for the oxidation of reduced sulfur, iron, and organic carbon) seems to be too slow to prevent oxygen from reaching much of the reduced uranium. Hence, biological reoxidation processes will have to be included in models that are being developed to assess the fate and transport of uranium during biostimulation.

Acknowledgements

This research was funded by the Environmental Remediation Sciences Program (ERSP), Office of Biological and

Environmental Research (OBER), US Department of Energy (DOE), Pacific Northwest National Laboratory Project 51882 'The Rifle, Colorado Integrated Field Research Challenge Site (IFRC)'. Financial support for this research was also provided by Brain Korea 21 Project (through the School of Earth and Environmental Sciences, Seoul National University) in 2009.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Extractable iron (0.5 N HCl, 24 h) in column sediments during the poststimulation period.

Fig. S2. Phylogenetic tree comparing 16S rRNA gene sequences for organisms extracted from groundwater and sediment from the flow-through columns.

Table S1. Bacterial numbers in the column sediments as a function of time during the postbiostimulation period.

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