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Microbial community dynamics in uranium contaminated subsurface sediments under biostimulated conditions with high nitrate and nickel pressure

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Abstract
Background, aim, and scope The subsurface at the Oak Ridge Field Research Center represents an extreme and diverse geochemical environment that places different stresses on the endogenous microbial communities, including low pH, elevated nitrate concentrations, and the occurrence of heavy metals and radionuclides, including hexavalent uranium [U(VI)]. The in situ immobilization of U(VI) in the aquifer can be achieved through microbial reduction to relatively insoluble U(IV). However, a high redox potential due to the presence of nitrate and the toxicity of heavy metals will impede this process. Our aim is to test biostimulation of the endogenous microbial communities to improve nitrate reduction and subsequent U(VI) reduction under conditions of elevated heavy metals.
Materials and methods Column experiments were used to test the possibility of using biostimulation via the addition of ethanol as a carbon source to improve nitrate reduction in the presence of elevated aqueous nickel. We subsequently analyzed the composition of the microbial communities that became established and their potential for U(VI) reduction and its in situ immobilization.
Results Phylogenetic analysis revealed that the microbial population changed from heavy metal sensitive members of the actinobacteria, α- and γ-proteobacteria to a community dominated by heavy metal resistant (nickel, cadmium, zinc, and cobalt resistant), nitrate reducing β- and γ-proteobacteria, and sulfate reducing Clostridiaceae. Coincidentally, synchrotron X-ray absorption spectroscopy analyses indicated that the resulting redox conditions favored U(VI) reduction transformation to insoluble U(IV) species associated with soil minerals and biomass.
Discussion This study shows that the necessary genetic information to adapt to the implemented nickel stress resides in the endogenous microbial population present at the Oak Ridge FRC site, which changed from a community generally found under oligotrophic conditions to a community able to withstand the stress imposed by heavy metals, while efficiently reducing nitrate as electron donor. Once nitrate was reduced efficient reduction and in situ immobilization of uranium was observed.
Conclusions This study provides evidence that stimulating the metabolism of the endogenous bacterial population at the Oak Ridge FRC site by adding ethanol, a suitable carbon source, results in efficient nitrate reduction under conditions of elevated nickel, and a decrease of the redox potential such that sulfate and iron reducing bacteria are able to thrive and create conditions favorable for the reduction and in situ immobilization of uranium. Since we have found that the remediation potential resides within the endogenous microbial community, we believe it will be feasible to conduct field tests.
Recommendations and perspectives Biostimulation of endogenous bacteria provides an efficient tool for the successful in situ remediation of mixed-waste sites, particularly those co-contaminated with heavy metals, nitrate and

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radionuclides, as found in the United States and other countries as environmental legacies of the nuclear age.

Keywords Bioremediation · Biostimulation · Microbial ecology · Nickel resistance · Nitrate reduction · Subsurface microbiology · Uranium reduction

1 Background, aim, and scope

The remediation of hazardous mixed-waste sites, particularly those co-contaminated with heavy metals, nitrate and radionuclides, is one of the most costly environmental legacies of the nuclear age currently faced by the United States and other countries. Groundwater and soils at the Field Research Center (FRC) at Oak Ridge National Laboratory are polluted with multiple contaminants including heavy metals, radionuclides, and nitrate (Petrie et al. 2003). An important in situ remediation approach being developed to reduce the bioavailability and prevent the further spread of uranium in groundwater involves promoting the activity of dissimilatory sulfate and iron reducing organisms to transform hexavalent uranium [U(VI)] into its reduced form, U(IV), which is effectively insoluble and immobile under the conditions at the site (Sani et al. 2004; Vrionis et al. 2005).

Nitrate serves as a competing and energetically favorable electron acceptor for metal-reducing bacteria in nitric acid-contaminated sediments (Dichristina 1992). Previous studies of microbial U(VI) reduction in sediments co-contaminated with nitrate have indicated that no net U(VI) reduction will occur until nitrate has been reduced (Finneran et al. 2002a, b; Senko et al. 2002). Unfortunately, nitrate reduction at the FRC site is inhibited due to the presence of several heavy metals, including Ni, Cd, Cr, and Hg (Sakadevan et al. 1999). Currently, this problem is addressed by ex situ treatment and removal of heavy metals and nitrate prior to in situ biostimulation to reduce U(VI) (Wu et al. 2006a, b). At mixed-waste sites where the concentrations of metal contaminants can reach toxic levels, the metal resistance of the endogenous microbial populations will be critical for the success of in situ biostimulation efforts. For example, while a number of microbes have been shown to carry out reductive precipitation of radionuclides (e.g., *Desulfovibrio* sp., *Geobacter* sp., and *Shewanella* sp.), the sensitivity of these organisms to non-reducible heavy metals could possibly limit their in situ activities. Work done by Sobecky et al. (Martinez et al. 2006) revealed the presence of heavy metal resistant genes within the endogenous microbial community at the FRC site and proof of horizontal gene transfer as a means to adapt the community to stresses imposed by heavy metals.

This study examines the potential of biostimulation (Istok et al. 2004; Nyman et al. 2006) by ethanol to improve the reduction of nitrate in the presence of heavy metals, followed by the successful reduction and in situ immobilization of uranium. Since nickel is one of the major contaminating metals at the FRC site and a representative for bivalent cations with common bacterial resistance mechanisms (also including Zn, Cd, Co), we focused our study on this metal.

2 Materials and methods

2.1 Soils

Contaminated subsurface soils were provided by the DOE Oak Ridge Field Research Center. The contaminated soils were collected from an area adjacent to an asphalt parking area that covers three former waste ponds (S-3 ponds) used during weapons production activities. The waste ponds and surrounding soils contain uranium and other radionuclides, nitric acid, organic solvents, and heavy metals. Soil cores were collected by the FRC site management team as previously described (Petrie et al. 2003). In our study, contaminated subsurface soil samples were obtained from the saturated zone at FB095 (Area 2; maximum depth 8.68 m) in 2005, where elevated U (5–60 ppm) and nitrate (<200–50,000 ppm) concentrations have been reported. We found as much as 100 mg/kg of uranium adsorbed to the solid phase material heterogeneously located in pockets throughout the sediment samples. The pH of groundwater samples was acidic ranging between 3.2 and 5.5. These samples were handled aseptically, preserved under an argon atmosphere, and shipped chilled at 4°C. Detailed descriptions of site geology, hydrology, and geochemistry are available on the DOE Natural and Accelerated Bioremediation website (http://public.ornl.gov/nabirfrc/other/frcconmod.pdf).

2.2 Columns

Flow-through aquifer columns (50 cm length, 5 cm outer diameter, 4 cm inner diameter; Flemish Institute for Technological Research, Mol, Belgium) made of Plexiglas and equipped with fourpling ports (10 cm—ort A, 20 cm—port B, 30 cm—port C, 40 cm—port D from the inlet) were used. Columns were packed with aquifer material from Area 2 within 3 months after collection and fed continuously with ethanol (10 g/l) dissolved in a mineral medium in an upflow method using a peristaltic pump. This mineral medium consisted of 10 mM KNO_3, 1 mM β-glycerophosphate, 1 mM $MgSO_4$ $7H_2O$, 0.1 mM $CaCl_2$ (pH 7). Glycerophospate was used to minimize the formation and precipitation of uranium phosphates at the pH used. The medium was purged with high purity argon to remove dissolved oxygen.

The wet soil cores were passed through a 5.6-mm sieve from Endecotts Ltd. (London, UK), homogenized and packed in the columns. Under these conditions, we expected the endogenous anaerobic microbial community to be heavily impacted. Therefore, we used one soil core to extract under anaerobic conditions the microbial community, which was subsequently used to inoculate the columns at a cell density of 10^{+4} g^{-1} of soil. This culture was obtained under anaerobic conditions by recovering the bacterial community from the soil particles with an isotonic solution of 10 mM $MgSO_4$ and a vortex procedure (Balkwill and Casida 1979). Each column received a different regime, which resulted in three setups (see Table 1). The abiotic control column had 1 M formaldehyde as a bactericidal agent added to the groundwater. The effective porosity for the columns was calculated to be approximately 30%.

2.3 Analytical techniques

Sulfate concentrations were determined turbidimetrically. Samples underwent precipitation with $BaCl_2$, forming $BaSO_4$, and were measured spectrophotometrically (Page 1982). Nitrate concentrations were determined via a reaction with brucine in strong sulfuric acid to produce a yellow color, which was measured spectrophotometrically according to Standard Methods for Examination of Water and Wastewater, 12th edition, American Public Health Association, Washington, D.C., pp. 542–544. Analyses of soluble uranium concentrations were performed with a Kinetic Phosphorescence Analyzer (KPA; Chemchek Instruments, Richland, WA, USA) according to the method of Brina and Miller (1992). Uranium standards were prepared from a 1,000 ppm U(IV) solution via dilution with double-deionized (DI) water. For sample measurements (triplicate), 1 ml aliquots of supernatant were filtered (0.2 mm), acidified with H_2SO_4 (3 mM final concentration), and diluted to a final volume of 4.0 ml with DI water and analyzed immediately. All measurements were performed at room temperature. Soluble nickel was determined by ICP-OES (Liberty 100, Varian Instruments) after 1:10 dilution with nitric acid.

Table 1 Overview of soil column conditions

	Column no.		
	C1	C2	C3
Short description	Abiotic control	+Ni	–Ni
1 M Formaldehyde	+		
120 ppm Ni	+	+	
Endogenous population[a]	+	+	+

Column C1 represents the abiotic control and was continuously fed 1 M formaldehyde. The difference between columns C2 and C3 is the presence or absence, respectively, of 120 ppm Ni (added as $NiCl_2$) in the artificial groundwater used to feed the columns. All three columns received 1% ethanol as a carbon source and were inoculated with the endogenous microbial community at a density of 10^{+4} cfu/g soil

[a] The endogenous microbial community was extracted from the homogenized soil core by vortexing in 10 mM $MgSO_4$ and subsequently used to inoculate the columns

2.4 BIOMET analysis

Luminometry assays to determine the bioavailable concentration of nickel were carried out using a Luminescan (Thermo Scientific) luminometer at 23°C, as previously described (Corbisier 1997; Tibazarwa et al. 2001; Everhart et al. 2006). For metal standards, duplicate samples were set up per microtiter assay. As negative controls, eight samples containing bidistilled water were included in the tests. Soil samples were suspended in reconstitutive medium (Corbisier 1997) and tested with the nickel biosensor strain *Cupriavidus metallidurans* AE2515. As a control, *C. metallidurans* AE864 (pMOL28; nickel-resistant), which shows constitutive light production, was used to monitor general toxicity and light quenching that could bias the interpretation of the Ni bioavailability test. The Ni bioavailability data collected with strain AE2515 were only considered valid when, at the same time, strain AE864 showed a signal to noise ratio between 0.8 and 1.2. For each assay, metal standard solutions were included in duplicate. The reconstituted soils were tested in duplicate in dilution series 1 to 1, 1 to 2, and 1 to 4. All experiments were repeated three times, and average bioavailability data plus standard deviations were calculated.

2.5 Micro-X-ray fluorescence/X-ray absorption near-edge structure analysis

Micro-X-ray fluorescence (XRF) and X-ray absorption near-edge structure (XANES) analysis were used to determine uranium and nickel spatial distribution, oxidation state, and solid phase identification (in the case of Ni). The samples of the columns were taken by pushing a Kapton capillary through a sample port, effectively removing a 3-mm diameter core across the diameter of the soil column. All sample preparations were performed in an Argon-purged glove bag, which also contained oxygen scrubbers. The capillary was sealed and maintained in an oxygen-scrubbed mason jar up until time of analysis. Micro-XRF two-dimensional maps at ~10 micron spatial resolution and XANES point spectra collected at the LIII-edge of uranium were performed at the National Synchrotron Light Source beamline X-27A X-ray microprobe. A 100-ppm U in calcite standard was used as the U(VI) standard, and a powdered uraninite (UO_2) was used as the U(IV) standard.

2.6 Microbial enumeration using the most probable number method

Denitrifying enrichment medium, iron reducing media, and Postgate B medium were prepared as described previously (Mergeay et al. 1985). All media contained yeast extract and lactate as the main electron donor. Acetate was chosen as a C-source since extended exposure to ethanol reduces the amount of iron reducing bacteria in most probable number (MPN) experiments (McLain 2004). The 25-ml glass serum vials were filled anaerobically, capped with a rubber stop, and sterilized by autoclaving. Sediment samples were taken with a gastight syringe from port B of each column and a dilution series was made anaerobically in 10 mM $MgSO_4$. Each dilution was injected using a gastight syringe into the corresponding MPN bottle. The MPN series were incubated at room temperature in the dark. As positive MPN controls, appropriate dilutions of overnight cultures of Pseudomonas stutzeri ATCC17591 (nitrate reducing bacterium), Shewanella putrefaciens ATCC8072 (iron reducing bacterium), and Desulfovibrio vulgaris ATCC7757 (sulfate reducing bacterium) were used.

2.7 Ni sensitivity assay

To determine the amount of bacteria resistant to Ni, sediment samples from the columns (port B) were spread plated on minimal media agar (1.5%) plates (Bastiaens et al. 2000) containing 1, 2, or 3 mM Ni. As C-source, a mixture of organic compounds was added (citrate, succinate, acetate, glucose, and lactate) in a wt/vol % of 0.05 each. The numbers of cfu were counted and compared to the non selective control (0 mM Ni^{+2}).

2.8 Soil DNA isolation

Column aquifer material was collected for DNA isolation. Approximately 1 g of soil was taken from the port vials located 20 cm from the columns' inlet, and the DNA was isolated using the PowerSoil™ DNA Isolation kit (MOBIO Laboratories Inc, Carlsbad, CA, USA). DNA was ready to use for polymerase chain reaction (PCR) analysis and other downstream applications.

2.9 Polymerase chain reaction and cloning

Community 16S rRNA genes were PCR amplified in mixtures containing 100 ng/µl DNA, 1× High Fidelity PCR buffer (Invitrogen, Carlsbad, CA, USA), 0.2 mM of each of the four deoxynucleoside triphosphates, 2 mM $MgCl_2$, 0.2 µM each of the forward and reverse primers, and one unit of High Fidelity Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) per 50 µl. The universal 1392R (5′-ACGGGCGGTGTGTRC-3′) and the Bacteria-specific 26F (5′-AGAGTTTGATCCTGGCT CAG-3′) were used for prokaryotic 16S rRNA gene (Amann et al. 1995) amplification. The following PCR conditions were applied: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 45°C for 45 s, and 72°C for 1.5 min, and completed with an extension period of 10 min at 72°C. PCR products were purified with QIAquick PCR purification columns (Qiagen, Valencia, CA, USA) and quantified by ethidium bromide-UV detection on a 1.5% agarose gel. The PCR products were cloned into pGEM-T (Promega, Madison, WI, USA) and transformed into competent host cells following the manufacturer's instructions. The *dsrB* gene was PCR-amplified using the forward primer DSRp2060F (without GC-clamp; Geets et al. 2006) and reverse primer DSR4R (Wagner et al. 1998). The applied PCR conditions were taken from Geets et al. (2006). Using DNA isolated from sulfate reducer *Desulfovibrio desulfuricans* as positive control, an annealing temperature of 57°C was found to be optimal. The *nreB* gene was amplified using primers and protocol according to Grass et al. (2001).

2.10 ARDRA screening and sequencing

For amplified 16S rDNA restriction analysis (ARDRA) and sequencing, the 16S rRNA gene inserts were PCR amplified as described above, except that cells of transformants were directly added to the PCR mixture. The pGEM-T vector specific M13 primers were used for PCR amplification of the inserts. Aliquots of the PCR products were digested overnight at 37°C with one unit of the 4-base-specific restriction endonuclease *Hpy*CH4 IV in 1× NEB buffer 1 (New England Biolabs, Beverly, MA, USA). Digestion products were separated by agarose gel (1.5%) electrophoresis, and visualized by ethidium bromide staining and UV illumination. ARDRA patterns were grouped, and clones with representative patterns were selected for sequencing.

Purified PCR products (QIAquick column) of the 16S rRNA gene inserts were sequenced using the Prism Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) with 100 ng of template DNA. The extended sequences were obtained with universal primers 26F and 1392R. DNA sequences were determined on a 16 Capillary DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

2.11 Phylogenetic analyses

Chimeric sequences were checked by the Check_Chimera program available at the Ribosomal Database Project

(RDP) II (Hugenholtz and Huber 2003). The operational taxonomic units were searched for homology using the programs Basic Local Alignment Search Tool and RDP, aligned with MUSCLE (Edgar 2004), and used to construct a phylogenetic tree by the neighbor-joining algorithm with JalView. Bootstrap analysis was performed with a round of 1,000 reassemblings (http://bioweb.pasteur.fr/seqanal/interfaces/clustalw.html).

2.12 Nucleotide accession numbers

The nucleotide sequences obtained in this study were deposited in the GenBank database and received accession numbers EU268569 to EU268631 (see also Fig. 5).

3 Results and discussion

3.1 Total microbial community analyses of the original soil

At the start of the experiment, total DNA was extracted from the homogenized soil which was used to pack the columns and from which the endogenous microbial community was isolated and used to inoculate the soil columns. Sequencing of 96 PCR amplified and cloned 16S rRNA gene fragments resulted in the identification of a population dominated by α-Proteobacteria, mainly Sphingomonadaceae, and γ-Proteobacteria, mainly Pseudomonadaceae, and Actinobacteria (Fig. 1, "original sample"). No growth was observed after plating soil extracts on Ni-containing (60, 120, 180 ppm) agar plates, indicating that if cultivable Ni-resistant strains were present, then they were present below detection. On nonselective plates (0 ppm Ni) between 10^{+7} and 10^{+8} CFU/g soil were counted. BIOMET analysis showed that the nickel bioavailability in the homogenized soil was below the detection limit (50 ppb), which is consistent with the lack of nickel-resistant bacteria in the cultivable fraction.

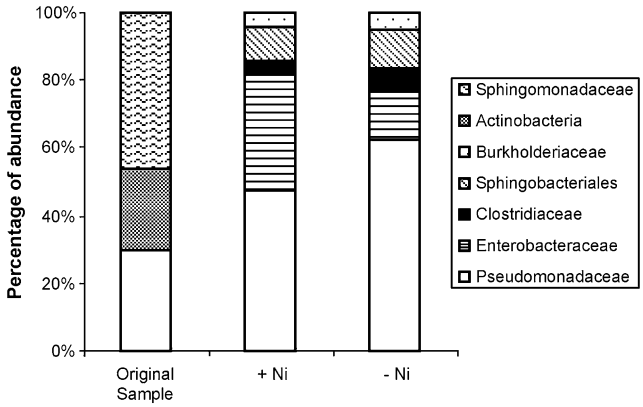

Fig. 1 Frequency distribution of *Hpy*CH4 IV restriction phylotypes from analysis of 100 cloned rDNA fragments from each column after 222 days of enrichment. A description of the column conditions can be found in Table 1

3.2 Reduction of U(VI), NO_3^- and SO_4^{2-}

During the column experiment, changes in pH and the concentrations of U, SO_4, and NO_3 were monitored in aqueous samples collected periodically at port B and in the effluent of each column. The pH of both the biostimulated columns and the abiotic control column increased from 6 to 8. We believe that the increase in pH for the abiotic control is due to the continuous application of groundwater containing basic cations such as Ca, Mg, and K. These ions replace acid cations such as Al and H, resulting in a liming effect.

Reduction of 620 ppm nitrate in the influent was measured as the loss of nitrate from aqueous samples collected periodically at port B and from the effluent of each column. During the first 50 days, nitrate reduction levels fluctuated and varied significantly among the biostimulated columns; however, beyond this period, nitrate reduction levels between 90% and 100% were observed in port B and the effluent of each biostimulated column.

The onset of reduction of 100 ppm sulfate, measured as the loss of sulfate, varied significantly among the biostimulated columns. After 50 days, however, sulfate reduction efficiencies leveled off between 60% and 80% in all biostimulated columns. The reduction of sulfate occurred along with the appearance of black metal sulfide precipitates. In contrast, sulfate losses in the abiotic control were not significant, and sulfide precipitation was not observed. The increase in sulfate-reducing bacteria at the moment when sulfate was efficiently being reduced was confirmed by PCR using *dsrB* gene specific primers and by cultivation dependent methods (see below).

The uranium concentration in the effluent of the columns decreased continuously from up to 17,000 ppb after 20 days to an average concentration of 160 (±120) ppb after 222 days of continuous biostimulation. The U concentration in the abiotic control column effluent remained constant at 3 (±2) ppm, which is one order of magnitude greater than the biostimulated columns. Micro-XANES point spectra of U in soil samples, which were extracted once the lowest pore water U concentrations were observed in the biostimulated columns, indicate reduction of a significant fraction of the insoluble U. It should be noted that reoxidation was observed over time at the beamline; therefore, post-sampling reoxidation could explain why complete reduction was not observed even though low NO_3^- concentration and sulfide formation are consistent with redox conditions known to result in complete U(VI) reduction (North et al. 2004). Another explanation could be that reoxidation could occur due to residual reactive Fe(III) driven by carbonate

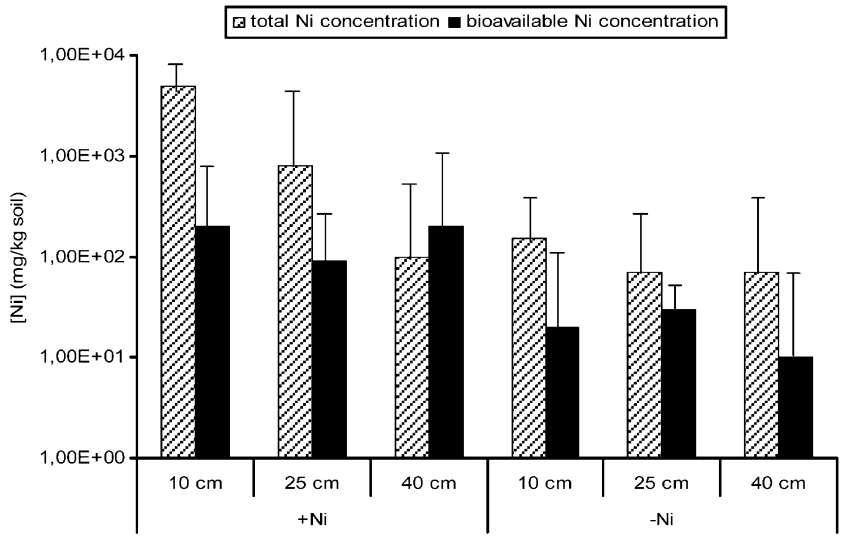

Fig. 2 Total and bioavailable concentrations of Ni in column sediments determined after 222 days. Samples were taken at three different locations of each column; at 10, 25, and 40 cm from the *inlet* of the column. The data obtained for column receiving Ni contaminated groundwater (+Ni) are compared to the column percolated with Ni-free groundwater (–Ni). Concentrations are presented in milligram equivalents of Ni per kilogram of dry sediment retrieved from the columns. Column conditions are described in Table 1. The *bars* represent standard deviations

accumulation (from microbial respiration) in the presence of high Ca^{2+} even in the absence of NO_3 and under low redox conditions (Wan et al. 2005).

3.3 Nickel bioavailability

Micro-XRF analyses revealed detectable Ni (46±8 ppm) in a subsample of the homogenized Area 2 FRC soil at the start of the experiment, which is consistent with field observations of Ni contamination at the site. Significant heterogeneity has been observed at the site (http://public.ornl.gov/nabirfrc/other/frcconmod.pdf); therefore, this background Ni concentration could vary in the columns. BIOMET analyses on subsamples of this material indicated that the bioavailable fraction was below detection (50 ppb). Although one of the biostimulated columns was amended with Ni-containing groundwater (120 ppm), aqueous Ni concentrations in port B pore water and column effluent never exceeded 40 ppm during the 222 day experiment. Figure 2 shows micro-XRF Ni soil concentrations and BIOMET Ni bioavailability in soil samples taken after 222 days of biostimulation at three different positions along each 50 cm long column: inlet (10 cm), middle (25 cm), and outlet (45 cm). The BIOMET values of the Ni-amended column indicate that a significant fraction of the Ni associated with the soil is bioavailable. Ni soil concentrations and bioavailability follow the same profile along the path of groundwater flow, decreasing with distance from the inlet. An intense blackening of the sediment was observed originating near the inlet of each biostimulated column due to the ongoing metabolism of sulfate-reducing bacteria and concomitant precipitation of sulfides. Ni K-edge micro-XANES spectra indicate extensive formation of Ni-sulfides and Ni-hydroxides in the soils exhibiting the highest Ni bioavailability in the reducing environment near the inlet of the Ni-amended column. Measurable levels of bioavailable Ni were also observed in the biostimulated column that had not been amended with Ni. BIOMET and micro-XRF measurements (see Fig. 2) indicate that geochemical changes resulting from biostimulation made a significant fraction of the original Ni bioavailable. According to Qureshi et al. (2003), who observed comparable leaching of Ni at neutral and acidic pH from peat soils, the most likely factors arising from microbial activity that mobilize trace elements are acidification and the release of dissolved organic compounds (DOC). The presence of DOC provides a mobile ligand that facilitates the mobility of trace elements, including Ni, at neutral pH. Ni bioavailability in the Ni-amended columns, however, was more than one order of magnitude greater than in the non-Ni-amended columns.

3.4 Total microbial community evaluation: cultivable fraction

Addition of Ni as a selection pressure had no significant effects on the total number of CFU throughout the duration of the experiment. To evaluate the adaptation of the community to the nickel stress, bacteria present in the columns were sampled at port B and enumerated on Ni-supplemented medium (Fig. 3).

For both viable columns, Ni-resistant bacteria were found on medium containing 60 ppm of Ni. In the endogenous microbial community, no cultivable Ni-resistant bacteria

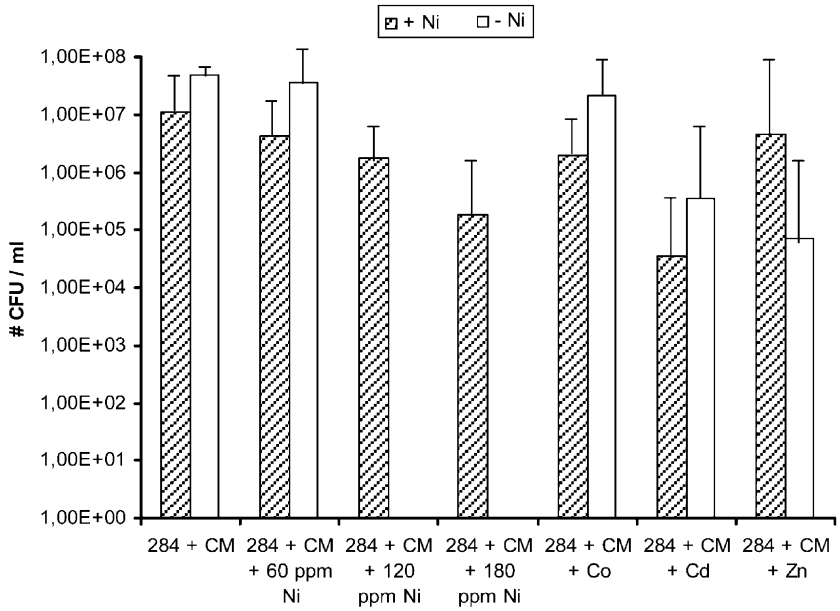

Fig. 3 Number of colony forming units growing on nonselective media and selective media supplemented with heavy metals Ni, Co, Cd or Zn. Samples were taken at 10 cm from the *inlet* of each column. Comparisons are presented for similar column conditions that received either Ni containing or Ni free groundwater. Growth in the presence of Ni was tested at concentrations 60, 120, and 180 ppm. Co, Cd, and Zn were supplied at a concentration of 50, 110, and 130 ppm, respectively. The *bars* represent standard deviations

were found, even though Ni was present in a non-bioavailable form in the homogenized soil used to pack the columns. It is, therefore, likely that these Ni-resistant bacteria were dormant and formed a minor fraction of the endogenous community. However, the ongoing metabolism in the columns resulted in changes of geochemical conditions, including the pH which, in turn, affected Ni solubility and bioavailability, even in the column that did not receive Ni-containing influent. This explains why after 222 days of operation, Ni became bioavailable in all columns. The increased Ni bioavailability also explains the observed changes and similarities in community composition in both biostimulated columns. It has been observed before (Ipsilantis and Coyne 2007) that even in small amounts, the presence of heavy metals can steer microbial community dynamics. However, bacteria growing in the presence of 120 and 180 ppm Ni were only detected in the material from the Ni-exposed column. This indicates the enrichment of Ni resistant bacteria with higher MIC values as a result of increased selection pressure.

Broad host resistance to nickel, limited to resistance levels of 180 ppm, is often encoded by the *nre* locus. However, in our case, it seems unlikely that the observed increase in Ni resistance is the direct effect of the expression of the *nre* locus, as we failed to detect the highly conserved *nreB* gene by PCR. In addition, cultivable bacteria were also found on selective media containing Cd, Co, or Zn, in numbers sometimes similar as observed for Ni-resistant strains (see Fig. 3). This suggests that within the endogenous microbial population at the FRC site, an inherent resistance to multiple heavy metals is residing, probably encoded by three component efflux systems.

Based on morphology, heavy metal resistance phenotypes, and 16S rRNA gene sequencing, three families dominated the cultivable community members in each column (see Fig. 1): Burkholderiaceae, Enterobacteraceae and Sphingobacteriales. Species belonging to the family of the Enterobacteraceae were more prominent in the column amended with Ni, while Pseudomonadaceae and Burkholderiaceae were observed in both Ni- and non-Ni-amended columns and were able to grow in the presence of the different heavy metals. Sphingobacteriales were more abundant in the non-Ni-amended column lacking the nickel selection, although they grew on media augmented with Co, Cd, Zn, and Ni (60 ppm only).

MPN counts (Fig. 4) of denitrifying, sulfate reducing (SRB) and iron reducing bacteria showed that their numbers increased over time from below detection to more than 10^{+8} bacteria ml^{-1}. In contrast to denitrifying and iron reducing bacteria, there was a lag-phase of 130 days before the SRB reached a value of more than 10^{+5} ml^{-1}. No microbial growth was observed in the MPN studies with the abiotic control column samples.

3.5 Total microbial community evaluation: non-cultivable fraction

After 222 days, soil samples from each column were taken at a height of 10 cm. DNA was extracted and used to amplify and

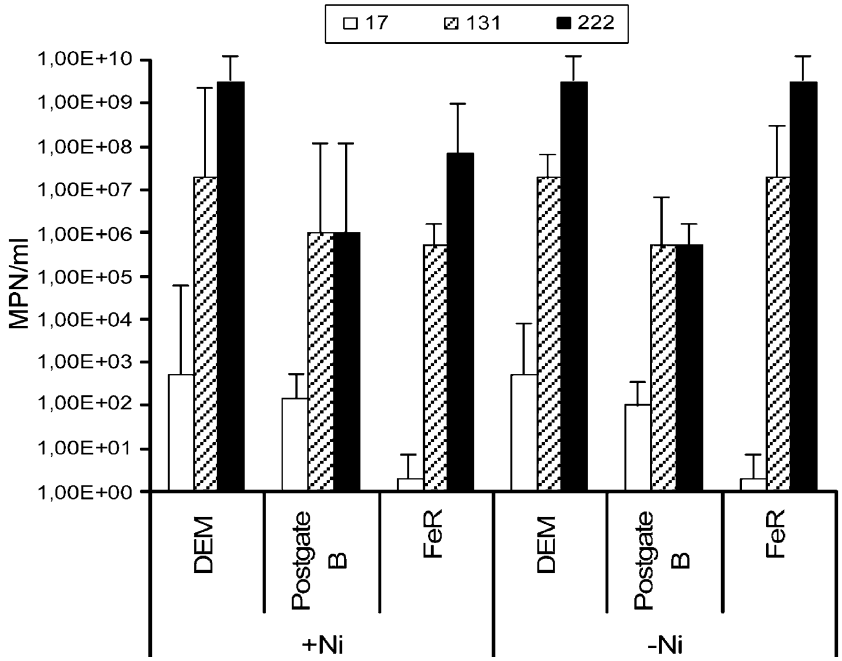

Fig. 4 Enumeration as a function of time of nitrate, sulfate, and iron reducing bacteria present in the viable columns by the MPN method. Results are presented in number of bacteria per ml of sludge (mixture of groundwater and sediment), extracted at 10 cm from the *inlet* of each column (port B). See Table 1 for column conditions. *DEM* Denitrifying enrichment medium, *FeR* Fe(III) reducing enrichment medium, *Postgate B* sulfate reducing enrichment medium

clone the 16S rRNA genes from the community members. For each library, 96 isolates were selected after blue-white screening, and their 16S rRNA gene inserts were PCR amplified and subjected to ARDRA analysis. The ARDRA patterns obtained for each column sample were compared and revealed no major differences in their distribution, indicating the presence of similar microbial groups among the columns (see Fig. 1). This confirms the results of the cultivation based community analysis. ARDRA analysis yielded 56 16S rRNA gene clones with unique restriction patterns. The sequenced clones could be assigned to four major lineages of bacteria: the β-, γ-Proteobacteria, the Clostridiaceae, and the Flavobacteriaceae. None of the clones were identical to 16S rRNA gene sequences from the cultivated bacteria. Strikingly, no Sphingomonadaceae were observed among the analyzed 16S rRNA gene clones, indicating the importance of complementary approaches for microbial community analysis. The phylogeny placement of the clones is shown in Fig. 5.

The largest number of the clones (30% and 36%, respectively) represented γ-Proteobacteria of the Pseudomonadaceae and Enterobacteriaceae. These clones also represented the most frequently observed ARDRA patterns. The Enterobacteriaceae included *Citrobacter* species, previously described in growth-decoupled biomineralization of heavy metals and radionuclides (Macaskie et al. 1992). The β-Proteobacteria contained 1 clone that belonged to the Burkholderiaceae. The Clostridial group contained ten different clones. The presence of sulfate reducing Clostridiaceae is consistent with the observed increase in sulfate reduction efficiency. This family harbors strains capable of reducing U(VI) (Suzuki et al. 2003). Finally, the members of the Flavobacteriaceae were also found to form an important group with eight closely related strains.

The combined results from the cultivation dependent and cultivation-independent microbial community show that as a consequence of the transition from microaerophilic and oligotrophic conditions present in the original Area 2 FRC sediments to anaerobic and nutrient-rich conditions present in the column experiments, significant changes in microbial community composition occurred from the original community, which was dominated by Actinobacteria, α-Proteobacteria (including Sphingomonadaceae), and γ-Proteobacteria. After enrichment, clone libraries of the column samples, ARDRA analysis, and cultivation dependent approaches indicated the dominant presence of nitrate reducing bacteria belonging to the Sphingobacteriales, Pseudomonadaceae, and Enterobacteriaceae, followed by members of Burkholderiaceae, a family to which the heavy metal resistant species *C. metallidurans* CH34 belongs (Taghavi et al. 2001). Future work should focus on increasing the number of sequenced clones, since the relatively low number used in this study might be the reason for the results deviating from those obtained by Brodie et al. (2006).

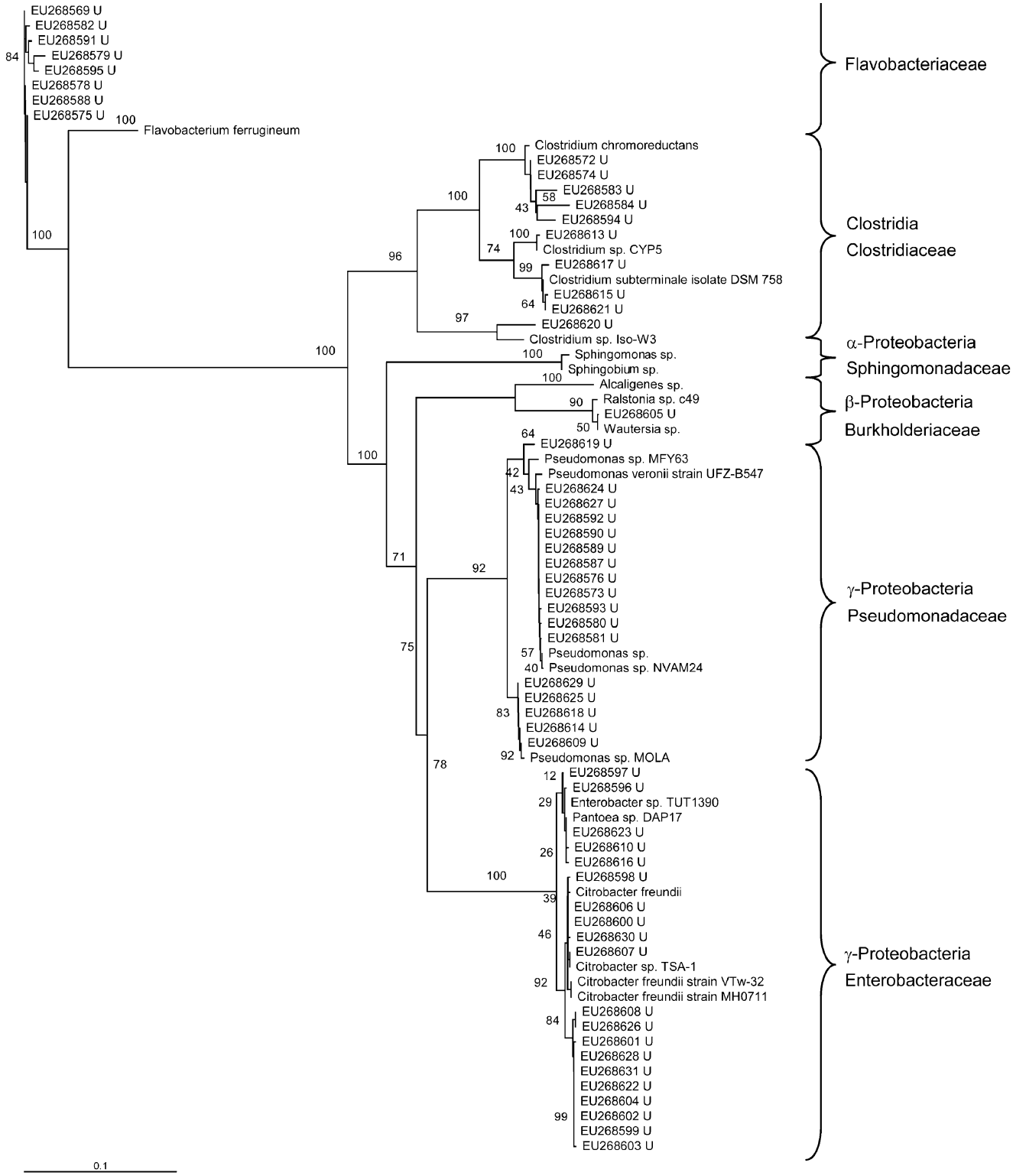

Fig. 5 Phylogenetic analysis of the dominant cultivable and non-cultured bacteria present in the columns. The phylogenetic tree is based on nearly complete 16S rRNA gene sequences for cultivable and noncultured isolates present in the columns. Sludge fractions were taken at port B from the viable columns filled with sediments from the FRC. The length of each branch is proportional to the estimated number of substitutions per position. The distance between two species is obtained by summing the lengths of the connecting horizontal branches using the *scale at the bottom*. Bootstrap values are indicated at the *nodes*

4 Conclusions and perspectives

Biostimulation of endogenous bacteria provides an efficient tool for the successful in situ remediation of mixed-waste sites, particularly those co-contaminated with heavy metals, nitrate, and radionuclides, as found in the United States and other countries as environmental legacies of the nuclear age. In this specific case, stimulating the metabolism of the endogenous bacterial population at the Oak Ridge FRC site by adding ethanol as a suitable carbon source resulted in efficient nitrate reduction, even under conditions of elevated nickel stress and in a decrease in the soil redox potential such that sulfate and iron-reducing bacteria are able to thrive and create conditions favorable for the reduction and in situ immobilization of uranium. Since our analysis of the microbial community was relatively limited, we believe it will be necessary to combine a small field experiment with a more detailed analysis of the microbial community composition. Field scale experiments at the FRC site are ongoing (North et al. 2004; Wu et al. 2006a; Wu et al. 2006b), so the technical know-how is present. Besides having operational equipment, an extensive geochemical and geophysical study of the subsurface has been undertaken. Since we have found that the remediation potential resides within the endogenous microbial community, we believe it will be feasible to conduct field tests.

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References

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in-situ detection of individual microbial cells without cultivation. Microbiol Rev 59(1):143–169

Balkwill DL, Casida LE (1979) Attachment to autoclaved soil of bacterial cells from pure cultures of soil isolates. Appl Environ Microbiol 37(5):1031–1037

Bastiaens L, Springael D, Wattiau P, Harms H, deWachter R, Verachtert H, Diels L (2000) Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. Appl Environ Microbiol 66(5):1834–1843

Brina R, Miller AG (1992) Direct detection of trace levels of uranium by laser-induced kinetic phosphorimetry. Anal Chem 64:1413–1418

Brodie EL, DeSantis TZ, Joyner DC, Baek SM, Larsen JT, Andersen GL, Hazen TC, Richardson PM, Herman DJ, Tokunaga TK, Wan JM, Firestone MK (2006) Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Appl Environ Microbiol 72 (9):6288–6298

Corbisier P (1997) Bacterial metal-lux biosensors for a rapid determination of the heavy metal bioavailability and toxicity in solid samples. Res Microbiol 148(6):534–536

Dichristina TJ (1992) Effects of nitrate and nitrite on dissimilatory iron reduction by *Shewanella Putrefaciens* 200. J Bacteriol 174 (6):1891–1896

Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5 (113):1–19

Everhart JL, McNear D, Peltier E, van der Lelie D, Chaney RL, Sparks DL (2006) Assessing nickel bioavailability in smelter-contaminated soils. Sci Total Environ 367(2–3):732–744

Finneran KT, Housewright ME, Lovley DR (2002a) Multiple influences of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediments. Environ Microbiol 4(9):510–516

Finneran KT, Forbush HM, VanPraagh CVG, Lovley DR (2002b) *Desulfitobacterium metallireducens* sp nov, an anaerobic bacterium that couples growth to the reduction of metals and humic acids as well as chlorinated compounds. Int J Syst Evol Microbiol 52(6):1929–1935

Geets J, Borrernans B, Diels L, Springael D, Vangronsveld J, van der Lelie D, Vanbroekhoven K (2006) DsrB gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria. J Microbiol Meth 66(2):194–205

Grass G, Fan B, Rosen BP, Lemke K, Schlegel HG, Rensing C (2001) NreB from *Achromobacter xylosoxidans* 31A is a nickel-induced transporter conferring nickel resistance. J Bacteriol 183(9):2803–2807

Hugenholtz P, Huber T (2003) Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. Int J Syst Evol Microbiol 53(1):289–293

Ipsilantis I, Coyne MS (2007) Soil microbial community response to hexavalent chromium in planted and unplanted soil. J Environ Qual 36(3):638–645

Istok JD, Senko JM, Krumholz LR, Watson D, Bogle MA, Peacock A, Chang YJ, White DC (2004) In situ bioreduction of technetium and uranium in a nitrate-contaminated aquifer. Environ Sci Technol 38(2):468–475

Macaskie LE, Empson RM, Cheetham AK, Grey CP, Skarnulis AJ (1992) Uranium bioaccumulation by a *Citrobacter* sp. as a result of enzymatically mediated growth of polycrystalline HUO_2PO_4. Sci 257(5071):782–784

Martinez RJ, Wang YL, Raimondo MA, Coombs JM, Barkay T, Sobecky PA (2006) Horizontal gene transfer of P-IB-type ATPases among bacteria isolated from radionuclide- and metal-contaminated subsurface soils. Appl Environ Microbiol 72 (5):3111–3118

McLain E (2004) Characterization of metal-reducing microbial communities from acidic subsurface sediments contaminated with uranium (VI). Dissertation presented at the Department of Oceanography, Florida State University, College of Arts and Sciences, http://etd.lib.fsu.edu/theses/available/etd-12222004–153409/unrestricted/01EMEprelims.pdf

Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P, Vangijsegem F (1985) Alcaligenes eutrophus CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J Bacteriol 162(1):328–334

North NN, Dollhopf SL, Petrie L, Istok JD, Balkwill DL, Kostka JE (2004) Change in bacterial community structure during in situ biostimulation of subsurface sediment cocontaminated with uranium and nitrate. Appl Environ Microbiol 70(8):4911–4920

Nyman JL, Marsh TL, Ginder-Vogel MA, Gentile M, Fendorf S, Criddle C (2006) Heterogeneous response to biostimulation for U (VI) reduction in replicated sediment microcosms. Biodegradation 17(4):303–316

Page AL (1982) Methods of soils analysis. Part 2—chemical and microbiological characteristics. American Society of Agronomy, Madison

Petrie L, North NN, Dollhopf SL, Balkwill DL, Kostka JE (2003) Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). Appl Environ Microbiol 69(12):7467–7479

Qureshi S, Richards BK, McBride MB, Baveye P, Steenhuis TS (2003) Temperature and microbial activity effects on trace element leaching from metalliferous peats. J Environ Qual 32 (10):2067–2075

Sakadevan K, Zheng H, Bavor HJ (1999) Impact of heavy metals on denitrification in surface wetland sediments receiving wastewater. Water Sci Technol 40:349–355

Sani RK, Peyton BM, Amonette JE, Geesey GG (2004) Reduction of uranium(VI) under sulfate-reducing conditions in the presence of Fe(III)-(hydr)oxides. Geochim Cosmochim Acta 68(12):2639–2648

Senko JM, Istok JD, Suflita JM, Krumholz LR (2002) In-situ evidence for uranium immobilization and remobilization. Environ Sci Technol 36(7):1491–1496

Suzuki Y, Kelly SD, Kemner KA, Banfield JF (2003) Microbial populations stimulated for hexavalent uranium reduction in uranium mine sediment. Appl Environ Microbiol 69(3):1337–1346

Taghavi S, Delanghe H, Lodewyckx C, Mergeay M, van der Lelie D (2001) Nickel-resistance-based minitransposons: new tools for genetic manipulation of environmental bacteria. Appl Environ Microbiol 67(2):1015–1019

Tibazarwa C, Corbisier P, Mench M, Bossus A, Solda P, Mergeay M, Wyns L, van der Lelie D (2001) A microbial biosensor to predict bioavailable nickel in soil and its transfer to plants. Environ Pollut 113(1):19–26

Vrionis HA, Anderson RT, Ortiz-Bernad I, O'Neill KR, Resch CT, Peacock AD, Dayvault R, White DC, Long PE, Lovley DR (2005) Microbiological and geochemical heterogeneity in an in situ uranium bioremediation field site. Appl Environ Microbiol 71(10):6308–6318

Wagner M, Roger AJ, Flax JL, Brusseau GA, Stahl DA (1998) Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. J Bacteriology 180(11):2975–2982

Wan J, Tokunaga TK, Brodie EL, Wang Z, Zheng Z, Herman D, Hazen TC, Firestone MK, Sutton SR (2005) Reoxidation of bioreduced uranium under reducing conditions. Environ Sci Technol 39(16):6162–6169

Wu WM, Carley J, Fienen M, Mehlhorn T, Lowe K, Nyman J, Luo J, Gentile ME, Rajan R, Wagner D, Hickey RF, Gu B, Watson D, Cirpka OA, Kitanidis PK, Jardine PM, Criddle CS (2006a) Pilot-scale in situ bioremediation of uranium in a highly contaminated aquifer 1 Conditioning of a treatment zone. Environ Sci Technol 40(12):3978–3985

Wu WM, Carley J, Gentry T, Ginder-Vogel MA, Fienen M, Mehlhorn T, Yan H, Caroll S, Pace MN, Nyman J, Luo J, Gentile ME, Fields MW, Hickey RF, Gu B, Watson D, Cirpka OA, Zhou J, Fendorf S, Kitanidis PK, Jardine PM, Criddle CS (2006b) Pilot-scale in situ bioremedation of uranium in a highly contaminated aquifer 2 Reduction of U(VI) and geochemical control of U(VI) bioavailability. Environ Sci Technol 40(12):3986–3995