Microbial Links between Sulfate Reduction and Metal Retention in Uranium- and Heavy Metal-Contaminated Soil

Jana Sitte,1 Denise M. Akob,1,2 Christian Kaufmann,1 Kai Finster,3 Dipanjan Banerjee,4 Eva-Maria Burkhardt,1 Joel E. Kostka,2 Andreas C. Scheinost,4 Georg Büchel,5 and Kirsten Küsel1*

Institute of Ecology, Friedrich Schiller University Jena, D-07743 Jena, Germany;1 Department of Oceanography, Florida State University, Tallahassee, Florida 32306; Department of Microbial Ecology, Institute for Biological Sciences, DK-8000 Aarhus C, Denmark;3 Institute of Radiochemistry, Forschungszentrum Dresden-Rossendorf, D-01314 Dresden, Germany, and The Rossendorf Beamline at ESRF, F-38043 Grenoble, France;4 and Institute of Earth Science, Friedrich Schiller University, D-07749 Jena, Germany

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Sulfate-reducing bacteria (SRB) can affect metal mobility either directly by reductive transformation of metal ions, e.g., uranium, into their insoluble forms or indirectly by formation of metal sulfides. This study evaluated in situ and biostimulated activity of SRB in groundwater-influenced soils from a creek bank contaminated with heavy metals and radionuclides within the former uranium mining district of Ronneburg, Germany. In situ activity of SRB, measured by the 35SO42− radiotracer method, was restricted to reduced soil horizons with rates of ≤142 ± 20 nmol cm−2 day−1. Concentrations of heavy metals were enriched in the solid phase of the reduced horizons, whereas pore water concentrations were low. X-ray absorption near-edge structure (XANES) measurements demonstrated that ~80% of uranium was present as reduced uranium but appeared to occur as a sorbed complex. Soil-based dsrAB clone libraries were dominated by sequences affiliated with members of the Desulfovibionales but also the Desulfobacterales, Syntrophobacteraceae, and Clostridiales. [13C]acetate- and [13C]lactate-biostimulated soil microcosms were dominated by sulfate and Fe(III) reduction. These processes were associated with enrichment of SRB and Geobacteraceae; enriched SRB were closely related to organisms detected in soils by using the dsrAB marker. Concentrations of soluble nickel, cobalt, and occasionally zinc declined ≤100% during anoxic soil incubations. In contrast to results in other studies, soluble uranium increased in carbon-amended treatments, reaching ≤1,407 nM in solution. Our results suggest that (i) ongoing sulfate reduction in contaminated soil resulted in in situ metal attenuation and (ii) the fate of uranium mobility is not predictable and may lead to downstream contamination of adjacent ecosystems.

Dissimilatory sulfate-reducing bacteria (SRB) play an important role in the sulfur cycle and the mineralization of organic matter in anoxic marine and freshwater environments (53). In addition, sulfate reduction can occur in oxygenated habitats where anoxic niches (8) and the expression of superoxide reductase activity (34) provide protection for SRB against oxygen toxicity. The rate-limiting step of sulfate reduction is catalyzed by the dissimilatory (bi-)sulfite reductase (encoded by the dsrAB gene). Phylogenetic investigations have shown that this key enzyme for sulfate and sulfite respiration was present in early ancestors of modern Bacteria and Archaea (66).

Dissimilatory sulfate reduction has been shown to be a terminal-electron-accepting process (TEAP) in acid mine drainage (AMD)-impacted and radionuclide- and metal-contaminated environments. Sulfate-reducing activities as well as SRB abundances show a wide range in these habitats (24, 29, 69). SRB are able to reductively transform metal ions, e.g., uranium and chromium, into insoluble and chemically inert forms via direct enzymatic reduction (41, 42). Sulfide, the end product of microbial sulfate reduction, may further contribute to metal attenuation through reduction of metal oxyanions and oxyanions, such as those of uranium and chromium (4, 19), or through precipitation of metal cations as sulfides (15, 20). In addition, SRB have the potential to enhance metal retention via extracellular binding, cellular uptake and accumulation of metals, oxidation/reduction processes, and surface-mediated mineral precipitation (20, 52). Metal stress for SRB in uranium-contaminated sediments (48, 63) and biofilms from Pb-Zn deposits (39) can be reduced by the formation of uraninite and metal sulfides.

Previous work in uranium-contaminated environments has emphasized the role of biostimulated SRB in mediating uranium and/or technetium reduction (3, 46, 63), although other metal contaminants are present (55, 63). The long-term stability of immobilized, reduced contaminants is a concern due to the potential for remobilization after carbon addition is stopped. Therefore, it is important to understand alternative remediation processes, such as those involved in natural attenuation. In the former uranium mining district of Ronneburg (Thuringia, Germany), leaching of low-grade black shale by acid mine drainage and sulfuric acid and pyrite oxidation resulted in serious large-scale contamination with heavy metals and radionuclides (28). Metal- and sulfate-enriched seepage waters and surface runoff infiltrated adjacent soils and surface waters, leading to elevated concentrations of sulfate, nickel,
copper, cadmium, zinc, arsenic, and uranium in creek bank soils (9). At the Ronneburg site, the presence of high levels of mixed contaminants provides a unique environment to look at complex processes involved in natural attenuation of contaminants. It is hypothesized that resident SRB contribute to natural uranium and heavy metal attenuation at the Ronneburg site. Thus, the objective of this study was to resolve the potential importance of SRB in contaminated creek bank soils both in situ and in biostimulated soil microcosms using stable isotope probing (SIP).

MATERIALS AND METHODS

Site description. The study site is located on the bank of Gessen Creek, one of the main drainage systems for the former leaching heaps within the former uranium mining site near Ronneburg (Thuringia, Germany; location E 4510121, N 5635807, Gauss-Krueger Potsdam coordinate system). In the lovie gleysol, two iron-rich groundwater-influenced oxidized horizons (BElc and Btlc) and two groundwater-influenced reduced horizons (Br1 and Br2) could be distinguished by color and textural contrasts, the humus top horizon (Ah) and a yellowish horizon (BElw/Ah) (6). The reduced horizons were gray (upper Br1, 82 to 103 cm in depth) and black (lower Br2, 102 to 110 cm in depth), respectively. The solid phase of Br2 had a high total sulfur and carbon content (up to 2.0% and 3.4%, respectively) and showed a low redox potential (Eh) of −30 mV (37). In contrast, Br1 had a sulfur content of only 0.4% and a redox potential of 60 mV.

Sampling procedure. Soil was aseptically sampled and stored in plastic bags in the dark at 4°C for transport and until further processing on the following day. For determination of the acid volatile sulfur (AVS) fraction, the soil was frozen at −20°C. Soil samples for total extraction were stored in 4°C in the dark prior to analysis. Pore water samples for determination of pH and redox potential and measurements of nitrate, sulfate, and soluble heavy metal concentrations were taken with Rhizon suction samplers (Eijkelkamp, Giessbeek, Netherlands) monthly from June to November 2007 from the soil profile at ~10-cm-depth intervals. The redox potential of pore water was measured directly after sampling, and soil waters were stored at 4°C overnight prior to subsequent analyses.

Determination of total soil metal concentrations. Soils from the Br1 and Br2 horizons were air dried and then finely ground to <63-μm sieve size. The ground Br1 and Br2 soils were digested with concentrated hydrofluoric acid, nitric acid, and perchloric acid at 150 to 170°C in a pressure digestion system (DAS, PicoTrace, Bovenden, Germany). Single elements were measured in the resulting solutions using inductively coupled plasma-optical emission spectroscopy (ICP-OES; Spectro Flam P FAV05; Spectro Analytical Instruments, Kleve, Germany) for Fe and inductively coupled plasma-mass spectrometry (ICP-MS; X-SeriesII; ThermoFisher Scientific, Bremen, Germany) for As, Cd, Co, Cu, Ni, U, and Zn.

X-ray absorption spectroscopy for analysis of solid uranium. X-ray absorption near-edge structure (XANES) spectra were collected for soils of Br1 and Br2 at the X-ray beamline (Grenoble, France). Samples were prepared under an anoxic atmosphere. Uranium L$_{mn}$-edge spectra were collected in fluorescence mode at 15 K using a closed-cycle He cryostat. In order to determine the relative proportions of U(IV) and U(VI) in these samples, linear combination fits of the XANES region of the spectra using reference spectra of U(IV) and U(VI) were performed. A reference spectrum of U(IV) aqueous complex was chosen for the U(IV) component, whereas a spectrum of U(VI) sorbed on clay mineral was selected for U(VI). Relative proportions of U(IV) and U(VI) obtained from the fits were within ±1% of the reported values. Additional details are described in a previous study (6).

Determination of AVS. Acid volatile sulfur (AVS) was determined by suspending 10 g of soil in 50% ethanol under a nitrogen flow. AVS was liberated as hydrogen sulfide by cold acid distillation for 1 h after addition of 8 ml of 30% (wt/vol) HCl. The released H$_2$S was collected in 2% (wt/vol) zinc acetate and measured spectrophotometrically according to the method described by Cline (12).

SRR. Sulfate reduction rates (SRR) were determined using the $^{35}$SO$_4^{2−}$-radiotracer technique (17). Rate determinations were conducted on all soil horizons in October 2007 and April 2008 in replicates of three and five, respectively. Soil (3 g) was transferred to sterile 7.5-ml serum bottles, which were sealed with butyl rubber stoppers flushed with sterile argon; and then diluted with 3 ml of sterile, anoxic water. Soil suspensions were amended with H$_2$SO$_4$ (Hartmann Analytica, Braunschweig, Germany) to a final activity of 100 kBq cm$^{-3}$ and incubated for 2 h at 15°C. Incubations were stopped by transferring the soil suspensions to plastic bottles containing 10 ml of 20% (wt/vol) zinc acetate. The soils were stored frozen at −20°C until further processing. The formation of sulfide was analyzed by combined chromium and acid distillation as described by Fossing and Jørgensen (17). Dry weight of soil was determined after the soil was dried at 105°C for 24 h to a constant weight.

Enumeration of sulfate-reducing bacteria. A three-tube most probable number (MPN) technique (14) using 10-fold serial dilutions was used to enumerate cultivable sulfate-reducing bacteria in soils from the reduced horizons. For selective growth, a modified Postgate C medium (7) was used with a final sulfate concentration of 10 mM and pH 6.2. The medium was amended with sodium acetate or lactate (final concentrations, 5 mM) as an electron donor. From both horizons, an additional MPN series was prepared to which heavy metals (0.3 μM CdCl$_2$, 38.6 μM ZnCl$_2$, 1.5 μM CuCl$_2$, 16.4 μM NiCl$_2$, 0.6 μM CoCl$_2$, and 23.2 μM AlCl$_3$) were added, reflecting the maximum pore water concentrations in the soil profile. The culture tubes were incubated at 16°C in the dark for 5 months. Positive growth was scored by a decrease in sulfate concentrations, and MPN values with 95% confidence limits were calculated according to the method of de Man (13). When the ratio between MPN values was above 8.87, the abundance of organisms was considered significant (2).

Soil microcosms. Anoxic soil incubation experiments were performed (i) to study microbial activity under anoxic conditions (soil from horizon Br1 or Br2) and (ii) to investigate active sulfate-reducing bacteria by using stable isotope probing (SIP; soil from Br2 only). Soil samples were collected in May and June 2007 from the Br2 horizon and in February and October 2008 from the Br1 horizon. With the exception of the second experiment using Br1 soil, microcosms were constructed by loading 20 g (fresh weight) of soil into 150-ml incubation bottles under a sterile argon atmosphere, and then the bottles were sealed with rubber stoppers and secured with aluminum caps. A mineral solution (50 mM Na$_2$CO$_3$/HCO$_3$ and 5 mM NaNO$_3$) was added, and stored in situ soil water concentrations was added in a ratio of 1.5 (weight of soil/volume) in a mineral solution. Seventy-five grams (fresh weight) of soil was prepared in 500-ml incubation bottles as described above for the second Br1 horizon experiment (experiment 2) with the sulfate concentrations again adjusted to match in situ conditions. The soil was diluted with mineral solution in a ratio of 1/4 (weight of soil/volume) containing 6 mM MgSO$_4$ only. Triplicate bottles for all experiments were amended to a final concentration of 5 mM glucose, 5 mM lactate, 5 mM acetate, or 10 mM ethanol as electron donor. Glucose and ethanol were not supplied in the SIP or the second Br1 experiment. 13C-labeled acetate or lactate (10 mM final concentration; >99 atom% $^{13}$C; Cambridge Isotopes) was used for the SIP microcosms. Reduced carbon concentrations, 3 mM acetate or lactate, were used for the second set of Br1 microcosms. Three replicates without an added carbon source served as a control. The pH of the microcosms was adjusted to 6.3 and 6.1 for Br1 and Br2, respectively, and the microcosms were then shaken for 1 h prior to incubation in the dark at 16°C. Microcosms were regularly sampled over a period of 31 to 37 days using anoxic, aseptic techniques to measure SO$_4^{2−}$, NO$_3^{−}$, Fe(II), and Mn (except for the Br1 microbial activity experiment). Soluble metal concentrations, pH, and redox potential were determined at the beginning and end of the anoxic incubation.

Analytical techniques. Functional genes. Determination of $^{35}$S-reductase (DSR) gene ($\delta$srAB) was performed using a forward and reverse DSR primer mix targeting a variety of sulfate-reducing bacteria (65; M. Pester, N. Bittner, P. Deevong, M. Wagner, and A. Loy, submitted for publication). The PCR mixture contained 1× MasterAmp PCR Premix D (Epi-
FIG. 1. Geochemistry (A to C) of the soil profile in June 2007 and depth profile of sulfate reduction rates (SRR) (D) at the bank of the contaminated Gessen Creek. SRR values are averages ± standard deviations (October 2007, n = 3; April 2008, n = 5). The corresponding soil horizons are given at the right side of the graphs.

For SSU rRNA gene clone library construction and phylogenetic analyses, purified PCR products were ligated into the TOPO TA cloning vector pCR XL according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Ligations were shipped to the Genome Sequencing Center at Washington University (St. Louis, MO) for transformation and bidirectional sequencing with vector-specific primers (M13F/R). Sequences were assembled, and vector sequences flanking the SSU rRNA gene inserts were removed using Geneious Pro version 4.6.4 (Biomatters, Auckland, New Zealand). Clones were grouped into phylogenies based on a sequence similarity cutoff of 97%, and previously identified sequences with high sequence similarity to the clones obtained in this study were determined using the BLAST algorithm against the GenBank database available from the National Center for Biotechnology Information (NCBI). All clone sequences were aligned with the alignment tool by Greengenes, and the nearest neighbors were identified using the Classify tool against the Greengenes database.

Nucleotide sequence accession numbers. Sequences from this study were published in the GenBank database under the accession numbers GU233963 to GU234006. Sequences generated in this study were also deposited in the GenBank database under the accession numbers GU235998 to GU236099.

RESULTS

Soil geochemistry. The average pH of the soil water measured monthly was neutral to slightly acidic, ranging from 5.8 to 7.0 over the whole profile (Fig. 1A). In general, the redox potential was low in both reduced horizons. Sulfate was enriched in the pore water (Fig. 1A) with average concentrations of 3.4 ± 3.2 mM and 3.1 ± 2.3 mM for Br1 and Br2, respectively. However, the range of sulfate concentrations for the reduced horizons was from 0.3 mM to 12.4 mM over the sampling period. Nitrate (Fig. 1A) was negligibly low in the reduced horizons (<20 μM), whereas it reached up to 1.2 mM in the upper horizons during the sampling period (data not shown). Both manganese and Fe(II) increased with depth, reaching highest concentrations in the horizon Br2 at 0.1 mM Mn and 2.2 mM Fe(II), while Fe(III) was negligible and declined within the oxidized horizons (Fig. 1B). Soluble heavy metal concentrations (U, Co, Ni, Zn, and Cu) peaked in the oxidized Bt horizon but were low in the deeper reduced horizons (Fig. 1C). The maximum uranium concentration of...
vibrionales (2% of total clones), and the families Desulfobacterales as a uranium mining site, a leachate-polluted aquifer, and related to uncultured SRB from pristine environments as well.

Sequences grouped into 39 OTUs, and all were closely constructed with 109 clones screened, resulting in a coverage of 76%. Sequences grouped into 39 OTUs, and all were closely

<table>
<thead>
<tr>
<th>Horizon</th>
<th>U</th>
<th>Zn</th>
<th>Ni</th>
<th>Cu</th>
<th>Co</th>
<th>As</th>
<th>Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Br1</td>
<td>343 ± 4</td>
<td>425 ± 10</td>
<td>170 ± 1</td>
<td>289 ± 2</td>
<td>31 ± 1</td>
<td>37 ± 1</td>
<td>5 ± 0</td>
</tr>
<tr>
<td>Br2</td>
<td>959 ± 3</td>
<td>565 ± 11</td>
<td>229 ± 1</td>
<td>325 ± 3</td>
<td>43 ± 0</td>
<td>55 ± 1</td>
<td>5 ± 0</td>
</tr>
<tr>
<td>BBodSchV</td>
<td>ND</td>
<td>60–200</td>
<td>15–70</td>
<td>20–60</td>
<td>ND</td>
<td>25</td>
<td>0.4–1.5</td>
</tr>
<tr>
<td>IAEA</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
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</table>

Values represent metal content in μg g⁻¹ (dry weight) of Br1 or Br2 soil and background soil levels.


278 nM was observed in horizon Br2 in June 2007. In general, arsenic concentrations were higher in the reduced horizons, reaching up to 180 nM, compared to the upper, oxidized horizons (Fig. 1C).

Acid volatile sulfur (AVS) was higher in the solid phase of the reduced horizons (52 and 126 mmol kg⁻¹ dry weight) soil⁻¹ at Br1 and Br2, respectively, whereas much lower concentrations were observed in the upper soil horizons (AVS ≤ 0.2 mmol kg⁻¹ dry weight) soil⁻¹. Creek bank soil contained high contents of metals in the solid phase and exceeded background levels in the majority of cases (Table 1). Br2 soil accumulated total uranium, zinc, nickel, and copper to a higher extent than did Br1 soil (Table 1). According to XANES measurement, soil of both horizons was highly enriched in reduced uranium species, with Br2 (83.8%) containing a slightly larger amount of U(VI) than Br1 (79.5%). In addition, a comparison of samples Br1 and Br2 to a uraninite (UO₂) reference spectrum showed a lack of features in the postedge region of the two samples, suggesting that U(VI) in these samples did not occur in a crystalline form similar to UO₂ but was more likely to be present as a sorbed complex (Fig. 2).

SRR. Sulfate-reducing activity was restricted to the reduced horizons (Fig. 1D) with sulfate reduction rates (SRR) of 142 ± 20 nmol cm⁻³ day⁻¹ and 48 ± 41 nmol cm⁻³ day⁻¹ for Br1 and Br2 in October 2007, respectively. Sulfate reduction rates determined in April 2008 showed a similar pattern, but total rates were only 5 nmol cm⁻³ day⁻¹. In contrast, activity was below detection in the upper, oxidized horizons.

Enumeration of sulfate-reducing bacteria. The abundances of cultivated SRB (Table 2) were similar in horizons Br1 and Br2 but differed slightly according to the amended electron donor in the enrichments. The highest abundances were observed in lactate-amended cultures of the Br2 horizon. SRB abundance in both horizons was slightly lower in the presence of heavy metals (Table 2). The difference was significant only in the lactate treatment for Br2.

dsrAB soil community. Partial dsrAB sequences were analyzed to identify soil-associated SRB. A clone library was constructed with 109 clones screened, resulting in a coverage of 76%. Sequences grouped into 39 OTUs, and all were closely related to uncultured SRB from pristine environments as well as a uranium mining site, a leachate-polluted aquifer, and sediment from a polluted harbor. dsrAB clones grouped within the families Desulfobacterales (64% of total clones), Desulfovibrionales (2% of total clones), and Syntrophobacterales (17% of total clones) within the Deltaproteobacteria and the Clostridiales (15% of total clones) within the Firmicutes (Fig. 3). The phylogenetic affiliation of a few clones (3% of total clones) remained uncertain (Fig. 3). With dsrAB primers, the non-SRB Carboxydothermus hydrogenoformans, which is known for sulfate reduction (23), was also detected.

Reducive redox processes in anoxic soil microcosms. Microbial activity was stimulated via carbon amendment in four experiments. In all of the microcosms, the pH increased over the incubation period and was slightly higher in biostimulated

![FIG. 2. XANES. Fitted XANES spectra of the Br1 and Br2 samples with reference spectra of U(VI) and U(IV), used for the fitting, as well as that of UO₂. In the spectra of samples Br1 and Br2, the open circles represent data points and the solid line is the fit to the data obtained from linear combination fitting using U(IV) and U(VI) components. The vertical dashed line indicate the U(VI) peak position, which is clearly separated from U(IV).](image-url)
TABLE 2. Enumeration of sulfate-reducing bacteria in reduced soil horizons in the bank of Gessen Creek with and without metals

<table>
<thead>
<tr>
<th>Treatment and electron donor</th>
<th>MPN (cells g [fresh wt] soil⁻¹)ᵃ</th>
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<tbody>
<tr>
<td>Without metals</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>2.0 × 10⁴ (0.4 × 10³-9.3 × 10⁴) A</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.0 × 10⁴ (0.4 × 10³-9.3 × 10⁴) A</td>
</tr>
<tr>
<td>With metals</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>4.0 × 10⁴ (0.8 × 10³-1.9 × 10⁴) A</td>
</tr>
<tr>
<td>Lactate</td>
<td>2.0 × 10⁴ (0.4 × 10³-9.3 × 10⁴) A</td>
</tr>
</tbody>
</table>

ᵃ Values represent abundances in cells g (fresh weight) of Br1 or Br2 soil⁻¹, determined in triplicate MPN serial dilutions after 5 months of incubation at 16°C. Values in parentheses represent the ranges of MPN values within 95% certainty. Capital letters after values represent significant differences of MPN values between metal treatments for the different soil horizons and electron donors.

...treatments (pH 7.3) than in the unamended controls (pH 6.6). The redox potential (Eₚ) declined in the biostimulated microcosms down to ~305 mV, whereas changes in Eₚ were negligible for the controls (data not shown).

Nitrate was rapidly reduced in all treatments within the first 2 to 4 days with negligible nitrite reduction rates (Fig. 4) due to low in situ concentrations. Manganese was reduced in micromolar-scale range within the first 10 to 16 days in Br1 soil and showed a slight decrease after a plateau phase (Fig. 4). In contrast, manganese concentrations of Br2 microcosms did not change significantly (data not shown). Formation rates of Fe(II) were not consistent throughout the different experiments, but some Fe(III) was reduced within the first 5 to 16 days for both soil horizons in two of four nonbiostimulated and in 5 of 12 biostimulated treatments if the initial Fe(II) soil content was low (Fig. 4 and data not shown). Net sulfate reduction was the dominant TEAP, and trends were similar in all experiments (Fig. 4 and data not shown). Net sulfate reduction began only after a 12- or 17-day lag phase, after slight Fe(III) reduction had ceased (Fig. 4). Due to an accumulation of sulfate in the Br2 control microcosms, a slight decreasing trend in sulfate was observed in the carbon-amended treatments after 5 days (Fig. 5A and B), but depletion was below detection. Kinetics of sulfate reduction were similar for Br1 and Br2 soil, and the highest stimulation was observed with the addition of acetate (rates up to 30.4 μmol g [fresh weight] soil⁻¹ day⁻¹), followed by lactate and ethanol. Methane was formed simultaneously as sulfate concentrations decreased in soil microcosms after 16 to 20 days with rates of ≤0.3 μmol g (fresh weight) soil⁻¹ day⁻¹ (Fig. 4) in acetate- or lactate-amended treatments.

Characterization of active microbial communities in anoxic soil microcosms. Addition of supplemental acetate and lactate stimulated SRB best, and consumption rates of these substrates were similar in all microcosm experiments (Fig. 5C and D for the SIP experiment). [¹³C]lactate was consumed slowly during the incubation, and a near-linear decrease was observed from days 18 to 34 (Fig. 5C). Most of the acetate was consumed concomitantly with sulfate reduction, and all acetate was consumed by day 34 (Fig. 5A and C). In the [¹³C]lactate-amended microcosms, lactate was consumed by day 6 of the incubation, yielding acetate and propionate as end products (Fig. 5D). Accumulated acetate was consumed slowly and appeared to be in parallel with sulfate reduction (Fig. 5B and D). DNA was detected in gradient fractions with buoyant den-...
Desulfotalea psychrophila, which reduces sulfate and utilizes lactate as a carbon substrate.

**Effect of anoxic incubation on dissolved metal concentrations.** Surprisingly, uranium was released to solution at the end of incubation in all carbon-amended treatments (Table 3 and data not shown). Concentrations of U reached up to 1,407 nM in the Br2 soil suspension amended with acetate (235-fold increase) and 165 nM in the Br1 soil suspension amended with lactate (6.6-fold increase). Nickel and cobalt were immobilized in all treatments, with up to 100% of the soluble metals removed from solution (Table 3 and data not shown). The addition of carbon led to a 3.0-fold-higher reduction in nickel concentrations at the end of the incubation. Similarly, cobalt concentrations were reduced to a 2.1-fold-higher extent than in the unamended controls. The dynamics of soluble zinc were not consistent among the treatments and experiments. A decline in soluble zinc, up to 95% of the starting concentration, was observed in soil microcosms for the Br1 and Br2 horizons (Table 3 and data not shown), whereas the soluble zinc concentrations in the second Br1 experiment declined in a similar manner for both the control and carbon-amended treatments (Table 3). The low soluble copper concentrations were constant or even declined in Br1 soil microcosms (Table 3 and data not shown). While little to no change in soluble arsenic concentrations was observed in the second Br1 experiment, arsenic concentrations were up to 5.7-fold higher at the end of incubation in the other microcosm experiments (Table 3).

**DISCUSSION**

Ongoing anaerobic microbial activities in Gessen Creek bank soil. Pore water profiles suggested that reduction of nitrate occurred mainly in the upper oxidized horizons, whereas soluble manganese and Fe(II) increased primarily in the reduced soil horizons Br1 and Br2. These horizons contained the highest concentrations of sulfate, which were highly variable with time. The accumulation of acid volatile sulfur and high in situ sulfate reduction rates in Br1 and Br2 soils indicated ongoing dissimilatory sulfate reduction in the creek bank soil. The in situ sulfate-reducing activity was slightly higher than what was previously found in contaminated soils and sediments of the Norilsk mining area (29) and was in the range of rates reported from unpolluted freshwater (25, 70) or marine (32, 57) ecosystems. A long, heavy rainfall prior to our measurements diluted sulfate concentrations, oxygenated the soil, and was likely responsible for the lower reduction rates observed in April 2008.

The detection of a diverse sulfate-reducing community via analysis of the functional marker gene dsrAB in the Br2 soil horizon supported our findings of ongoing sulfate reduction. The majority of clones were related to the Desulfobacterales, and the closest relatives of our clones were freshwater-associated SRB that were found in uranium mining tailings (11), metal-contaminated aquifers (21), or intertidal river soil (47). SRB abundance was within the same range as that in samples from other uranium-contaminated subsurface sediments (48), deciduous forest soils (59), or lake sediments (35, 38). Abundances of resident SRB were only slightly inhibited in the presence of metals at maximum in situ pore water concentrations. This indicates that the resident, cultivatable SRB com-
community is adapted to the level of metal stress in the Gessen Creek bank soils.

**Microbial activities during anoxic soil incubation.** Sulfate reduction was an important TEAP in biostimulated, anoxic microcosms after 12 to 17 days of incubation, with amendment by acetate and lactate resulting in the highest sulfate reduction rates. *In situ* SRR were in the nanomole range, suggesting that sulfate reducers were active at a low level also during the first days of incubation but masked due to the high sulfate concentrations in the treatments. The increase in sulfate concentra-

tions that was observed in the Br2 control microcosms at the beginning of incubation may have been caused by the desorption of sulfate bound or adsorbed to Fe(III)-(hydr)oxides (56). Because nitrate and manganese were present at low concentrations, nitrate reduction and manganese reduction were likely not substantial energy-generating processes. Microbial Fe(III) reduction was a significant TEAP next to sulfate reduction, although not steadily since Fe(II) formation rates were low and highly variable among treatments and replicates. However, Fe(II) may also have been formed from abiotic reduction coupled to sulfide oxidation, since a high reduced sulfur content was present in the Br2 soil. Although we did not detect significantly higher Fe(II)-forming activities compared to the unamended control (data not shown), *Geobacter* was active in the lactate- and acetate-amended treatments. In Btlc soil, stimulated Fe(III) reduction yielded the presence of the genera *Geobacter*, *Geothrix*, and *Pelosinus* (6). This observation was similar to previous field and laboratory experiments at other uranium-contaminated sites, where Fe(III)- and metal-reducing bacteria were of great importance for bioremediation (26, 27).

Active biostimulated sulfate-reducing bacteria were shown to be related to SRB known for complete or incomplete acetate utilization (10) and the capability of oxidizing propionate, which was observed in [13C]lactate treatments (68). *Desulfobacca acetoxidans* and *Desulfocapsa thiozymogenes*-related clones have been previously isolated from uranium and Pb/Zn mining sites (11, 62), indicating the presence of two metal- and radionuclide-tolerant species. The high abundance of *Geobacter* spp. found in the active communities may also have been involved in reduction of humic substances and elemental sulfur (40, 61), since soil showed high carbon and sulfur contents.

**Metal retention potential.** An accumulation of metals in the solid phase was not associated with high soluble concentrations of metals, indicating an additional capacity for metal attenuation for the inherent soil-associated microorganisms and different soil properties. A decrease in soluble nickel and cobalt was observed independently of biostimulation, as expected based on previous work on sulfate reduction in batch experiments (7, 33). It is hypothesized that the decrease in nickel and cobalt was related to the formation of metal sulfides in the soil incubation experiments. Therefore, ongoing, *in situ* sulfate-

![FIG. 6. Frequencies of active bacterial phylogenetic lineages detected in SSU rRNA gene clone libraries from [13C]acetate (A and B)- and [13C]lactate (C and D)-amended microcosms. Calculations were made based on the total number of clones associated with phylotypes of sequenced representatives.](image)

![TABLE 3. Soluble metal concentration at the beginning (T₀) and end (Tₚ) of incubation of Br1 and Br2 soil suspensions](table)
reducing activity would be sufficient to precipitate nickel and cobalt as sulfides (16, 33). However, passive mechanisms, such as metal binding to sites on bacterial cell surfaces and to metal-complexing groups of extrapolymeric substances (20), cannot be ruled out. Metal dynamics for zinc, copper, and arsenic were not uniform throughout the anoxic incubations of the soil and were therefore in contrast to previous work that showed metal sulfide formation with these cations and anions under sulfate-reducing conditions (39, 50, 60). As(VI)-reducing bacteria, such as Desulfoforsorosinus auripigmenti, likely contributed to the observed increase in soluble arsenic (51). Therefore, sulfate-reducing bacteria have the potential to attenuate metal cations in the investigated field site, which was indicated by the metal geochemistry.

In contrast to what other studies have shown (e.g., references 1 and 71), we observed that soluble uranium concentrations increased under anoxic, sulfate-reducing conditions. This was unexpected because anaerobic metabolism of bacteria has been shown to promote reduction in soluble U by indirect and direct mechanisms (5, 42, 45). However, as was seen in this study, an increase in uranium concentration was previously observed during sulfate reduction in uranium-contaminated aquifers (3) or in pure cultures of Desulfovibrio desulfuricans G20 (58). It is also possible that bacteria from the soil produced siderophores, thereby promoting the dissolution of UO2 (18), or that microbially mediated formation of carbonates could have resulted in highly stable carbonate-U(VI) complexes (67). However, carbonate-U(VI) complexes are unlikely to have been formed, due to a lack of carbonates in the soil, and also XANES data did not indicate the presence of carbonate-U(VI) complexes in the soil. Surface-bound uranyl ions [U(IV)], ~20% of the uranium in the soil solid phase, may have been detached from Fe(III)-mineral phases, which have an affinity for uranium (49) and which were bacterially reduced upon amendment of the microcosms. For example, the Fe(III)-mineral phase illite was likely to be present in Br1 and Br2 and could provide sorption surfaces for hexavalent actinides (30).

Conclusions. Active SRB were identified in reduced soils within the bank of Gessen Creek in the former uranium mining site of Ronneburg. SRB were shown to be adapted to the presence of metals and radionuclides and to influence the retention of contaminants. In particular, nickel and cobalt, but occasionally zinc and copper, were retained during sulfate reduction, indicating that soil-associated SRB contributed to in situ metal attenuation, which could explain the high solid metal contents and the low concentrations of metals in the pore water. Uranium was released during anaerobic microbial activities acting as potential sources of contaminants for downstream ecosystems. The increase in soluble uranium concentrations is in contrast to what has been seen during biostimulation of iron- and sulfate-reducing bacteria at other uranium-contaminated sites (3, 27). Our results show that site-specific geochemistry and variable in situ microbial communities can affect the success of biostimulation as a strategy for the enhancement of metal retention. In reduced soils of the Gessen Creek bank, ongoing sulfate reduction is providing a means for natural attenuation of nickel and cobalt contaminants but does not lessen the risk of downstream uranium contamination.

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