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Influence of Heterogeneous Ammonium Availability on Bacterial Community Structure and the Expression of Nitrogen Fixation and Ammonium Transporter Genes during in Situ Bioremediation of Uranium-Contaminated Groundwater

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The influence of ammonium availability on bacterial community structure and the physiological status of *Geobacter* species during in situ bioremediation of uranium-contaminated groundwater was evaluated. Ammonium concentrations varied by 2 orders of magnitude (<4 to 400 μM) across the study site. Analysis of 16S rRNA sequences suggested that ammonium may have been one factor influencing the community composition prior to acetate amendment with *Rhodoferax* species predominating over *Geobacter* species with higher ammonium and *Dechloromonas* species dominating at the site with lowest ammonium. However, once acetate was added and dissimilatory metal reduction was stimulated, *Geobacter* species became the predominant organisms at all locations. Rates of U(VI) reduction appeared to be more related to acetate concentrations rather than ammonium levels. In situ mRNA transcript abundance of the nitrogen fixation gene, *nifD*, and the ammonium transporter gene, *amtB*, in *Geobacter* species indicated that ammonium was the primary source of nitrogen during uranium reduction. The abundance of *amtB* was inversely correlated to ammonium levels, whereas *nifD* transcript levels were similar across all sites examined. These results suggest that *nifD* and *amtB* expression are closely regulated in response to ammonium availability to ensure an adequate supply of nitrogen while conserving cell resources. Thus, quantifying *nifD* and *amtB* transcript expression appears to be a useful approach for monitoring the nitrogen-related physiological status of subsurface *Geobacter* species. This study also emphasizes the need for more detailed analysis of geochemical and physiological interactions at the field scale in order to adequately model subsurface microbial processes during bioremediation.

Introduction

Rational optimization of subsurface bioremediation strategies requires an understanding of factors influencing the activity of the microorganisms involved in remediation processes (*1*). Nitrogen, for example, is an essential nutrient in microbial metabolism. Thus, ammonium or other sources of fixed nitrogen are sometimes added during the bioremediation of organic contaminants to ensure that fixed nitrogen availability does not limit the activity of microorganisms involved in remediation transformations (*2*). In metal-contaminated aquifers, stimulating dissimilatory metal reduction using carbon donor amendments has shown promise as an approach to immobilize uranium from the groundwater (*3*−*6*). To date, studies on the influence of nitrogen species on uranium bioremediation have primarily focused on nitrification by ammonium-oxidizing bacteria, the role of nitrate as an electron acceptor for the oxidation of U(IV) to U(VI), or how nitrate serves as an alternative competing electron acceptor for metal-reducing microorganisms (*5, 7*−*9*). There does not appear to have been much investigation as to the influence of natural ammonium availability on the abundance and activity of indigenous anaerobic microorganisms involved in uranium bioremediation processes.

The availability of fixed nitrogen is important to the survival of microorganisms because when it is in limited supply, it is necessary for microorganisms to employ other mechanisms to obtain it or produce it themselves using processes such as nitrogen fixation. One method for elucidating environmental factors that may influence the growth and activity of microorganisms is by quantifying mRNA transcripts for key metabolic genes. Transcript abundance has previously been used to quantify genes involved in remediation processes (*10, 11*) and to diagnose the physiological status of bacteria during in situ bioremediation (*12*−*14*). Recently, this approach has been effective for understanding the central metabolism physiology and the oxidative and heavy metal stress response of *Geobacteraceae* involved in acetate-stimulated U(VI) bioremediation (*13, 15, 16*). Theoretically, nutrient limitations of *Geobacter* species during in situ bioremediation could be assessed by quantifying transcript abundance for genes involved in nitrogen fixation or ammonium acquisition.

The ability of *Geobacter* species to fix atmospheric nitrogen (*17*−*19*) suggests that they should be able to grow in subsurface environments when fixed nitrogen is unavailable. One of the genes that codes for the dinitrogenase protein, NifD, which is involved in nitrogen fixation (*20, 21*), is well-conserved among *Geobacter* species (*22*). It was also shown to be repressed with the addition of ammonium during acetate-stimulated growth in sediments (*18*), suggesting it may be important during growth in the subsurface.

While the uptake of ammonium by *Geobacter* species has not been studied in detail, it has been shown in other bacteria to diffuse across cell membranes at high concentrations and be actively imported at concentrations less than 1 mM (*23*−*26*). Our preliminary analysis of available *Geobacter* species genome sequences indicated that they possess two

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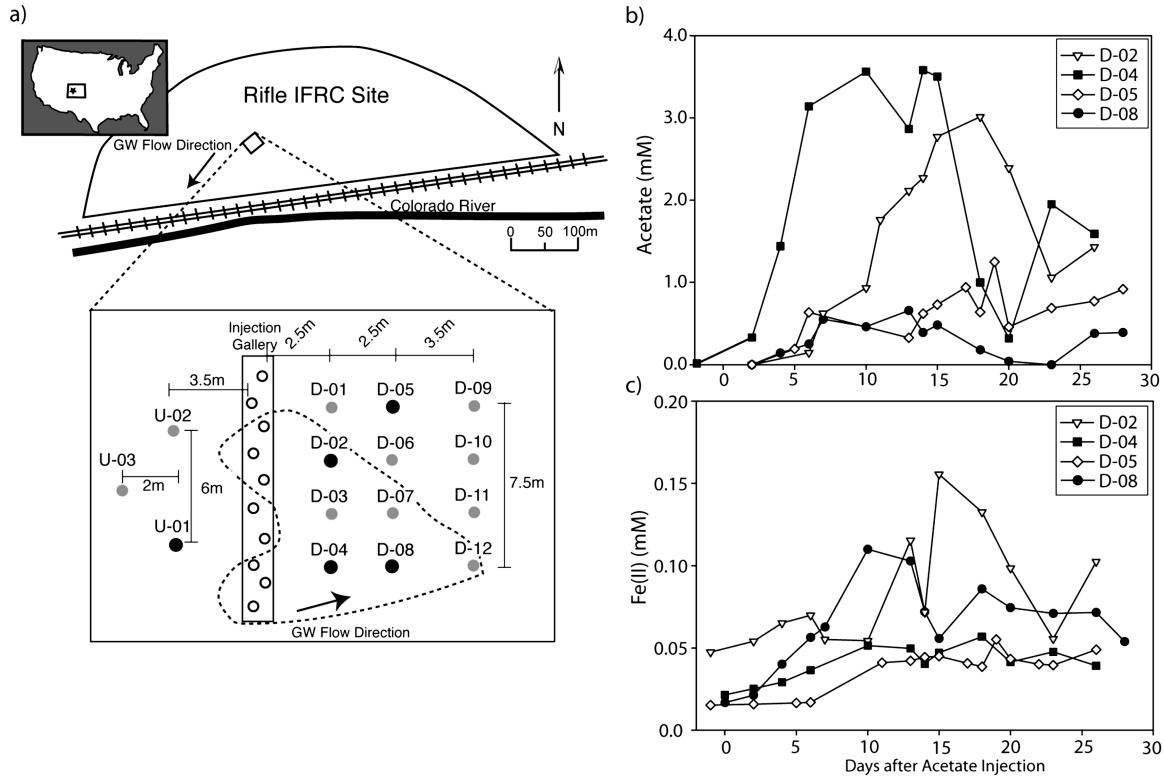

FIGURE 1. (a) Conceptual site layout of the experimental design at Rifle, CO. Monitoring locations discussed in the text are shown in black. The dotted line indicates boring locations where organic matter was observed in soil cores. (b) and (c) Spatiotemporal fluctuations of dissolved acetate and Fe(II) concentrations collected from four locations during groundwater amendment.

putative importers, the ammonium transporter (AmtB) and the Rhesus-family proteins (Rh). The gene that codes for AmtB is present in a wide diversity of bacterial species (*23, 26*), and its protein sequence was highly conserved (>80% similarity) across the available *Geobacter* species genomes.

Because *Geobacter* species can potentially employ genes for both ammonium uptake and nitrogen fixation, we hypothesized that they may be able to out-compete other bacteria without the ability to fix nitrogen under ammonium-limiting conditions during acetate-stimulated U(VI) bioremediation. Therefore, when variable ammonium concentrations were observed prior to acetate amendment across a small experimental plot in a uranium-contaminated aquifer in Rifle, CO, it offered an opportunity to study their in situ response at the field scale. Our results relate the abundance of *amtB* and *nifD* gene transcripts and the bacterial community composition to ammonium levels in the groundwater and its implications for uranium bioremediation.

Methods

Site Hydrogeology. During 2007, a bioremediation experiment was conducted as part of the Rifle Integrated Field Research Challenge (IFRC) challenge of the U.S. Department of Energy (see ref *27* for a review of uranium mill tailings geochemistry and microbiology). The aquifer is located within a flood plain of the Colorado River, with a 7 m thick unconfined sandy gravel alluvium (saturated thickness 2.4 m) underlain by the relatively impermeable Wasatch Formation. An acetate:bromide solution (50 mM:20 mM) mixed with groundwater was injected into the subsurface through an array of injection wells to provide ~5 mM acetate as an electron donor over the course of 30 days. The average velocity during the experiment was 0.5 m/d (hydraulic conductivity 35 m/d, porosity 0.25, and gradient 0.004 m/m), resulting in a 2-pore volume turnover in the monitoring array, which consisted of 12 downgradient, 10 injection, and three upgradient wells (Figure 1a).

Groundwater Sampling and Analysis. Samples for geochemical analysis were collected approximately every two days between August 10 and September 5, 2007. Prior to sampling, the first 12 L of groundwater was purged using a peristaltic pump. After purge, samples were field filtered, using 0.2 μm pore size PTFE (Teflon) filters (Alltech Associates, Inc., Deerfield, IL), and analyzed as follows. Nitrate, nitrite, acetate, and bromide were measured using an ion chromatograph (ICS-1000, Dionex) equipped with a AS22 column and a carbonate−bicarbonate eluent (4.5 mM/1.4 mM). Ammonium was measured using an indophenol−hypochlorite method (*28*) and a Genesys 6 spectophotometer (Thermospectronic, Madison, WI). U(VI) was measured using a kinetic phosphorescence analyzer (*29*), and Fe(II) samples were preserved with 10 M HCl and measured using the Ferrozine method (*30*).

Approximately 2 L of groundwater was filtered through 0.2 μm pore-size Sterivex filters (Millipore Corporation, Bedford, MA) for 16S rRNA microbial community analysis. Samples for mRNA abundance were obtained by concentrating 10 L of groundwater on a 0.2 μm, 293 mm diameter Supor membrane filter (Pall Corporation, East Hills, NY). Filters were removed into whirl paks (Nasco, Fort Atkinson, WI), flash frozen in an ethanol−dry ice bath, and stored at −80 °C until extraction. A total of 6 (D-05), 8 (D-08), and 9 (D-02 and D-04) samples that spanned the experimental period were analyzed for mRNA abundance.

Nucleic Acid Extractions and Clone Library Construction. Nucleic acids for microbial community analysis were extracted from filters using the FastDNA SPIN kit (Bio 101, Inc., Carlsbad, CA). The 16S rRNA gene was amplified with bacterial primers 8F and 519R (*31, 32*), using PCR reagents and cycling parameters described previously (*3*). Polymerase chain reaction (PCR) products were cloned into the TOPO TA vector pCR 2.1 and chemically competent TOP10 cells (Invitrogen, Carlsbad, CA). Inserts from at least 90 clones for each library were amplified with the M13F primer and

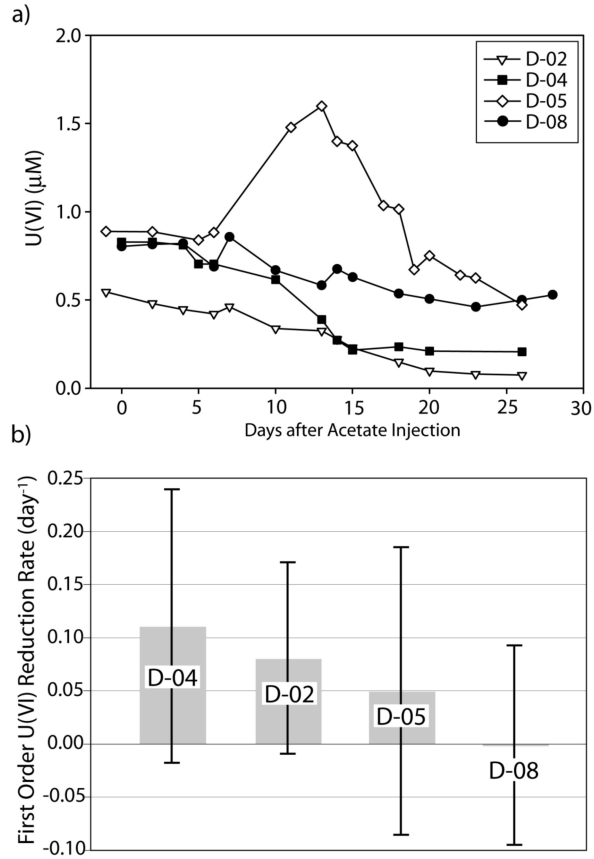

FIGURE 2. (a) Spatiotemporal changes in dissolved U(VI) concentrations for four monitoring locations at the Rifle, CO, aquifer during acetate amendment. (b) Average (bars ± std) first-order U(VI) reduction rates calculated between subsequent sampling events during the amendment experiment.

sequenced at the Univeristy of Massachusetts (UMass), Amherst Sequencing Facility. Alignments were constructed using the Lasergene software (DNASTAR, Inc., Madison, WI) and clone library sequences were compared to those compiled in GenBank using BLAST (*33*). Phylogenetic analysis was conducted using the neighbor-joining method. Sequences for phylogenetic analysis were deposited into GenBank under accession numbers FJ875700-FJ875723.

Filters for mRNA abundance were crushed with liquid nitrogen, resuspended in 800 μL of TPE buffer (100 mM Tris-HCl, 100 mM KH_2PO_4, 10 mM EDTA; pH 8.0), and extracted using a modified phenol−chloroform method as previously described (*16, 18*). Total RNA was separated from other nucleic acids with the Rneasy RNA cleanup kit (Qiagen) and then treated with Dnase (Ambion, Austin, TX). RNA quality was visualized on a 1% agarose gel and then quantified for concentration and purity using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE).

Quantification of mRNA Gene Transcripts. Degenerate primers designed to amplify *Geobacteraceae* genes were developed by constructing alignments from the following seven genomes: *Geobacter uraniireducens*, *G. sulfurreducens* (*34*), *G. lovleyi*, *G. bemijensis*, *G. metallireducens*, *Pelobacter carbinolicus*, *and P. propionicus*, which are available from the U.S. Department of Energy (DOE) Joint Genome Institute Web site (www.jgi.doe.gov). Alignments were developed for an ammonium transporter Geo*amtB*649F/1298R 5′-ATGTTTGCCATGATTACCGT-3′ and 5′-AAGGCRTCVAAGGGARTCGTC-3′, nitrogenase Geo*nifD*225F/560R (*18*), and a housekeeping gene (*recA*) designated Geo*recA*283F/730R 5′-GAGCATGCHCTSGAYATCGGCTA-3′ and 5′-ACCTTGTTCTTMACHTGYTT-3′. Genes were amplified from the environment using reagents and cycling parameters described previously (*22*).

PCR products from *amtB*, *nifD*, and *recA* genes were cloned and sequenced using the methods described above. At least 40 inserts for each library were sequenced to verify specificity. Primers for RT-qPCR that targeted ~150 bp regions were developed from degenerate alignments as described previously (*22*) and included Geo*amtB*312F/508R 5′-AAGCCGAACCAGCCGAAC-3′ and 5′-ACGGCTGGCTCTTCAAGATG-3′, Geo*nifD*58F/242R 5′-ATTTTCTCCAGCTCGCCGTC-3′ and 5′-AACGACACCATCCGCGACC-3′, and Geo*recA* 147F/292R 5′-ACTCCGTCGCCGCCCTG-3′ and 5′-TGATGAAGATGACGCAGCAGT-3′.

Sample cDNA was generated from mRNA transcripts with the DuraScript Enhanced Avian RT synthesis kit (Sigma Aldrich). Transcript abundance was quantified using the Applied Biosystems 7500 Real-Time PCR system (PE Biosystems, Foster City, CA). Reactions (25 μL total volume) consisted of 12.5 μL of 2x Power SYBR green master mix (Applied Biosystems), 5 μL of 1:10 diluted template cDNA, and 200 pmol primers. Thermal cycling parameters consisted of an activation step (50 °C for 2 min) and a denaturation step (95 °C for 10 min), followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. Standard curves covering 8 orders of magnitude (10^1-10^8) were developed using genomic DNA amplified using degenerate *nifD, amtB,* or *recA* primers that covered the RT-qPCR insert. Standards were run concurrently with cDNA samples, along with two negative controls: RNA template not subjected to reverse transcription and reaction solution without template.

Statistical Methods. Data distributions were tested for normality using the Shaprio−Wilkes test. Ammonium and U(VI) data met normality assumptions ($W > 0.05$), however, expression data did not. Therefore, one-way analysis of variance (ANOVA) was used to compare geochemical data, while the Kruskal−Wallis method was used to compare transcript abundance. Where statistical differences were observed among monitoring locations at the $\alpha = 0.05$ level, pairwise comparisons were made using either the *t*-test or Wilcoxen test. Temporal trends were tested using Pearson's correlation with a $\alpha = 0.05$ significance level. Analyses were conducted in JMP version 5.1 (SAS Institute, Cary, NC).

Rates of U(VI) reduction were computed between subsequent sampling events using a first-order reaction rate equation, $C = C_0e^{-kt}$, where C_0 and C represent initial and final U(VI) concentrations, respectively, t represents the time between samples, and k is the first order reaction rate.

Results

Acetate, Bromide, Fe(II), U(VI), and Ammonium Dynamics. Monitoring wells D-02, D-04, D-05, and D-08 (Figure 1a) were selected for detailed time course analysis because initial sampling demonstrated that they represented ammonium concentrations that spanned several orders of magnitude. Acetate appearance in the wells suggested that groundwater flow and microbial consumption were not uniform across the plot (Figure 1b). Acetate arrival was observed earlier at D-04 than at the other three locations, and levels reached higher concentrations in the first row of wells (D-02 and D-04) than in D-05 and D-08 located in the second flow transect (Figure 1b). After 10 days of injection, acetate additions were stopped, and the injection zone was flushed with acetate-free groundwater for seven days before amendments resumed for an additional 13 days. The temporary halt in amendment was reflected in decreasing acetate concentrations at all four wells between day 15 and 23. Once amendments resumed, acetate again increased in all four locations (Figure 1b).

Bromide concentrations generally followed acetate fluctuations, except at location D-05, where higher bromide

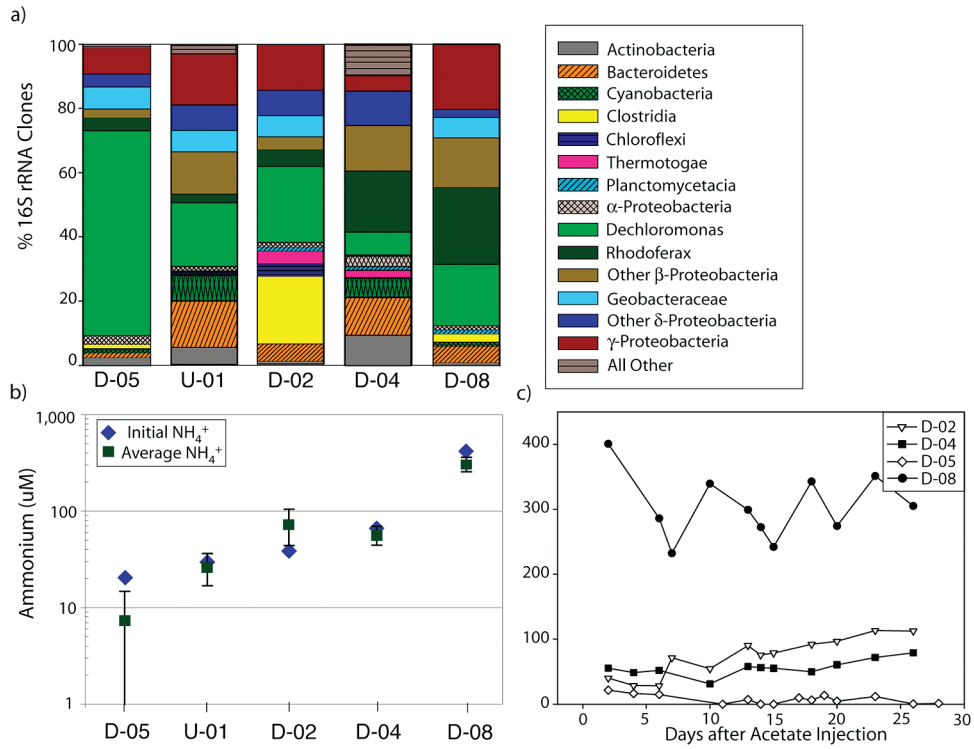

FIGURE 3. (a) Microbial community composition based on 16S rRNA clone sequences extracted from the groundwater at five monitoring locations before acetate injection began. (b) Ammonium concentrations observed prior to acetate injection (initial, blue ♦) and average concentrations (green ■ ± std) for locations corresponding to 16S rDNA clone libraries. (c) Spatiotemporal ammonium fluctuations during acetate amendment.

concentrations relative to acetate concentrations suggested that relatively more acetate had been consumed at this location (Figure S1 of the Supporting Information). As expected from previous studies (*3, 29*), acetate amendment stimulated dissimilatory metal reduction as evidenced by an accumulation of dissolved Fe(II) (Figure 1c) and a loss of U(VI) at all four locations (Figure 2a). U(VI) reduction was stimulated to the lowest concentrations in D-02, where some of the higher acetate concentrations were delivered (Figure 2a).

Significant differences were observed in ammonium levels across the experimental plot, with concentrations varying by 2-orders of magnitude between the wells (Figure 3b). Well D-08 had significantly higher ammonium levels, whereas D-05 had significantly lower levels than those of all other locations (p-value < 0.02) (Figure 3b). While locations D-02 and D-04 showed increases in ammonium during the amendment, no significant trends were observed (Figure 3c). Consistent with the expected lack of nitrate and nitrite under dissimilatory metal-reducing conditions, other sources of inorganic nitrogen were below detection (<10 μM) in all wells throughout the experiment.

Microbial Community Dynamics. Differences in groundwater ammonium concentrations may have been one factor contributing to variations in the microbial community composition before and during acetate amendment. Analysis of 16S rRNA gene sequences extracted from the groundwater indicated that microorganisms within the β and δ-proteobacteria classes were among the most abundant prior to and during the iron-reducing phase of acetate amendment (Figure 3a). The 16S rDNA clone sequences related to *Dechloromonas* species (88−99% similarity) were abundant at all five sites prior to the addition of acetate to the groundwater (Figure S3 of the Supporting Information) and represented more than 60% of sequences in D-05, which had the lowest ammonium levels (Figure 3a). Temporal samples from three locations that spanned low, intermediate, and high ammonium levels showed a decline in relative abundance of *Dechloromonas* species following acetate additions by day nine and a recurrence after 18 days in D-08, which had the highest ammonium levels (Figure S2 of the Supporting Information).

Relative proportions of *Geobacter* and *Rhodoferax* species were comparable at the sites with lower ammonium levels prior to acetate addition, including D-05, U-01, and D-02 (Figure 3a). However, at locations with higher initial ammonium, including D-04 and D-08, *Rhodoferax* species (91−98% similarity) were more abundant prior to acetate injection (Figure 3a and Figure S3 of the Supporting Information) but declined in relative abundance after a week of acetate amendment (Figure S2 of the Supporting Information). As expected from previous studies (*3*−*6*), *Geobacter* species (88−98% similarity) became the predominant community members after acetate amendment, regardless of ammonium concentrations (Figure S2 of the Supporting Information). Consistent with studies of other uranium contaminated sites (*9, 18, 35*), clones with high similarity to other β-proteobacteria, including *Nitrosomonadaceae, Comamonadaceae*, and *Oxalobacteraceae* families were detected in 2−10% of clone sequences prior to acetate amendment (Figure 3a).

In Situ Expression of Ammonium Transporter and Nitrogen Fixation Genes in *Geobacter* Species. In order to learn more about the in situ metabolism of subsurface *Geobacter* species, the number of transcripts for the ammonium transporter gene, *amtB*, and the nitrogen fixation gene, *nifD*, was quantified during the biostimulation experiment. In general, the number of *nifD* transcripts was slightly less abundant than those for the housekeeping gene *recA*, resulting in median *nifD* and *recA* levels between 0.28 and 0.43 (Figure 4a). Overall *nifD* abundance levels were not statistically different among the four monitoring locations (p-value = 0.43) (Figure 4a). A previous study (*18*) demonstrated that *Geobacter* species living in sediments have

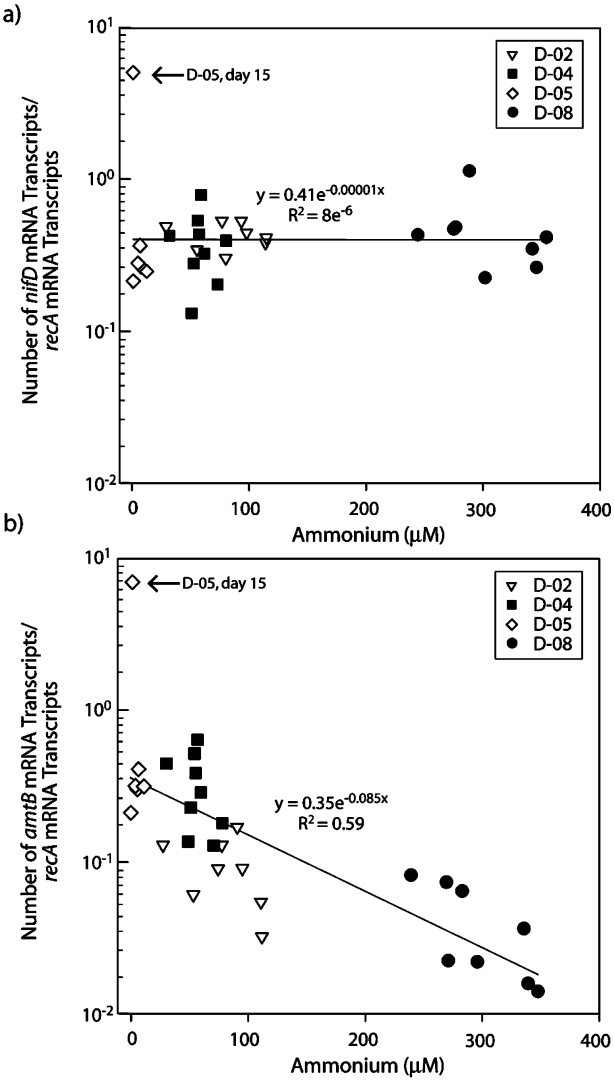

FIGURE 4. (a) Relationships between ammonium concentrations and transcript abundance of *Geobacteraceae nifD* relative to *recA* genes and (b) *amtB* relative to *recA* in four monitoring locations during an acetate amended bioremediation experiment. Points represent the mean of triplicate RT-qPCR reactions. Lines represent a standard least-squares best-fit regression and its coefficient of determination (R^2).

comparable *nifD* and *recA* transcript levels when nitrogen fixation is repressed with the addition of 100 μM ammonium concentrations. Thus, the relatively low abundance of *Geobacter nifD* transcripts detected in the groundwater indicated that ammonium levels were high enough to sustain the population during U(VI) bioremediation.

In contrast to *nifD* trends, transcript abundance for the ammonium transporter gene, *amtB*, was statistically different among the four monitoring locations (p-value < 0.001), showing a significant inverse correlation between *amtB* in the subsurface *Geobacter* community and ammonium availability (p-value < 0.001) (Figure 4b). Median *amtB* levels quantified in locations D-04 and D-05 were 10-fold higher than transcript abundance levels recorded for D-08 and 3-fold higher than those for D-02 (p-value < 0.002) (Figure 4b). Even among locations with higher levels of ammonium, median *amtB* abundance levels in D-02 were about 2-fold higher than those in D-08 (p-value = 0.02).

During periods of variations in the ammonium concentration, declines in ammonium availability often resulted in increased abundance of *amtB* and *nifD* transcripts and vice versa (Figure S4 of the Supporting Information). This relationship was particularly evident for *amtB* abundance in D-08, where a significant inverse correlation was observed ($\rho = -0.75$, p-value = 0.03). Interestingly, the relationship between *amtB* and *nifD* transcript abundance became stronger as ammonium concentrations decreased, with strong correlations observed in D-04 and D-05 ($\rho > 0.92$, p-value < 0.001) (Figure S4 of the Supporting Information).

A further example of this paired *nifD* and *amtB* response occurred at location D-05 with the depletion of ammonium on day 15 (Figure 3a,b and Figure S4 of the Supporting Information). Levels of *nifD* and *amtB* increased more than 20-fold in D-05 when ammonium was below detection (detection limit approximately 2 μM), while *recA* levels remained relatively unchanged (Figure S4 of the Supporting Information). Transcript abundance of *nifD* and *amtB* decreased again when small amounts of ammonium subsequently became available in the groundwater. The relative transcript abundance of *nifD* and *recA* on day 15 was similar to what was previously reported for *Geobacter* species under nitrogen-fixing conditions (*18*) and indicates a switch in physiology to enhance nitrogen fixation.

Rates of U(VI) Reduction. Increased availability of fixed nitrogen in the form of ammonium did not appear to stimulate higher U(VI) removal during biostimulation. In contrast, the highest observed ammonium concentrations were associated with the lowest average U(VI) reduction rate in monitoring well D-08 (Figure 2b). The mean U(VI) reduction rate in D-08 averaged less than zero over the 30 day experiment, significantly lower than the average rate of 0.11 day^{-1} observed in D-04 (p-value < 0.05). Concentrations of U(VI) in D-05 appeared to rapidly decrease after day 15, which corresponded to a 20-fold increase in *nifD* and *amtB* transcript abundance (Figure 2a and Figure S3 of the Supporting Information). However, U(VI) reduction rates calculated over the 30 day period for D-05 and rates calculated after day 15 were still lower than the average U(VI) reduction rate observed in D-04 (Figure 2b), suggesting the lack of ammonium alone in D-05 was probably not responsible for decreasing U(VI) during the second half of the experiment.

Discussion

This study revealed that there was significant heterogeneity in ammonium concentrations within the monitoring array of this uranium bioremediation field study. Variations in groundwater ammonium concentrations are believed to be related to differences in organic matter and solid phase carbon content across the D-05 to D-08 transect (*36*). Sediment cores extracted during drilling of D-08 had visible layers of darkened organic materials dispersed within zones of clay, gravely silts, and sands. Similar zones of organic matter were observed to a lesser degree in cores collected from D-02 and D-04 (Figure 1a). In contrast, neither organic-rich nor clay-rich sediments were observed at D-05, which contained only slight variations in gravel and silt soil units. Sediment-derived organic matter near D-08 probably resulted in forms of organic nitrogen in equilibrium with the groundwater. Heterogeneity of ammonium concentration may also be reflective of differential preservation of ammonium, resulting from milling operations (*27*).

Ammonium availability and qualitative observations of solid-phase organic matter content appeared to influence the microbial community composition, in particular the β-proteobacteria, who are thought to be important agents of nitrogen cycling in other uranium-contaminated sites (*6, 9, 18, 35*) prior to the addition of acetate to stimulate dissimilatory metal reduction. *Dechloromonas* species were observed in considerable abundance in 16S rDNA clone libraries, regardless of ammonium concentrations. Clone sequences dominated by *Dechloromonas denitrificans* were

observed concurrent with low ammonium in locations D-05 and U-01, whereas clone sequences closely related to more than half a dozen different *Dechloromonas*, and *Rhodoferax* species were observed in monitoring wells with higher initial ammonium levels. Certain *Dechloromonas* species are known denitrifiers (*37*) and can couple acetate oxidation to nitrate and/or nitrite reduction. In addition, they have previously been detected at other uranium-contaminated sites containing elevated nitrate concentrations (*6, 35*). Their initial prevalence prior to acetate amendment combined with the unexplained phenomenon of increasing U(VI) concentrations during the first week of injection in monitoring well D-05 suggests that they may play an important role in possibly inhibiting U(VI) reduction during the transition to a dissimilatory Fe(III)-reducing environment.

Geobacter and *Rhodoferax* species were the most abundant microorganisms detected in this study that are known to couple the oxidation of acetate with the reduction of metals (*38, 39*). However, known *Geobacter* and *Rhodoferax* species differ in their ability to fix atmospheric nitrogen. For example, available *Geobacter* species genomes contain sequences for proteins involved in nitrogen fixation (*22*), and this capacity has been verified in *G. metallireducens* (*17*). In contrast, no nitrogen fixation genes were found in a search of the complete genome sequence of *R. ferrireducens*. These results suggest that prior to acetate amendment, ammonium may influence the ability of *Rhodoferax* species to compete with other bacteria in metal-reducing environments when levels of fixed sources of nitrogen are lower. Regardless of ammonium concentrations, the addition of acetate resulted in a predominance of *Geobacter* species during the active phase of U(VI) reduction, suggesting that other physiological factors such as the ability to grow rapidly via acetate oxidation coupled to Fe(III) reduction, are also important in the subsurface environment. While this community survey is insufficiently detailed to directly link ammonium levels to bacterial composition, this interesting dynamic between *Dechloromonas*, *Geobacter*, and *Rhodoferax* species and available nutrients has important implications for U(VI) bioremediation and warrants further investigation.

Analysis of transcript abundance for the ammonium transporter gene, *amtB*, and the nitrogen fixation gene, *nifD* suggested that with the exception of one time-point in one of the four monitoring locations, ammonium uptake rather than nitrogen fixation was the primary source of nitrogen for the growth of *Geobacter* species during in situ uranium bioremediation. Ammonium concentrations greater than 5 μM are apparently high enough to repress *nifD* expression in the environment. As would be expected from previous studies (*18, 19*), expression of *nifD* and presumably other genes involved in nitrogen fixation appeared to be closely regulated in the subsurface.

The observed inverse relationship between *amtB* transcript abundance and ammonium levels is consistent with the current understanding of *amtB* transport patterns observed in other bacteria (*23, 24, 26*) and appears to be an adaptive response, with *Geobacters* reducing the energy invested in transporter synthesis when ammonium is plentiful. However, when ammonium concentrations are low or at levels below about 50 μM in the environment, the production of more transporters is essential in order to enhance the capacity for ammonium uptake. Thus, tracking the expression of *nifD* and particularly *amtB* appears to be a useful approach to monitoring the nitrogen-related physiological status of *Geobacter* species in subsurface environments.

There was no clear relationship between ammonium availability and the extent of U(VI) reduction in the field experiment. In fact, the location with the highest mean U(VI) reduction rate was D-04, which had lower accumulated Fe(II) and ammonium concentrations that ranged between 30 to 80 μM during amendment. Interestingly, transcript abundance of *amtB* and *nifD* in D-04 were statistically indistinct from D-05, which had the lowest observed ammonium levels. Acetate amendment was also observed first and reached higher concentrations in D-04. Although ammonium has been added to stimulate bioremediation of organics contaminants (*2*), a previous study showed the addition of ammonium to *Geobacter* species growing in sediments did not stimulate Fe(III) reduction (*18*). Adding ammonium might not stimulate rates of dissimilatory metal reduction because when *Geobacter* species have to fix nitrogen, higher rates of respiration are required, and hence, more metal reduction occurs in order to generate the additional reducing equivalents and ATP that nitrogen fixation requires. As a result, U(VI) reduction rates in D-04 could be a reflection of concentrations of acetate delivered to this area or the general level of bacterial growth and activity relative to other monitoring locations rather than the ability of *Geobacter* species to out-compete other species under conditions of limited nitrogen. Such considerations emphasize the need for a comprehensive analysis of the physiological status of microorganisms involved in bioremediation processes via large-scale analysis of gene expression or other approaches.

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Supporting Information Available

Spatiotemporal trends in bromide concentrations and ratios of bromide relative to acetate levels (Figure S1), 16S rRNA microbial community composition and phylogenetic relationships (Figures S2 and S3, respectively), and spatiotemporal *nifD*, *amtB*, and *recA* transcript expression patterns (Figure S4). This information is available free of charge via the Internet at http://pubs.acs.org.

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