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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Abstract

The impact of ammonium availability on microbial community structure and the physiological status and activity of *Geobacter* species during *in situ* bioremediation of uranium-contaminated groundwater was evaluated. Ammonium concentrations varied by as much as two orders of magnitude (<4 to 400 µM) across the study site. Analysis of 16S rRNA gene sequences suggested that ammonium influenced the composition of the microbial community prior to acetate addition with *Rhodoferax* species predominating over *Geobacter* species at the site with the highest ammonium, and *Dechloromonas* species dominating at sites 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 the concentration of acetate that was delivered to each location rather than the amount of ammonium available in the groundwater. In situ mRNA transcript abundance of the nitrogen fixation gene, *nifD*, and the ammonium importer gene, *amtB*, in *Geobacter* species indicated that ammonium was the primary source of nitrogen during in situ uranium reduction, and that the abundance of *amtB* transcripts was inversely correlated to ammonium levels across all sites examined. These results suggest that *nifD* and *amtB* expression by subsurface *Geobacter* species are closely regulated in response to ammonium availability to ensure an adequate supply of nitrogen while conserving cell resources. Thus, quantifying *nifD* and *amtB* expression appears to be a useful approach for monitoring the nitrogen-related physiological status of Geobacter species in subsurface environments during bioremediation. This study also emphasizes the need for more detailed analysis of geochemical/physiological interactions at the field scale, in order to adequately model subsurface microbial processes.

Introduction

Rational optimization of subsurface bioremediation strategies requires an understanding of what factors might influence the activity of the microorganisms involved in bioremediation 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 in order to ensure that the availability of fixed nitrogen 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 the role of nitrate as an electron acceptor for the oxidation of U(IV) to U(VI), or serving as an alternative, competing electron acceptor for metal-reducing microorganisms (5, 7, 8). There does not appear to have been much investigation as to the influence of natural ammonium availability on the abundance and activity of indigenous 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 (9, 10), and to diagnose the physiological status of bacteria during *in situ* bioremediation (11-13). Recently, this approach has been effective for understanding the central metabolism physiology, oxidative, and heavy metal stress response of *Geobacteraceae* involved in acetate-stimulated U(VI) bioremediation (12, 14, 15). Theoretically, limitations due to fixed nitrogen during *in situ* bioremediation could be assessed by quantifying transcript abundance for ammonium importers, or for genes involved in nitrogen fixation.

The ability of *Geobacter* species to fix atmospheric nitrogen (16-18) 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 (19, 20), is well conserved among *Geobacter* species (21). It was also shown to be repressed with the addition of ammonium during acetate-stimulated growth in sediments (17), suggesting it may be important during growth in subsurface sediments.

While the uptake of ammonium by *Geobacter* species has not been studied in detail, it has been shown in other bacteria to be passively transported across cell membranes at high concentrations, and actively transported at concentrations less than 1 mM (22-25). Our preliminary analysis of available *Geobacter* species genome sequences indicated that they possess two putative ammonium transporters, 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 (22, 25), 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 we observed variable ammonium concentrations across a small experimental plot in a uranium-contaminated aquifer, 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 microbial community composition to ammonium levels in the groundwater, and its implications for uranium bioremediation.

Methods

Site Hydrogeology

During 2007, a bioremediation experiment was conducted near a former ore processing facility as part of the Uranium Mill Tailings Remedial Action (UMTRA) program and Rifle Integrated Field Research (IFC) challenge of the U.S. Department of Energy. The aquifer is located within the 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 to provide ~5 mM acetate as an electron donor out the course of 30 days. The average linear velocity during the experiment was 0.9 m/d (hydraulic conductivity 35 m/d; porosity 0.19; gradient 0.005 m/m) (3), resulting in about a 2-pore volume turnover in the experimental 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 from groundwater wells approximately every other day between August 10th through September 5th, 2008. Prior to sampling, the first 12 l of groundwater was purged through dedicated well tubing connected to a peristaltic pump. After purge, geochemical samples were collected and 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 concentrations were measured using an ion chromatograph (ICS-1000, Dionex) equipped with a AS22 column and a carbonate/bicarbonate eluent (4.5mM/1.4 mM). Ammonium was measured using an indophenol-hypochlorite method (26) and a Genesys 6 spectophotometer (Thermospectronic, Madison, WI). U(VI) was measured using a kinetic phosphorescence analyzer as described by others (27), and Fe(II) samples were preserved with 10 M HCl and measured using the Ferrozine method (28). Unless UMASS did its own Fe(II) analysis, the method we used for Fe(II) analysis during Winchester was the phenanthroline method. I wouldn't worry about correcting this at this point, but I include it to be thorough. Reference: Standard methods for the examination of water and wastewater, American Public Health Association, American Water Works Association, Water Pollution Control Federation; Mary Ann H. Franson, managing editor; 17th ed, Washington, DC; American Public Health Association, 1989.

Approximately 2 1 groundwater was then filtered through 0.2 µm pore-size Sterivex filters (Millipore Corporation, Bedford, MA) for analysis of 16S rRNA gene sequences. 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). Nucleic acid filters were removed into sterile whirl pak bags (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 analysis.

Nucleic Acid Extractions for Microbial Community Analysis and Transcript Abundance

Microbial community analysis of groundwater was conducted by extracting nucleic acids from filters with the FastDNA SPIN kit (Bio 101 Inc., Carlsbad, CA). The 16S rRNA gene was

amplified with bacterial primers 8F and 519R (29, 30) using PCR reagent mixtures and cycling parameters described previously (3). 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 sequenced at the UMASS, Amherst Sequencing Facility. Sequences were compared to those compiled in GenBank using the BLAST suite of programs (31).

Filters for mRNA transcript analysis were crushed with liquid nitrogen, separated into 2 ml tubes, re-suspended in 800 µl of TPE buffer (100 mM Tris-HCl, 100 mM KH₂PO₄, 10 mM EDTA; pH 8.0), and nucleic acids were extracted using a modified phenol-chloroform extraction method as previously described (*15, 17*). The total RNA was separated from other nucleic acids with the Rneasy RNA cleanup kit (Qiagen) then treated with DNA-free Dnase (Ambion, Austin, TX). RNA quality was visualized on a 1% agarose gel, 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* (32), *G. lovleyi, G. bemijensis, G. metallireducens, Pelobacter carbinolicus, and P. propionicus,* available on the DOE Joint Genome Institute website (www.jgi.doe.gov). Alignments were developed for three genes, including an ammonium transporter (*amtB*), designated Geo*amtB*649F/1298R 5'-ATGTTTGCCATGATTACCGT-3' and 5'-AAGGCRTCVAAGGGARTCGTC-3'; nitrogenase (*nifD*), designated Geo*nifD*225F/560R described in previous work (17); and a housekeeping gene (*recA*) designated Geo*recA*283F/730R

5'-GAGCATGCHCTSGAYATCGGC-3' and 5'-ACCTTGTTCTTMACHTGYTT-3'. Genes were amplified from the environment using mixture concentrations and cycling parameters described previously (21).

PCR products from amtB, nifD, and recA genes were cloned and sequenced using methods described above for 16S rRNA clone libraries. At least 40 inserts for each library were sequenced to verify gene specificity, and alignments were constructed using the Lasergene software (DNASTAR, Inc, Madison, WI). Primers for RT-qPCR that targeted ~150 bp regions were developed from degenerate alignments as described previously (21). Primer pairs include Geo*amtB*312F/508R 5'-AAGCCGAACCAGCCGAAC-3' and 5'-ACGGCTGGCTCTTCAAGATG-3'; GeonifD58F/242R 5'-ATTTTCTCCAGCTCGCCGTC-3' 5'-AACGACACCATCCGCGACC-3', and GeorecA 147F/292R 5'and ACTCCGTCGCCGCCCTG-3' and 5'-TGATGAAGATGACGCAGCAGT-3'.

Sample cDNA was generated from mRNA transcripts with the DuraScript Enhanced Avian RT single-strand synthesis kit (Sigma Aldrich). The number of mRNA transcripts was quantified using the Applied Biosystems 7500 Real-Time PCR system (PE Biosystems, Foster City, CA). Reactions (25 μ l total volume) consisted of 12.5 μ l 2 x POWR SYBR green master mix (Applied Biosystems), 5 μ l of 1:10 diluted template cDNA, and 200 pmol of the appropriate primer pair. The thermal cycling parameters consisted of an activation step of 50°C for 2 min, a denaturation step of 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Standard curves that covered 8-orders of magnitude (10¹-10⁸) were developed using serially-diluted genomic DNA extracted from environment samples and amplified using degenerate *nifD*, *amtB* or *nifD* primers that covered the RT-qPCR insert. Standard curves were run concurrently

with cDNA samples along with two types of negative controls: RNA template that had not been subjected to reverse transcription, and reaction solution without template.

Statistical Methods

Data distributions were tested for normality prior to statistical analysis using the Shaprio-Wilkes test. Ammonium and U(VI) concentrations 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 non-parametric Kruskal-Wallis method was used to compare transcript abundances. Where statistical differences were observed amongst monitoring locations at the α =0.05 level, pairwise comparisons were made using either the *t*-test or Wilcoxen test. Temporal trends were tested using pairwise comparisons with a significance level set at α =0.05. Statistical 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 for the two sampling events, *t* represents the time between samples, and *k* is the calculated 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 amendment 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 the other three locations and levels reached higher maximum concentrations in the first row of monitoring 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 thirteen days. The temporary cease in amendment addition was reflected in decreasing acetate concentrations at all four wells between day 15 and 23. Once amendments resumed, acetate again increased in all four monitoring locations (Figure 1b).

Bromide concentrations generally followed acetate fluctuations, except at location D-05, where higher bromide relative to acetate concentrations suggested that relatively more acetate was being consumed at this location (Figure S1). As expected from previous studies (*3*, *27*), the 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). During the first 30 days of the experiment, U(VI) reduction was stimulated to the lowest concentrations in D-02, located in the first transect downgradient from the injection gallery, where some of the higher acetate concentrations were delivered (Figure 2a).

Significant differences were observed in ammonium levels across the small experimental plot, with concentrations varying by over 2 orders of magnitude between the monitoring locations (Figure 3b). Well D-08 had significantly higher ammonium levels whereas D-05 had significantly lower levels than all other locations (p<0.02) (Figure 3b). While locations D-02 and D-04 showed slight 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 ($<10 \mu$ M) in all wells throughout the experiment.

Microbial community dynamics

Differences in ammonium concentrations in the groundwater 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 most closely related to *Geobacteraceae*, *Rhodoferax* and *Dechloromonas* species were among the most abundant prior to and during the iron-reducing phase of acetate injection (Figure 3a). 16S clones sequences related to *Dechloromonas* species (88-99% similarity) were abundant at all five sites prior to the addition of acetate to the groundwater, representing more than 60% of sequences in D-05, which had the lowest ammonium levels. Temporal samples from three locations that spanned low, intermediate, and higher ammonium levels showed a decline in relative abundance of *Dechloromonas* species following acetate additions by day nine (Figure S2a), and a recurrence after 18 days in D-08, which had the highest ammonium levels (Figure S2b).

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 about 4 fold more abundant prior to acetate injection (Figure 3a-b), but declined in relative abundance in by day 9 (Figure S2a). As expected from previous studies (*3-6*), upon acetate amendment *Geobacter* species (88-98% similarity) became the predominant community members regardless of ammonium concentrations (Figure S2).

In Situ Expression of Ammonium Transporter and Nitrogen Fixation Genes in Geobacter species In order to learn more about the *in situ* metabolism of the *Geobacter* species during biostimulation, the number of transcripts for the ammonium transporter gene, *amtB*, and the nitrogen fixation gene, *nifD*, in the subsurface *Geobacter* community was quantified. In general, the number of *nifD* transcripts was slightly less abundant than those for the housekeeping gene *recA*, resulting in median *nifD/recA* levels between 0.28 and 0.43 (Figure 4a). Overall *nifD* abundance levels were not statistically different among the four monitoring locations (Kruskal Wallis, p=0.43) (Figure 4a). A previous study (*17*) demonstrated that *Geobacter* species living in sediments have comparable *nifD* and *recA* transcript levels when nitrogen fixation is repressed with the addition of environmentally relevant concentrations (100 μ M) of ammonium. Thus, the relatively low abundance of *Geobacter nifD* transcripts detected in the groundwater indicated that ammonium concentrations were high enough to sustain the *Geobacter* population during *in situ* uranium bioremediation.

In contrast to the overall *nifD* trends, transcript abundance for the ammonium uptake gene, *amtB*, were statistically different amongst the four monitoring locations (p<0.001), showing an apparent inverse correlation between *amtB* in the subsurface *Geobacter* community and ammonium availability (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 D-02 (p<0.002) (Figure 4b). Even between locations with higher levels of ammonium, median *amtB* abundance levels in D-02 were ca. 2-fold higher than in D-08 (p=0.02).

During periods of substantial variations in the ammonium concentration, declines in ammonium availability often resulted in increased abundance of *amtB* and *nifD* transcripts and

vice versa (Figure S3). This relationship was particularly evident for *amtB* abundance in D-08, where a significant inverse correlation was observed (ρ =-0.75, p-value=0.03). Interestingly, the relationship between *amtB* and *nifD* transcript abundance became stronger as ammonium concentrations decreased, with significant correlations observed in both D-04 and D-05 (ρ >0.92, p-values<0.001) (Figure S3).

A further example of this paired *nifD* and *amtB* response occurred at location D-05 with the depletion of ammonium on day 15 (Figures 3a-b and S3). Levels of both *nifD* and *amtB* increased more than 20-fold in D-05 when ammonium was below detection (detection limit approximately 2 μ M) while *recA* levels remained relatively unchanged (Figure S3). Transcript abundance of both *nifD* and *amtB* decreased again when small amounts of ammonium (4-13 μ M) 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 (*17*), and indicates a switch in their 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). Mean U(VI) reduction rates in D-08 averaged less than zero during the 30-day experiment, and were significantly lower than the average rate of 0.11 day⁻¹ observed in D-04 (p<0.05). Concentrations of U(VI) in monitoring location D-05 appeared to drastically decrease after day 15, which corresponded to a 20-fold increase in both *nifD* and *amtB* transcript

abundance (Figures 2b and S3). 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 that the lack of ammonium alone in D-05 was probably not responsible for the decrease in U(VI) concentrations during the second half of the experiment.

Discussion

This study revealed that there was remarkable heterogeneity in ammonium concentrations within the relatively small treatment plot 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 (*33*). 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. Decaying organic materials near D-08 probably resulted in sediment-bound forms of organic nitrogen that are in equilibrium concentration with the surrounding groundwater.

Ammonium availability and differences in solid-phase carbon appeared to influence the composition of the subsurface microbial community prior to the addition of acetate to stimulate dissimilatory metal reduction. *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 (*34, 35*). 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 (21), and this capacity has been verified in *G. metallireducens* (16). In contrast, no nitrogen-fixation genes were found in a search of the complete genome sequence of *R. ferrireducens*. These results suggest that ammonium may influence the ability of *Rhodoferax* species to compete with other bacteria in metal-reducing environments, and may be why clone sequences closely related to *Rhodoferax* species have not been specifically reported in previous Rifle studies. 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.

Dechloromonas species were observed in considerable abundance in 16S 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 species were observed in monitoring wells with higher initial ammonium levels. Some Dechloromonas species are known denitrifiers (36, 37) and can couple acetate oxidation to nitrate and/or nitrite reduction. In addition, they have previously been detected at another uranium-contaminated site which also contains elevated nitrate concentrations (6). 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 (N.B. This is most likely the result of uranium desorption accompanying the initial increase in bicarbonate following acetate injection) suggests that they may play important role in possibly

inhibiting U(VI) reduction during the transition to a dissimilatory Fe(III)-reducing environment. This intriguing dynamic between available ammonium, *Dechloromonas*, *Geobacter*, and *Rhodoferax* species 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 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 μ M are apparently high enough to repress *nifD* expression in the environment. As would be expected from previous studies (*17, 18*), expression of *nifD*, and presumably other genes involved in nitrogen fixation appeared to be closely regulated.

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 (22, 23, 25), 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 μ M 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 monitoring location with the highest mean U(VI) reduction rate was D-04, where ammonium concentrations ranged between about 30 to 80 μ M

throughout the 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 amendments were also observed first and reached their highest concentrations in D-04. Although ammonium has been added to stimulate bioremediation of organic contaminants (2), a previous study showed the addition of ammonium to *Geobacter* species growing in subsurface sediments did not stimulate Fe(III) reduction (17). 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 amendment delivered to this area, or the general level of growth and metabolic activity relative to other monitoring locations, rather than the ability of Geobacter species to out compete other species under conditions of nitrogen fixation. Such considerations emphasize the need for a comprehensive analysis of the physiological status of the microorganisms involved in important bioremediation processes via large-scale analysis of gene expression or other approaches.

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Supporting Information

Spatiotemporal trends in bromide concentrations, ratios of bromide relative to acetate levels, 16S rRNA microbial community composition, and *nifD*, *amtB*, and *recA* transcript expression are found in more detail in supporting information. This information is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Lovley, D. R., Cleaning up with genomics: Applying molecular biology to bioremediation. *Nature Reviews Microbiology* **2003**, *1*, (1), 35-44.

(2) Shin, W. S.; Pardue, J. H.; Jackson, W. A.; Choi, S. J., Nutrient enhanced biodegradation of crude oil in tropical salt marshes. *Water Air and Soil Pollution* **2001**, *131*, (1-4), 135-152.

(3) Anderson, R. T.; Vrionis, H. A.; Ortiz-Bernad, I.; Resch, C. T.; Long, P. E.; Dayvault, R.; Karp, K.; Marutzky, S.; Metzler, D. R.; Peacock, A., et al., Stimulating the *in situ* activity of *Geobacter* species to remove uranium from the groundwater of a uranium-contaminated aquifer. *Applied and Environmental Microbiology* **2003**, *69*, (10), 5884-5891.

(4) Cardenas, E.; Wu, W. M.; Leigh, M. B.; Carley, J.; Carroll, S.; Gentry, T.; Luo, J.; Watson, D.; Gu, B.; Ginder-Vogel, M., et al., Microbial communities in contaminated sediments, associated with bioremediation of uranium to submicromolar levels. *Applied and Environmental Microbiology* **2008**, *74*, (12), 3718-3729.

(5) Istok, J. D.; Senko, J. M.; Krumholz, L. R.; Watson, D.; Bogle, M. A.; Peacock, A.; Chang, Y. J.; White, D. C., In situ bioreduction of technetium and uranium in a nitrate-contaminated aquifer. *Environmental Science & Technology* **2004**, *38*, (2), 468-475.

(6) North, N. N.; Dollhopf, S. L.; Petrie, L.; Istok, J. D.; Balkwill, D. L.; Kostka, J. E., Change in bacterial community structure during *in situ* biostimulation of subsurface sediment cocontaminated with uranium and nitrate. *Applied and Environmental Microbiology* **2004**, *70*, (8), 4911-4920.

(7) Finneran, K. T.; Housewright, M. E.; Lovley, D. R., Multiple influences of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediments. *Environmental Microbiology* **2002**, *4*, (9), 510-516.

(8) Gu, B. H.; Wu, W. M.; Ginder-Vogel, M. A.; Yan, H.; Fields, M. W.; Zhou, J.; Fendorf, S.; Criddle, C. S.; Jardine, P. M., Bioreduction of uranium in a contaminated soil column. *Environmental Science & Technology* **2005**, *39*, (13), 4841-4847.

(9) Chin, K. J.; Esteve-Nunez, A.; Leang, C.; Lovley, D. R., Direct correlation between rates of anaerobic respiration and levels of mRNA for key respiratory genes in *Geobacter* sulfurreducens. Applied and Environmental Microbiology **2004**, *70*, (9), 5183-5189.

(10) Rahm, B. G.; Richardson, R. E., Correlation of respiratory gene expression levels and pseudo-steady-state PCE respiration rates in *Dehalococcoides ethenogenes*. *Environmental Science & Technology* **2008**, *42*, (2), 416-421.

(11) Fleming, J. T.; Sanseverino, J.; Sayler, G. S., Quantitative relationship between naphthalene catabolic gene frequency and expression in predicting PAH degradation in soils at town gas manufacturing sites. *Environmental Science & Technology* **1993**, *27*, (6), 1068-1074.

(12) O'Neil, R. A.; Holmes, D. E.; Coppi, M. V.; Adams, L. A.; Larrahondo, M. J.; Ward, J. E.; Nevin, K. P.; Woodard, T. L.; Vrionis, H. A.; N'Guessan, A. L., et al., Gene transcript analysis of assimilatory iron limitation in *Geobacteraceae* during groundwater bioremediation. *Environmental Microbiology* **2008**, *10*, (5), 1218-1230.

(13) Baelum, J.; Nicolaisen, M. H.; Holben, W. E.; Strobel, B. W.; Sorensen, J.; Jacobsen, C. S., Direct analysis of *tfdA* gene expression by indigenous bacteria in phenoxy acid amended agricultural soil. *Isme Journal* **2008**, *2*, (6), 677-687.

(14) Holmes, D. E.; O'Neil, R. A.; Chaven, M. A.; N'Guessan, L. A.; Vrionis, H. A.; Perpetua, L. A.; Larrahando, M. J.; DiDonato, R. J.; Liu, A.; Lovley, D. R., Transcriptome of *Geobacter uraniireducens* growing in uranium-contaminated subsurface sediments. *Isme Journal* **2008**, *3*, 216-230.

(15) Mouser, P. J.; Holmes, D. E.; Purpetua, L. A.; DiDonato, R. J.; Postier, B.; Liu, A.; Lovley, D. R., Quantifying expression of *Geobacter* spp. oxidative stress genes in pure culture and during *in situ* uranium bioremediation. *ISME Journal* **2009**, *doi:10.1038/ismej.2008.126*.

(16) Bazylinski, D. A.; Dean, A. J.; Schuler, D.; Phillips, E. J. P.; Lovley, D. R., N2-dependent growth and nitrogenase activity in the metal-metabolizing bacteria, *Geobacter* and *Magnetospirillum* species. *Environmental Microbiology* **2000**, *2*, (3), 266-273.

(17) Holmes, D. E.; Nevin, K. P.; Lovley, D. R., In situ expression of *nifD* in *Geobacteraceae* in subsurface sediments. *Applied and Environmental Microbiology* **2004**, *70*, (12), 7251-7259.

(18) Methe, B. A.; Webster, J.; Nevin, K.; Butler, J.; Lovley, D. R., DNA microarray analysis of nitrogen fixation and Fe(III) reduction in *Geobacter sulfurreducens*. *Applied and Environmental Microbiology* **2005**, *71*, (5), 2530-2538.

(19) Lammers, P. J.; Haselkorn, R., Sequence of the *nifD* gene coding for the alpha-subunit of dinitrogenase from the *Cyanobacterium Anabaena*. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **1983**, 80, (15), 4723-4727.

(20) Mortenson, L. E.; Thorneley, R. N. F., Structure and function of nitrogenase. *Annual Review of Biochemistry* **1979**, *48*, 387-418.

(21) Holmes, D. E.; Nevin, K. P.; Lovley, D. R., Comparison of 16S rRNA, *nifD*, *recA*, *gyrB*, *rpoB* and *fusA* genes within the family *Geobacteraceae* fam. nov. *International Journal Of Systematic And Evolutionary Microbiology* **2004**, *54*, 1591-1599.

(22) Andrade, S. L. A.; Einsle, O., The Amt/Mep/Rh family of ammonium transport proteins. *Molecular Membrane Biology* **2007**, *24*, (5-6), 357-365.

(23) Hoch, M. P.; Fogel, M. L.; Kirchman, D. L., Isotope fractionation associated with ammonium uptake by a marine bacterium. *Limnology and Oceanography* **1992**, *37*, (7), 1447-1459.

(24) Khademi, S.; O'Connell, J.; Remis, J.; Robles-Colmenares, Y.; Miericke, L. J. W.; Stroud, R. M., Mechanism of ammonia transport by Amt/MEP/Rh: Structure of AmtB at 1.3.5 angstrom. *Science* **2004**, *305*, (5690), 1587-1594.

(25) Kleiner, D., Bacterial ammonium transport. *Fems Microbiology Reviews* **1985**, *32*, (2), 87-100.

(26) Solorzano, L., Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnology Oceanography* **1969**, *14*, 799.

(27) Finneran, K. T.; Anderson, R. T.; Nevin, K. P.; Lovley, D. R., Potential for bioremediation of uranium-contaminated aquifers with microbial U(VI) reduction. *Soil Sediment and Contamination* **2002**, *11*, 339-357.

(28) Lovley, D. R.; Phillips, E. J. P., Organic-matter mineralization with reduction of ferric iron in anaerobic sediments. *Applied and Environmental Microbiology* **1986**, *51*, (4), 683-689.

(29) Eden, P. E.; Schmidt, T. M.; Blakemore, R. P.; Pace, N. R., Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *International Journal of Systematic Bacteriology* **1991**, *41*, 324-325.

(30) Lane, D. J.; Pace, B.; Olsen, G. J.; Stahl, D.; Sogin, M. L.; Pace, N. R., Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proceedings of the National Academy of Science of the United States of America* **1985**, *82*, 6955-6959.

(31) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J., Basic local alignment search tool. *Journal of Molecular Biology* **1990**, *215*, 403-410.

(32) Methe, B. A.; Nelson, K. E.; Eisen, J. A.; Paulsen, I. T.; Nelson, W.; Heidelberg, J. F.; Wu, D.; Wu, M.; Ward, N.; Beanan, M. J., et al., Genome of *Geobacter sulfurreducens*: Metal reduction in subsurface environments. *Science* **2003**, *302*, (5652), 1967-1969.

(33) Campbell, K. M.; Qafuku, N. P.; Kukkadapu, R.; Peacock, A.; Lesher, E.; Figueroa, L.; Williams, K. H.; Wilkins, M. J.; Davis, J. A.; Long, P. E., Characterizing the extent and role of natural subsurface bioreduction in a uranium-contaminated aquifer. *Geochimica et Cosmochimica Acta* in review.

(34) Finneran, K. T.; Johnsen, C. V.; Lovley, D. R., *Rhodoferax ferrireducens* sp. nov., a psychrotolerant, facultatively anaerobic bacterium that oxidizes acetate with the reduction of Fe(III). *International Journal Of Systematic And Evolutionary Microbiology* **2003**, *53*, (3), 669-673.

(35) Lovley, D. R.; Holmes, D. E.; Nevin, K. P., Dissimilatory Fe(III) and Mn(IV) reduction. In *Advances in Microbial Physiology, Vol. 49*, 2004; Vol. 49, pp 219-286.

(36) Achenbach, L. A.; Michaelidou, U.; Bruce, R. A.; Fryman, J.; Coates, J. D., *Dechloromonas agitata* gen. nov., sp nov and *Dechlorosoma suillum* gen. nov., sp nov., two novel

environmentally dominant (per)chlorate-reducing bacteria and their phylogenetic position. *International Journal of Systematic and Evolutionary Microbiology* **2001**, *51*, 527-533.

(37) Horn, M. A.; Ihssen, J.; Matthies, C.; Schramm, A.; Acker, G.; Drake, H. L., *Dechloromonas denitrificans* sp nov., *Flavobacterium denitrificans* sp nov., *Paenibacillus anaericanus* sp. nov and *Paenibacillus terrae* strain MH72, N2O-producing bacteria isolated from the gut of the earthworm *Aporrectodea caliginosa*. *International Journal of Systematic and Evolutionary Microbiology* **2005**, *55*, 1255-1265.

Figure Captions

Figure 1. a) Conceptual site layout of 2007 experimental design at Rifle, CO. Monitoring locations discussed in the text are shown in black. The dotted line indicates boring locations where darkened organic matter were observed in soil cores during drilling. b) and c) spatiotemporal fluctuations of dissolved acetate and Fe(II) concentrations collected from four monitoring locations during an acetate amended bioremediation experiment.

Figure 2 a) Spatiotemporal changes in dissolved U(VI) concentration for four monitoring locations at the Rifle, CO aquifer during an acetate stimulated bioremediation experiment. b) Average (bars<u>+</u>std) for first-order U(VI) reduction rates calculated between subsequent sampling events during the experiment.

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, diamond) and average concentrations (squares±std) in monitoring locations corresponding to 16S clone libraries. c) spatiotemporal ammonium fluctuations for four monitoring locations during an acetate amended bioremediation experiment.

Figure 4. a) Relationships between ammonium concentrations and transcript abundance of *Geobacteraceae nifD* relative to *recA* genes and b) *amtB* relative to *recA* genes 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

across all four locations, with a the mean square error shown as R^2 , and ρ representing Pearson's correlation coefficient.



Figure 1.





Figure 3.



Figure 4.