UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

CHARACTERIZATION OF SUBSURFACE MICROBIAL COMMUNITIES INVOLVED IN BIOREMEDIATION OF URANIUM AND NITRATE

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

Degree of

DOCTOR OF PHILOSOPHY

By

ANNE MARIE SPAIN
Norman, Oklahoma
2009

CHARACTERIZATION OF SUBSURFACE MICROBIAL COMMUNITIES INVOLVED IN BIOREMEDIATION OF URANIUM AND NITRATE

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY

_____
Dr. Lee R. Krumholz, Chair

_____
Dr. Joseph M. Suflita

_____
Dr. Michael J. McInerney

_____
Dr. Ralph S. Tanner

_____
Dr. Elizabeth C. Butler

I dedicate this to my mom,
Kristan Spain

Acknowledgements

The work presented in this dissertation thesis has been aided by the efforts of several people. First, I would like to thank each of my committee members, each of whom I have learned much from. Dr. Joe Suflita, Dr. Michael McInerny, Dr. Ralph Tanner, and Dr. Elizabeth Butler: not only have I learned much from each of you in classes and in department seminars, but also I have also gained a lot from your students who have used what they have learned from you to help me with my research efforts in various ways. The cooperation and fellowship among faculty and graduate students at the University of Oklahoma has been of extreme value to me, and is one of the things that drew me here for my graduate studies. And so again, I thank each of you for your positive attitudes, and your genuine willingness to help me as well as all graduate students here at OU.

And of course, I would like to acknowledge and thank the support of my major advisor, Dr. Lee Krumholz. It has been a trying seven years, full of hard work and stress. Dr. Krumholz has always pushed me when I have needed it the most, and has never made it unclear what his expectations of me were. He has pushed me to strive for the best science I can possibly achieve, within reason, and because of his expectations, I don't feel I could have learned how to be a better scientist at any other research institution. Dr. Krumholz has given me a lot of freedom in my academic and research pursuits, and while there has been some struggle associated with that freedom, the process has been a rewarding, gratifying, and humbling experience. He has also taught me that with science, some balance can exist along with personal happiness, as he has always stressed the importance of a balanced life. To sum it up, I

may not have chosen to come to OU if not for Lee Krumholz; when I came for my interview visit, I knew that he was the right advisor for me. Sartre states that "to choose one's adviser is another way to commit oneself," and I could not be more happy or grateful for the choice that I have made and for what has been given to me.

I would also like to thank my fellow graduate students with whom I have shared my graduate school experience. Namely, Dr. Kristen Savage, Dr. Kelli Palmer, and Dr. Matthew Traxler – all of you have been my partners in this graduate program, and I am so happy to have been able to form true friendships with each of you. I also would like to thank Dr. John Senko for his role in training me when I first started and for all of his helpful conversations, and Dr. Mostafa Elshahed for his role as a fill-in advisor at times and for helping me so much along the way in the lab and with writing. As well, I would like to thank John Frink, who, as a dedicated undergraduate in the lab assisted me with several tasks, the most time-consuming and appreciated of which was the set up and monitoring of pure and mixed culture groundwater experiments. And I would like to thank Neil Wofford for all of his assistance with experiment design and troubleshooting. And of course, all other past and members of the Krumholz lab and OU Micro graduate student community – working with you has been a priveledge.

I would also like to thank the support of my undergraduate advisor, Dr. Elizabeth Wheeler Alm, from Central Michigan University. As with Dr. Krumholz, I feel like I have learned about the kind of scientist and human being I want to be from you; you have been a true example for me, and a good friend since I have left CMU.

Lastly, I need to thank the following family and friends who have helped me along the way. My mother, Kristan Spain, and sister, Jessica Shore (and family!) for

their years of encouragement, emotional support, for understanding and reluctantly accepting my absence in times when I have not been able to be a part of family functions, and for visiting me at the times I have needed you the most. My brother, Mason Spain and my father, John Spain for their adoring attitudes and for always believing that I can be the best. And my cousin and long-time BFF, Lauren Rossi Harroun for her support and consistent presence throughout my life. My undergraduate friends, Margaret Fedder, Darlene Rosteck, and Matthew Kutch for their continuing presence in my life, and for giving me the feelings of family, love and support. And my friends and current and former roommates here in Norman, particularly Annissa and Avery Huckabee, Dusty Wheatley, Kristen Savage, Lauren Bray, Lauren Ehrsam, Katherine DeWitt, Joshua Cooper, and Genevieve Stotler, for providing me with consistency and balance during my time spent here.

Table of Contents

List of Tables

Appendix I

Appendix II

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Appendix I

Appendix II

Preface

This dissertation contains four chapters and two appendices, each of which contains a reference bibliography section formatted to the style of the journal *Applied and Environmental Microbiology*.

Chapter 1 is a literature review, describing the importance of nitrate-reducing bacteria in regards to the bioremediation of uranium- and nitrate- co-contaminated groundwater and describes the field site from which sediment and groundwater samples (namely in Chapters 3 and 4) came, the Oak Ridge Integrated Field Research Challenge Site, or the OR-IFRC, located in Oak Ridge Tennessee. Much of the work presented in this chapter comes published data from 16S rRNA gene clone libraries generated from this site. I provided some data presented in this chapter, including some of the site groundwater geochemical analyses (pH and soluble aluminum analysis) and some 16SrRNA, nirK, and nirS clone library data (which are presented in detail in Chapter 3). This chapter is in preparation for submission as a Minireview article, to *Applied and Environmental Microbiology*, *Environmental Microbiology*, or to the *ISME Journal*.

Chapter 2 includes a set of sediment microcosm experiments, designed to look at U(VI) reduction by different populations stimulated by sulfate-reducing conditions, iron-reducing conditions, and methanogenic conditions. Sediment and groundwater samples used in this study came from the Norman Landfill Environmental Site, operated by US Geological Survey Toxic Substances Hydrology Program. In this study, I was involved in sediment and groundwater collection, microcosm set-up and sub-sampling, and all geochemical analysis. Aaron Peacock at the Center for

Biomarker Analysis at the University of Tennessee, Knoxville, performed polar lipid fatty acid (PLFA) extraction and analyses from sediments, as well as the multivariate statistical analyses to correlate geochemical and PLFA data. This work is in preparation for submission as a short note or full research article to *Environmental Microbiology*.

Chapter 3 is based on a manuscript written for and accepted by the journal *Applied and Environmental Microbiology*. The manuscript was published in the August, 2007 issue, 73(15): 4892-4904. The work presented in this chapter describes denitrifying microbial community compositions of OR-IFRC high nitrate sediments undergoing biostimulation with ethanol, and describes the cultivation *Castellaniella* denitrifying isolates, which are believed to be important in *in situ* nitrate removal at the site. Field samples (sediment and groundwater) were provided to me by Dave Watson, the Field Research Manager at the OR-IFRC. Dr. Jonathan Istok was primarily involved in field experiment design and operation of push-pull tests. I performed the cultivation and molecular work and analyses of sequence data, while sequencing was carried out at the Advanced Center for Genome Technology at the University of Oklahoma (Norman, OK) or at the Oklahoma Medical Research Foundation (Oklahoma City, OK). Aaron Peacock at the Center for Biomarker Analysis at the University of Tennessee, Knoxville, performed PLFA extraction and analyses from sediment samples.

Chapter 4 includes characterization of denitrifying bacteria belonging to three genera (*Castellaniella*, *Rhizobium*, and *Pseudomonas*) isolated from the OR-IFRC and proposes roles of each isolate in denitrification during bioremediation with ethanol as

an electron donor. Dr. John Senko cultivated the *Rhizobium* and *Pseudomonas* strains when he was working as a graduate student at OU, and I was involved in the cultivation of *Castellaniella* isolates, and the characterization of all isolates described in this chapter. Dr. Yiran Dong, a former graduate student with Dr. Elizabeth Butler, kindly performed the non-linear regression analyses used to generate Michaelis Menten kinetic coefficients (V_{max} and K_m) from rate data that I had provided. The work in this chapter is currently in preparation for submission as a full report to *Applied and Environmental Microbiology*.

Appendix I is based on a manuscript written for and accepted by *The ISME Journal*. The manuscript was published online in Aprill, 2009 and in print in Volume 3**,** pages 992–1000. The work presented in this chapter describes the composition and diversity of P*roteobacteria* in soils, and describes some of the non-cultivatble Proteobacteria lineages with respect to their ecological distribution. In this study, I performed all *Proteobacteria*-affiliated 16S rRNA gene sequences binned from a large (13,001 clones) clone library generated from soil sampled from the Kessler Farm Field Laboratory in central, OK. The dataset used in this study was generated by Dr. Mostafa Elshahed and is published in a separate article: Elshahed, M. S., Youssef, N. H., Spain, A. M., Sheik, C., Najar, F.Z., Sukharnikov, L. O., Roe, B. A., Davis, J. P., Schloss, P. D., Bailey, V. L., and L. R. Krumholz. 2008. Novelty and uniqueness patterns of rare members of the soil biosphere. Appl Environ Microbiol. 74(17): 5422-5428.

Appendix II is based on a portion of a chapter written for the 2nd edition of *Bergey's Manual for Systematic Bacteriology* describing the phylum *Fibrobacteres*.

This chapter will appear in Volume 4: The *Bacteroidetes, Planctomycetes, Chlamydiae, Spirochaetes, Fibrobacteres, Fusobacteria, Acidobacteria, Verrucomicrobia, Dictyoglomi* and *Gemmatimonadetes* (editors include**:** Noel R. Krieg, James T. Staley, Brian Hedlund, Bruce J. Paster, Naomi Ward, Wolfgang Ludwig and William B. Whitman). The work that I did for this chapter includes 16S rRNA gene analysis of all *Fibrobacteres*-affiliated gene sequences available from GenBank (published and unpublished), the description of the taxonomic divisions within the phylum, and analysis of the ecological distribution (based on cultivated isolates and environmental 16S rRNA gene clones) of this phylum. Other work presented in the Bergey's Manual *Fibrobacteres* chapter (but not in this dissertation) concerned physiology of species within the *Fibrobacteres* phylum and enymes involved in fiber degradation; these portions were written by Dr. Lee Krumholz and Dr. Cecil Forsberg. This chapter was reviewed and accepted for publication by the editor Dr. James T. Staley, and will be published later in 2009.

Abstract

Mining and enrichment of uranium (U) for nuclear weapons and energy has left this radionuclide an important groundwater contaminant in the United States and worldwide. Migration of U in groundwater can be prevented by U immobilization which can be achieved through microbial reduction of soluble U(VI) to insoluble U(IV) upon electron donor addition. One of the major concerns regarding U bioremediation is the presence of nitrate, which serves as a competitive electron acceptor in the subsurface, inhibiting or retarding U(VI) reduction efforts; as well, intermediates of nitrate respiration (or denitrification) can lead to U(IV) oxidation and remobilization. Experiments performed in this study aimed to characterize subsurface microbial communities involved in U and nitrate reduction. In microcosm experiments, we stimulated sulfate-reducing, Fe(III)-reducing, and methanogenic populations in landfills sediment and found that U(VI) reduction occurred under each set of conditions, and that molybdate inhibited U(VI) reduction, regardless of the occurrence of sulfate reduction, or presence of sulfate or stimulation of sulfate-reducing bacteria. From *in situ* field experiments designed for bioremediation of acidic high-nitrate and U-contaminated aquifers (at a site, located in Oak Ridge, TN, designated the OR-IFRC) with ethanol as an electron donor, we found through molecular and cultivation techniques that *Betaproteobacteria*, namely species within the genus *Castellaniella*, are stimulated upon ethanol addition and are likely involved in *in situ* nitrate removal. Lastly, through the isolation and characterization of denitrifying bacteria from OR-IFRC biostimulated sediment and groundwater, we found that different isolates are likely involved in different stages of denitrification in

OR-IFRC groundwater. Specifically, we have isolates belonging to the genera *Pseudomonas*, *Castellaniella*, and *Rhizobium* are important in nitrate reduction, nitrite reduction, and nitrous oxide reduction, respectively. Lastly, we have shown that pH and nitrite tolerance are likely key factors contributing to the growth and survival of *Castellaniella* species in acidic, high nitrate aquifers at the OR-IFRC.

CHAPTER 1

Introduction and Literature Review: The Importance and Composition of Nitrate-Reducing Bacteria at the Oak Ridge Integrated Field Research Challenge Site

Site Description and the Effects of Nitrate on Uranium Bioremediation

The Integrated Field Research Challenge site in Oak Ridge, Tennessee provides an ideal location to study uranium bioreduction under different geochemical conditions. The Y-12 plant is part of the DOE's nuclear weapons complex, which has served to enrich for and process uranium, and provide the US military with nuclear fuel (17). Radionuclide contamination seeping into shallow groundwater (GW) aquifers at the site has generated a need for bioremediation and the development of bioremdiation technology. The research operation, in Oak Ridge, Tennessee, is termed the Integrated Field Research Challenge (OR-IFC).

The S-3 Ponds are the source of contamination at the OR-IFC. These are waste-disposal ponds built in 1951 to dispose of liquid waste containing nitric acid, radionuclides, heavy metals, and various organic contaminants at an approximate rate of ten million gallons per year until 1983. Of the radionuclides present, uranium (U) and technetium (Tc) are primary concerns, but these can be immobilized in the subsurface by bioreduction of the oxidized forms, U(VI) and Tc(VII), to insoluble reduced U(IV) and Tc(IV) minerals by sulfate- and dissimilatory metal-reducing bacteria (19-24, 39). The process involves release of organic electron donors (e.g.

ethanol or acetate) into injection wells within or upstream of the contaminated aquifer, resulting in the microbiologically-mediated reduction of radionuclides. GW contamination at the OR-IFC, however, is heterogeneous due to differential flow paths of contaminants (Summarized in Figure 1). As such, radionuclide contamination is often, though not always, accompanied by high levels of nitrate. For example, typical GW nitrate concentrations in Area 2 at the OR-IFC range from 1-4 mM, whereas in Areas 1 and 3, nitrate concentrations are typically much higher, often exceeding 100 mM (Table 1). Area 1 is the location from which the research described in Chapter 3 was conducted and from which the microorganisms described in Chapter 4 were isolated. In Area 1, GW tends to be acidic (pH 3-6.8), and primary contaminants include not only nitrate, U, and Tc, but aluminum and nickel as well (Table 1). While the inverse relationship between pH and nitrate concentrations in OR-IFC GW has been well-documented (3, 8, 43), we also found that soluble aluminum concentrations are magnitudes of order higher at low pHs than at circumneutral pHs (Table 1); these correlations may be particularly relevant to the physiology of microorganisms involved in GW bioremediation whose nitrate, nitrite, metal and pH tolerance mechanisms warrant further understanding.

Nitrate influences U(VI) reduction and U(IV) stability in aquifers co-contaminated with uranium and high [NO_3^-]. Nitrate as a co-contaminant will inhibit U(VI) reduction as nitrate acts as a competing terminal electron acceptor during the oxidation of organic compounds. Therefore, U(VI) reduction typically proceeds only after nitrate has been reduced to low levels (9, 16, 37). However, when nitrate concentrations are low and nitrite accumulation is limited, concurrent nitrate and U

reduction is possible (16). Reduction of U(VI) has also been documented to occur in the presence of nitrate when fermentative bacterial populations are enriched (25). However, microbial populations in biostimulated high-nitrate areas at the OR-IFC are composed of denitrifying genera (Table 2) and denitrification is the primary fate of nitrate upon *in situ* biostimulation (through addition of organic electron donors) of high-nitrate aquifers (16). During the denitrification process, intermediates that accumulate, such as nitrite, may lead to the re-oxidation of previously bioreduced U(IV) (9, 29, 37). Nitrite accumulation ranging in concentration from 10 mM to as high as 130 mM has been documented during biostimulation of Area 1 wells containing 142 mM nitrate (16, 38). Thus, if the ultimate goal is U immobilization by bioreduction, it is critical to stimulate the denitrification process in aquifers co-contaminated with nitrate; however, accumulation of denitrification intermediates must be controlled to ensure U(IV) stability.

Nitrate-Reducing Bacteria at the OR-IFC

In situ denitrifying populations are important in bioremediation of acidic U and NO_3^- co-contaminated sediments. Bioremediation of aquifers co-contaminated with nitrate and U is possible with additions of electron donor to the subsurface (4, 16, 41). During a field scale bioremediation effort in Area 3 of the OR-IFC, nitrate was first removed from GW in an above-ground denitrifying bioreactor prior to being returned to the subsurface for *in situ* U immobilization (41). In Area 1, nitrate reduction followed by U and Tc immobilization has been demonstrated via single well push-pull tests through direct addition of ethanol to the subsurface, without pre-treatment for nitrate removal (4, 16, 38). Nitrite accumulations can be extremely high, and in these instances, U(IV) is susceptible to remobilization (16). In an above-ground model of a bio-barrier treating acidic high-nitrate GW, it was shown that nitrate and Tc(VII) reduction occurred concomitantly, and U concentrations in the effluent of the model remained below EPA levels for a sustained length of time (27), suggesting that denitrification, Tc(VII)- and U(VI)- immobilization can be promoted *in situ* in a bio barrier receiving ethanol additions. Near the inlet of the model, U precipitated in the denitrifying zone as recalcitrant $(UO_2)_3(PO_4)_2 \cdot 4H_2O_{(s)}$, suggesting a possible role of denitrifying populations in direct U(VI) immobilization without bioreduction (27). Likewise, biomass from a denitrifying fluidized bed reactor (FBR) as well as a denitrifying isolate from this bioreactor have been shown to immobilize U(VI), though the mechanism of immobilization has not been determined (32, 40). These studies, along with studies showing that aerobic bacteria from the OR-IFC can bioprecipitate U(VI) (5, 26), suggest that remediation of acidic high-nitrate U-contaminated GW can

be achieved by electron donor stimulation of *in situ* microbial populations and that denitrifying populations may even play a role in U(VI) immobilization.

Denitrifying communities from pristine and contaminated sites at the OR-IFC are complex, consisting of numerous genera primarily from the *Beta-*, *Alpha-* and *Gammaproteobacteria*. 16SrRNA analysis has been carried out at a number of locations within the OR-IFC in an attempt to describe the microbial community structure of sediment and GW. This work has demonstrated the presence of a number of nitrate-reducing and denitrifying genera (Table 2). The vast majority of these belong to the *Betaproteobacteria* (19 genera detected), *Gammaproteobacteria* (two genera detected), and *Alphaproteobacteria* (six genera detected), while clones from *Paenibacillus* and *Anaeromyxobacter*, and *Flavobacterium* have also been detected (Table 2). From high-nitrate acidic GW in Area 3, *Azoarcus* was found to predominate along with *Pseudomonas*, *Ralstonia* and *Rhizobium* (12); however, in a study of attached microbial communities to mineral coupons deployed in low pH, high-nitrate GW wells from Area 3, *Alcaligenes/ Castelaniella* clones were dominant with *Aquaspirillum*, *Pseudomonas*, and *Burkholderia* also being detected (35). These genera along with clones belonging to *Acidovorax*, *Herbaspirillum*, *Comamonas*, *Rhizobium/Agrobacterium*, and *Thiobacillus* were detected in sediment and mineral coupons deployed in Area 1, another low pH, high-nitrate site (36). Although a variety of genera have been detected through molecular analysis, most are from groups that are easily cultivatable with representatives available in culture collections.

A recent study designed to differentiate active and inactive microbial populations showed that in Area 1 sediments, 43% of the active microbial community

(organisms detected in rRNA-derived clone libraries) belonged to nitrate-reducing genera, including *Castellaniella/Alcaligenes*, *Burkholderia*, and *Ralstonia*, and *Bradyrhizobium* (3). Nitrate concentrations increased with depth, with a corresponding decrease in pH. At the lowest depth, *Burkholderia* was dominant among RNA clones, suggesting that this genus is active under high-nitrate, low pH conditions. Also, *Pseudomonas*, the most widely detected genus in 16S rRNA gene clone libraries from FRC sediment and GW (see Table 2), was not among the "active" members of the microbial community from Area 1 sediment (3). *Betaproteobacteria*, on the other hand, seem to be not only active *in situ* (3), but are collectively dominant in sediment-associated communities from the high nitrate sites, Areas 1 and 3 (8, 35, 36).

In contaminated Area 2 sediment, where pH is circumneutral and nitrate concentrations are considerably lower than in Areas 1 and 3 (Figure 1), *Dechloromonas*, *Dechlorosoma*, *Zooglea*, *Pseudomonas*, and *Paenibacillus* were among the identified nitrate-reducing bacteria (1, 40). In Area 2 batch microcosms in which nitrate- and technetium-reduction were stimulated by electron donor addition, *Paenibacillus* was also detected in clone libraries during nitrate reduction and *Rhizobium*/*Agrobacterium* was detected following nitrate reduction (18). In uncontaminated background (pristine) sediments, nitrate-reducing genera detected include *Burkholderia*, *Ralstonia*, *Acidovorax*, *Dechloromonas*, *Aquaspirillum*, *Pseudomonas*, and *Sphingomonas*, many of the same genera found in Areas 1, 2, and 3. However, it seems clear that nitrate-reducing communities, identified through 16S rRNA gene surveys, in Areas 1 and 3, where nitrate concentrations are high, differ

from those in low-nitrate Area 2. Thus, pH and its effect on GW geochemistry may shape the types of nitrate-reducing bacteria found in sediments.

Denitrifying bacteria are a phylogenetically diverse functional group, and although several nitrate-reducing genera have been detected at the OR-IFC through 16S rRNA gene surveys, it is unclear whether those clones identified are derived from bacterial species capable of denitrification. To address this question, two studies have utilized cloning and sequencing of the dissimilatory nitrite reductase genes *nir*K and *nir*S as a tool to identify denitrifying bacteria from the OR-IFC (38, 43) and several possible denitrifying genera have been identified (Table 3). The dominant *nir*K-containing clone from several GW samples was related to the *nir*K genes from *Rhizobium* and *Hyphomicrobium* isolates (43) and the dominant *nir*S clones were related to *nir*S genes from *Pseudomonas stutzeri* and *Alcaligenes faecalis*. Other *nir*S clones from Area 2 (low nitrate) were related to *Thiobacillus* and *Azospirillum* (43), whereas *nir*S clones from Area 1 (high nitrate, low pH) sediment were related to *Azoarcus*, *Dechloromonas*, *Ralstonia*, *Magnetospirillum*, and *Thauera* (38), providing further evidence that denitrifying communities differ between low- and high nitrate-contaminated sites. The use of *nir*S and *nir*K to classify environmental clones, however, is not widely accepted, as current primer sets will not amplify genes from all known denitrifiers. Although *nir*S sequence-derived phylogeny is typically congruent with 16S rRNA phylogeny at the genus and family level, *nir*K genes are not, suggesting that horizontal gene transfer among denitrifying bacteria may be very common (12). Due to the limited number of studies on functional genes and the complex nature of denitrifying communities at the FRC, there is a need for more

sequencing of *nir*S and *nir*K genes from GW, sediments, as well as denitrifying isolates and the use of genomic sequences to develop new primer sets. By sequencing *nirS* and *nirK* gene sequences from OR-IFC denitrifying isolates and comparing the resulting phylogeny to 16S rRNA phylogeny, identifying *nir*K and *nir*S environmental clones from the same site will be more feasible.

Growth of Castellaniella and other Betaproteobacteria is stimulated during in situ biostimulation of high-nitrate aquifers with ethanol. While *Alpha-*, *Beta-*, and *Gammaproteobacteria* have all been identified as potential important nitrate-reducers at the OR-IFC, few studies have focused on identifying which denitrifiers may be involved in nitrate reduction during *in situ* biostimulation. In a recent field study, ethanol additions to acidic high-nitrate and uranium contaminated GW in Area 1 resulted in the increase in *Betaproteobacteria* in stimulated sediments vs. a control, which was dominated by *Gammaproteobacteria* (38). Two biostimulated sediment cores and one control core were sampled during nitrate reduction and *Castellaniella* sequences dominated 16S rRNA gene clone libraries from one biostimulated sediment core, whereas *Burkholderia* sequences dominated the other. In both biostimulated samples, *nir*K gene sequences identical to an OR-IFC *Castellaniella* isolate 4.5A2 dominated both *nir*K clone libraries, indicating this genus' involvement during *in situ* nitrate reduction following biostimulation with ethanol. Furthermore, in a separate Area 1 field biostimulation experiment (4), *Castellaniella* 16S rRNA gene clones dominated (>90%) sand and minerals in multilevel samplers deployed in wells receiving high-nitrate ethanol-amended GW whereas a broader diversity of

microorganisms were detected in pre-stimulation incubations of sterile sand in these multilevel sampler (MLS) wells (36).

Other organisms that have been detected in high-nitrate ethanol-stimulated sediment and GW include *Burkholderia*, *Ralstonia*, *Comamonas*, *Acidovorax*, *Dechloromonas*, *Azoarcus*, *Ferribacterium*, *Aquaspirillum*, *Pseudomonas*, *Sphingomonas*, *Rhizobium*, *Magnetospirillum*, *Phyllobacterium*, and *Paenibacillus* (Table 2). Among these organisms, however, it remains unclear whose growth is stimulated upon ethanol addition and which, if any, are active in nitrate reduction. Furthermore, the extent to which any single denitrifying species may contribute to nitrate reduction rates and the amounts of denitrification intermediates that each produce is unknown. In order to properly address these concerns, it will first be necessary to determine which denitrifying species are active *in situ* during biostimulation. While it is has been shown *Castellaniella* is actively involved in nitrate reduction, other species of denitrifying bacteria are likely involved as well. Studies suggest that denitrifying community composition may directly affect the kinetics of denitrification (6, 13). The nature of the relationship between denitrifying species (i.e. competition vs. cooperation) in a complex community may help to determine the effect species diversity may have on ecosystem function.

Microbial community structure shifts during nitrate reduction and bioremediation of U-contaminated GW. Among the studies done on microbial communities stimulated during nitrate reduction, many have focused on the community at one time point during stimulation, offering only a snapshot of community structure. In an above-ground bio-barrier model treating acidic high-nitrate FRC GW with ethanol,

different denitrifying populations were detected at different locations in the column, suggesting unique nitrate-reducing populations may be stimulated upon ethanol additions depending on pH and nitrate concentrations in GW (27). Also, community shifts were also observed along the length of the model, even though denitrification was the primary TEAP occurring (27). Thus, the denitrifying community shifts as denitrification proceeds in high nitrate GW.

Similarly, in a denitrifying fluidized bed reactor treating high nitrate GW from Area 3, microbial community shifts were observed over 118 days of operation. A diverse mixed inoculum (from Area 2 GW and denitrifying sludge from the Y-12 wastewater treatment plant) was used for this experiment, but within 12 days of operation, microbial diversity drastically decreased, with *Azoarcus* sp. dominating (96.5%) 16S rRNA gene clone libraries. However, as the experiment continued and nitrate concentrations decreased, diversity began to increase. These experiments were not quantitative as data were from clone sequences of mixed communities. Thus, only by isolating and characterizing representative species of those detected *in situ* can we understand the growth, competition, and interactions between these species during the denitrification process.

The goals in the following chapters are threefold: first, the impact of U(VI) reduction by different microbial communities stimulated by different TEAPs is discussed; second, the nitrate-reducing populations in Area 1 GW undergoing bioremediation for nitrate and U(VI) removal are characterized; and lastly, by isolating and characterizing representative species of those detected *in situ*, we intend

to understand the growth, competition, and interactions between these species during the denitrification process.

References

1. **Abulencia, C. B., D. L. Wyborski, J. A. Garcia, M. Podar, W. Chen, S. H. Chang, H. W. Chang, D. Watson, E. L. Brodie, T. C. Hazen, and M. Keller.** 2006. Environmental whole-genome amplification to access microbial populations in contaminated sediments. Appl Environ Microbiol **72:**3291-301.

2. **Akob, D. M., H. J. Mills, T. M. Gihring, L. Kerkhof, J. W. Stucki, A. S. Anastacio, K. J. Chin, K. Kusel, A. V. Palumbo, D. B. Watson, and J. E. Kostka.** 2008. Functional diversity and electron donor dependence of microbial populations capable of U(VI) reduction in radionuclide-contaminated subsurface sediments. Appl Environ Microbiol **74:**3159-70.

3. **Akob, D. M., H. J. Mills, and J. E. Kostka.** 2007. Metabolically active microbial communities in uranium-contaminated subsurface sediments. FEMS Microbiol Ecol **59:**95-107.

4. **Baldwin, B. R., A. D. Peacock, M. Park, D. M. Ogles, J. D. Istok, J. P. McKinley, C. T. Resch, and D. C. White.** 2008. Multilevel samplers as microcosms to assess microbial response to biostimulation. Ground Water **46:**295-304.

5. **Beazley, M. J., R. J. Martinez, P. A. Sobecky, S. M. Webb, and M. Taillefert.** 2007. Uranium biomineralization as a result of bacterial phosphatase activity: insights from bacterial isolates from a contaminated subsurface. Environ Sci Technol **41:**5701-7.

6. **Cavigelli, M. A., and G. P. Robertson.** 2000. The functional significance of denitrifier community composition in a terrestrial ecosystem. Ecology **81:**1402-1414.

7. **Dougan, W. K., and A. L. Wilson.** 1974. The absorptiometric determination of aluminium in water. A comparison of some chromogenic reagents and the development of an improved method. Analyst **99:**413-30.

8. **Fields, M. W., T. Yan, S. K. Rhee, S. L. Carroll, P. M. Jardine, D. B. Watson, C. S. Criddle, and J. Zhou.** 2005. Impacts on microbial communities and cultivable isolates from groundwater contaminated with high levels of nitric acid-uranium waste. FEMS Microbiol Ecol **53:**417-28.

9. **Finneran, K. T., M. E. Housewright, and D. R. Lovley.** 2002. Multiple influences of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediments. Environ Microbiol **4:**510-6.

10. **Gentile, M., T. Yan, S. M. Tiquia, M. W. Fields, J. Nyman, J. Zhou, and C. S. Criddle.** 2006. Stability in a denitrifying fluidized bed reactor. Microb Ecol **52:**311-21.

11. **Gentile, M. E., C. M. Jessup, J. L. Nyman, and C. S. Criddle.** 2007. Correlation of functional instability and community dynamics in denitrifying dispersed-growth reactors. Appl Environ Microbiol **73:**680-90.

12. **Heylen, K., D. Gevers, B. Vanparys, L. Wittebolle, J. Geets, N. Boon, and P. De Vos.** 2006. The incidence of nirS and nirK and their genetic heterogeneity in cultivated denitrifiers. Environ Microbiol **8:**2012-21.

13. **Holtan-Hartwig, L., P. Dorsch, and L. R. Bakken.** 2000. Comparison of denitrifying communities in organic soils: kinetics of NO−3 and N2O reduction. Soil Biology and Biochemistry **32:**833-843.

14. **Hwang, C., W. Wu, T. J. Gentry, J. Carley, G. A. Corbin, S. L. Carroll, D. B. Watson, P. M. Jardine, J. Zhou, C. S. Criddle, and M. W. Fields.** 2009. Bacterial community succession during in situ uranium bioremediation: spatial similarities along controlled flow paths. Isme J **3:**47-64.

15. **Hwang, C., W. M. Wu, T. J. Gentry, J. Carley, S. L. Carroll, C. Schadt, D. Watson, P. M. Jardine, J. Zhou, R. F. Hickey, C. S. Criddle, and M. W. Fields.** 2006. Changes in bacterial community structure correlate with initial operating conditions of a field-scale denitrifying fluidized bed reactor. Appl Microbiol Biotechnol **71:**748-60.

16. **Istok, J. D., J. M. Senko, L. R. Krumholz, D. Watson, M. A. Bogle, A. Peacock, Y. J. Chang, and D. C. White.** 2004. In situ bioreduction of technetium and uranium in a nitrate-contaminated aquifer. Environ Sci Technol **38:**468-75.

17. **Kersters, K., P. De Vos, M. Gillis, J. Swings, P. Vandamme, and E. Stackebrandt.** 2006. Introduction to the *Proteobacteria*, p. 3-37, The Prokaryotes, vol. 5. Springer New York.

18. **Li, X., and L. R. Krumholz.** 2008. Influence of nitrate on microbial reduction of pertechnetate. Environ Sci Technol **42:**1910-1915.

19. **Liu, C., Y. A. Gorby, J. M. Zachara, J. K. Fredrickson, and C. F. Brown.** 2002. Reduction kinetics of Fe(III), Co(III), U(VI), Cr(VI), and Tc(VII) in cultures of dissimilatory metal-reducing bacteria. Biotechnol Bioeng **80:**637-49.

20. **Lovley, D. R., S. J. Giovannoni, D. C. White, J. E. Champine, E. J. Phillips, Y. A. Gorby, and S. Goodwin.** 1993. *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. Arch Microbiol **159:**336-44.

21. **Lovley, D. R., and E. J. Phillips.** 1992. Reduction of uranium by *Desulfovibrio desulfuricans*. Appl Environ Microbiol **58:**850-6.

22. **Lovley, D. R., and E. J. P. Phillips.** 1992. Bioremediation of uranium contamination with enzymatic uranium reduction. . Environmental Science and Technology **26:**2228-2234.

23. **Lovley, D. R., E. J. P. Phillips, Y. A. Gorby, and E. R. Landa.** 1991. Microbial reduction of uranium. Nature **350**.

24. **Lovley, D. R., E. E. Roden, E. J. P. Phillips, and J. C. Woodward.** 1993. Enzymic iron and uranium reduction by sulfate-reducing bacteria. . Marine Geology **113:**41-53.

25. **Madden, A. S., A. C. Smith, D. L. Balkwill, L. A. Fagan, and T. J. Phelps.** 2007. Microbial uranium immobilization independent of nitrate reduction. Environ Microbiol **9:**2321-30.

26. **Martinez, R. J., M. J. Beazley, M. Taillefert, A. K. Arakaki, J. Skolnick, and P. A. Sobecky.** 2007. Aerobic uranium (VI) bioprecipitation by metal-resistant bacteria isolated from radionuclide- and metal-contaminated subsurface soils. Environ Microbiol **9:**3122-33.

27. **Michalsen, M. M., Aaron D. Peacock, Amanda N. Smithgal, David C. White, Anne M. Spain, Yamil Sanchez-Rosario, Lee R. Krumholz, Shelly D. Kelly, Kenneth M. Kemner, James McKinley, Steve M. Heald, Mary Anna Bogle, David B. Watson and Jonathan D. Istok.** 2009. Treatment of nitric acid-, U(VI)-, and Tc(VII)-contaminated groundwater in intermediate-scale physical models of an in situ biobarrier. Environ Sci Technol **Online Ahead of Print, February 6, 2009.**

28. **Michalsen, M. M., A. D. Peacock, A. M. Spain, A. N. Smithgal, D. C. White, Y. Sanchez-Rosario, L. R. Krumholz, and J. D. Istok.** 2007. Changes in microbial community composition and geochemistry during uranium and technetium bioimmobilization. Appl Environ Microbiol **73:**5885-96.

29. **Moon, H. S., J. Komlos, and P. R. Jaffe.** 2007. Uranium reoxidation in previously bioreduced sediment by dissolved oxygen and nitrate. Environ Sci Technol **41:**4587-92.

30. **Moreels, D., G. Crosson, C. Garafola, D. Monteleone, S. Taghavi, J. P. Fitts, and D. van der Lelie.** 2008. Microbial community dynamics in uranium contaminated subsurface sediments under biostimulated conditions with high nitrate and nickel pressure. Environ Sci Pollut Res Int **15:**481-91.

31. **North, N. N., S. L. Dollhopf, L. Petrie, J. D. Istok, D. L. Balkwill, and J. E. Kostka.** 2004. Change in bacterial community structure during in situ biostimulation of subsurface sediment cocontaminated with uranium and nitrate. Appl Environ Microbiol **70:**4911-20.

32. **Nyman, J. L., T. L. Marsh, M. A. Ginder-Vogel, M. Gentile, S. Fendorf, and C. Criddle.** 2006. Heterogeneous response to biostimulation for U(VI) reduction in replicated sediment microcosms. Biodegradation **17:**303-16.

33. **Peacock, A. D., Y. J. Chang, J. D. Istok, L. Krumholz, R. Geyer, B. Kinsall, D. Watson, K. L. Sublette, and D. C. White.** 2004. Utilization of microbial biofilms as monitors of bioremediation. Microb Ecol **47:**284-92.

34. **Petrie, L., N. N. North, S. L. Dollhopf, D. L. Balkwill, and J. E. Kostka.** 2003. Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). Appl Environ Microbiol **69:**7467-79.

35. **Reardon, C. L., D. E. Cummings, L. M. Petzke, B. L. Kinsall, D. B. Watson, B. M. Peyton, and G. G. Geesey.** 2004. Composition and diversity of microbial communities recovered from surrogate minerals incubated in an acidic uranium-contaminated aquifer. Appl Environ Microbiol **70:**6037-46.

36. **Sanchez-Rosario, Y., A. M. Spain, and L. Krumholz.** In preparation. Microbial communities from multilevel sampler systems filled with synthetic matrix. University of Oklahoma, Norman, OK.

37. **Senko, J. M., J. D. Istok, J. M. Suflita, and L. R. Krumholz.** 2002. In-situ evidence for uranium immobilization and remobilization. Environ Sci Technol **36:**1491-6.

38. **Spain, A. M., A. D. Peacock, J. D. Istok, M. S. Elshahed, F. Z. Najar, B. A. Roe, D. C. White, and L. R. Krumholz.** 2007. Identification and isolation of a *Castellaniella* species important during biostimulation of an acidic nitrate- and uranium-contaminated aquifer. Appl Environ Microbiol **73:**4892-904.

39. **Wu, Q., R. A. Sanford, and F. E. Loffler.** 2006. Uranium(VI) reduction by *Anaeromyxobacter dehalogenans* strain 2CP-C. Appl Environ Microbiol **72:**3608-14.

40. **Wu, W.-M., B. Gu, M. W. Fields, M. Gentile, Y.-K. Ku, H. Yan, S. Tiquias, T. Yan, J. Nyman, J. Zhou, P. M. Jardine, and C. S. Criddle.** 2005. Uranium (VI) reduction by denitrifying biomass. Bioremediation Journal **9:**49-61.

41. **Wu, W. M., J. Carley, T. Gentry, M. A. Ginder-Vogel, M. Fienen, T. Mehlhorn, H. Yan, S. Caroll, M. N. Pace, J. Nyman, J. Luo, M. E. Gentile,**

M. W. Fields, R. F. Hickey, B. Gu, D. Watson, O. A. Cirpka, J. Zhou, S. Fendorf, P. K. Kitanidis, P. M. Jardine, and C. S. Criddle. 2006. Pilot-scale in situ bioremedation of uranium in a highly contaminated aquifer. 2. Reduction of U(VI) and geochemical control of U(VI) bioavailability. Environ Sci Technol **40:**3986-95.

42. **Wu, W. M., J. Carley, J. Luo, M. A. Ginder-Vogel, E. Cardenas, M. B. Leigh, C. Hwang, S. D. Kelly, C. Ruan, L. Wu, J. Van Nostrand, T. Gentry, K. Lowe, T. Mehlhorn, S. Carroll, W. Luo, M. W. Fields, B. Gu, D. Watson, K. M. Kemner, T. Marsh, J. Tiedje, J. Zhou, S. Fendorf, P. K. Kitanidis, P. M. Jardine, and C. S. Criddle.** 2007. In situ bioreduction of uranium (VI) to submicromolar levels and reoxidation by dissolved oxygen. Environ Sci Technol **41:**5716-23.

43. **Yan, T., M. W. Fields, L. Wu, Y. Zu, J. M. Tiedje, and J. Zhou.** 2003. Molecular diversity and characterization of nitrite reductase gene fragments (*nirK* and *nirS*) from nitrate- and uranium-contaminated groundwater. Environ Microbiol **5:**13-24.

Table 1. Historical and collected geochemical GW data from various well locations in Area 1. All historical data is publicly available from the OR-IFC website: http://www.esd.ornl.gov/orifrc/. Soluble Al and pH were also measured in our lab from GW samples collected in October, 2002. Soluble Al was measured by a colorimetric procedure, modified from Dougan and Wilson (7), and pH was measured using an electrode.

Well ID	Historical Info	Publicly available historical GW data						Measured data	
		pH	$[NO_3^-]$ (mM)	$[SO_4^{-2}]$ (mM)	[U] (μM)	[Tc] (pCi/L)	[Ni] (mM)	pH	$[Al]_{sol}$ (mM)
FW015	C	3.37	153.61	0.083	28.11	18,500	0.15	3.36	32.2
FW021	Used as test GW for several Area 1 single well push-pull tests (16, 38)	3.05	142.29	1.271	5.80	19,009	NA	3.6	12.4
FW031	C (16)	5.68	62.74	0.070	0.04	2053	NA	4.84	0.251
FW033	G (16) I (Chapter 4)	5.85	14.28	0.653	0.27	2237	NA	6.72	$4.8x10^{-5}$
FW032	G (16) I (Chapter 4)	5.22	23.26	0.007	0.07	1606	NA	7.16	$1.7x10^{-3}$
FW034	E (16, 38)	6.79	0.77	0.747	0.47	66	NA	7.48	$2.5x10^{-3}$
FW028	E (38) I (Chapter 4)	4.40	167.17	0.056	2.22	12,125	NA	4.34	1.01
FW016	C (38)	3.92	11.40	0.427	2.58	NA	NA	5.84	$2.1x10^{-2}$

C = Control; well has never received electron donor
G = Glucose-amended well used in single well push pull tests
E = Ethanol-amended
I = Isolate obtained from this well, or sediment adjacent. Isolate characterization information is described in Chapter 4.

Table 2. Presence of nitrate-reducing or denitrifying genera in 16S rRNA gene or 16S rRNA libraries generated from OR-IFRC unstimulated (U) or biostimulated (A= acetate-stimulated; E=ethanol-stimulated; G=glucose-stimulated; L=lactate-stimulated) groundwater (GW), sediments (Sed), and fluidized bed reactors (FBR). Bold font denotes detection from RNA-derived clone libraries.

Genus	Detection Sources
Betaproteobacteria	
Burkholderia	Fe(III)-Reducing enrichments with background Sed (A) (34)
	Background Sed (U) (31)
	Hematite coupons deployed in background (U) (35)
	Area 1 Sed (U) (3)
	Area 1 Sed (E) (38)
	Area 1 Sed column treating acidic high-NO_3^- GW (E) (27)
	Hematite coupons deployed in Area 3 (U) (35)
	Area 3 GW (E) (14)
	Inoculum for Area 3 FBR (Y-12 wastewater sludge and Area 2 GW (15)
Alcaligenes/	Microbial samplers deployed in Area 1 (A and U) (33)
Castellaniella/	Area 1 Sed (G) (31)
Achromobacter	Area 1 Sed (E) (38)
	Area 1 Sed (U) (3)
	Area 1 Sed column treating acidic high-NO_3^- GW (E) (27)
	Sand, hematite, goethite, and gypsum deployed in Area 1 MLS (U, E) (36)
	Area 2 Sed microcosms (U) (2)
	Hematite coupons deployed in Area 3 (U) (35)
	Area 3 GW (E) (14)
	Inoculum for Area 3 FBR (Y-12 wastewater sludge and Area 2 GW) (15)
	Denitrifying FBR treating synthetic GW using FBR inoculum (L, E) (11)
Denitrobacter	Sand deployed in Area 1 MLS (U) (36)
Ralstonia	Background Sed (U) (31)
	Microbial samplers deployed in Area 1 (U) (33)
	Area 1 Sed (U) (3, 31)
	Area 1 GW (U) (8)
	Area 1 Sed column treating acidic high-NO_3^- GW (E) (27)
	Area 2 Sed column receiving synthetic GW (E) (30)
	Area 2 Sed microcosms (U, G) (2)
	Area 3 GW (U) (8)
	Area 3 GW (E) (14)
Acidovorax/	Background Sed (U) (31)
Diaphorobacter	Background GW (U) (8)
	Hematite coupons deployed in background (U) (35)
	Area 1 GW (U) (8)
	Area 1 Sed (U) (3)
	Area 1 Sed column treating acidic high-NO_3^- GW (E) (27)
	Sand deployed in Area 1 MLS (U, E) (36)
	Area 2 Sed microcosms (U, E) (2)
	Area 3 Sed flushed with low NO_3^- FBR-treated GW (E) (42)
	Area 3 GW (E) (14)
	Inoculum for Area 3 FBR (Y-12 wastewater sludge and Area 2 GW) (15)
	Area 3 fluidized bed reactor (E) (15)
	Denitrifying FBR treating synthetic GW using Area 2 inoculum (L, E) (10)
	Denitrifying FBR treating synthetic GW using FBR inoculum (L, E) (11)

Comamonas/	Sand deployed in Area 1 MLS (U and E) (36)
Delftia	Area 3 GW (E) (14)
	Denitrifying FBR treating synthetic GW using FBR inoculum (L, E) (11)
Herbaspirillum	Sand deployed in Area 1 MLS (U) (36)
	Area 3 GW (E) (14)
Dechloromonas	Background Sed (U) (31)
	Hematite coupons deployed in background (U) (35)
	Microbial samplers deployed in Area 1 (A, G) (33)
	Area 1 Sed (G) (31)
	Area 3 GW (E) (14)
	Inoculum for Area 3 FBR (Y-12 wastewater sludge and Area 2 GW) (15)
	Area 3 fluidized bed reactor (E) (15)
	Denitrifying FBR treating synthetic GW using Area 2 inoculum (L, E) (10)
Dechlorosoma	Area 3 GW (E) (14)
	Inoculum for Area 3 FBR (Y-12 wastewater sludge and Area 2 GW) (15)
	Denitrifying FBR treating synthetic GW using Area 2 inoculum (L, E) (10)
Azoarcus	Area 1 GW (U) (8)
	Area 3 GW (U) (8)
	Area 3 GW (E) (14)
	Area 3 fluidized bed reactor (E) (15)
Zoogloea	Denitrifying FBR treating synthetic GW using Area 2 inoculum (L, E) (40)
	Area 3 GW (E) (14)
	Inoculum for Area 3 FBR (Y-12 wastewater sludge and Area 2 GW) (15)
Ferribacterium	Background Sed in an Area 2 aboveground bio-barrier model (E) (28)
	Sand deployed in Area 1 MLS (E) (36)
	Area 3 Sed flushed with low NO3- FBR-treated GW (E) (42)
	Area 3 GW (E) (14)
Thiobacillus	Sand deployed in Area 1 MLS (U) (36)
Aquaspirillum	Background Sed (U) (35)
	Microbial samplers deployed in Area 1 (A, E) (33)
	Sand deployed in Area 1 MLS (U and E) (36)
	Hematite coupons deployed in Area 3 (U) (35)
	Area 3 Sed (U) (1)
	Area 3 GW (E) (14)
Hydrogenophaga	Area 3 GW (E) (14)
	Denitrifying FBR treating synthetic GW using Area 2 inoculum (L, E) (10)
Gammaproteobacteria	
Pseudomonas	Fe(III)-Reducing enrichments with background Sed (A) (34)
	Background Sed (U) (35)
	Background GW (U) (8)
	Area 1 Sed (U, G, and E) (3, 31, 38)
	Area 1 GW (U) (8)
	Area 1 Sed column treating acidic high-NO_3^- GW (E) (27)
	Sand and goethite deployed in Area 1 MLS (U and E) (36)
	Area 2 Sed (U) (1)
	Area 2 Sed column receiving synthetic GW (E) (30)
	Area 2 Sed microcosms (U, E) (2)
	Hematite coupons deployed in Area 3 (U) (35)
	Area 3 GW (U) (8)
	Area 3 Sed (U) (1)
	Area 3 fluidized bed reactor (E) (15)
	Denitrifying FBR treating synthetic GW using FBR inoculum (L, E) (11)
Acinetobacter	Area 1 Sed (U) (3)

Alphaproteobacteria	
Sphingomonas	Microbial samplers deployed in Area 1 (U) (33)
	Background Sed (U) (31)
	Area 1 Sed (U, G, E) (3, 31)
	Area 1 Sed columntreating acidic high-NO_3^- GW (E) (27)
	Area 3 GW (E) (14)
Rhizobium/	Area 3 GW (U) (8)
Bradyrhizobium/	**Area 1 Sed (U)** (3)
Agrobacterium	Area 1 Sed column treating acidic high-NO_3^- GW (E) (27)
	Sand and goethite deployed in Area 1 MLS (U) (36)
	Area 2 Sed in NO_3^- and Tc-reducing microcosms (E) (18)
	Area 3 GW (E) (14)
Magnetospirillum	Background Sed in an Area 2 aboveground bio-barrier model (E) (28)
	Sand and hematite deployed in Area 1 MLS (E) (36)
Phyllobacterium	Area 1 Sed column treating acidic high-NO_3^- GW (E) (27)
	Area 3 GW (E) (14)
Other Bacteria	
Paenibacillus	Area 1 Sed (U, G, E) (31)
	Sand deployed in Area 1 MLS (E) (36)
	Area 2 Sed in NO3- and Tc-reducing microcosms (E) (18)
Anaeromyxobacter	Fe(III)-Reducing enrichments with background Sed (A) (34)
	Area 1 Sed (U, G, E) (31)
Flavobacterium	Area 1 Sed (E) (38)
	Denitrifying FBR treating synthetic GW using FBR inoculum (L, E) (11)

Table 3. Denitrifying genera identified* from OR-IFC *nir*K and *nir*S clone libraries.

Genus	Detection Sources	*nir*K/*nir*S libraries	Biostimulated? (Y/N, electron donor)	Reference
Rhizobium	Background GW Area 1 GW Area 2 GW Area 3 GW	*nir*K	No	(43)
Nitrosomonas	Area 1 GW	*nir*K	No	(43)
	Area 1 sediment	*nir*K	Yes, ethanol	(38)
Castellaniella/ Alcaligenes	Area 1 sediment	*nir*K	Yes, ethanol No	(38)
Ochrobactrum	Area 1 sediment	*nir*K	No	(38)
Pseudomonas	Background GW Area 1 GW Area 2 GW Area 3 GW	*nir*S	No	(43)
	Area 1 sediment	*nir*S	Yes, ethanol	(38)
Thiobacillus	Area 2 GW	*nir*S	No	(43)
Azospirillium	Area 2 GW	*nir*S	No	(43)
Azoarcus	Area 1 sediment	*nir*S	Yes, ethanol	(38)
Dechloromonas	Area 1 sediment	*nir*S	Yes, ethanol	(38)
Ralstonia	Area 1 sediment	*nir*S	Yes, ethanol	(38)
Magnetospirillum	Area 1 sediment	*nir*S	Yes, ethanol	(38)
Thauera	Area 1 sediment	*nir*S	Yes, ethanol	(38)

*Genus identified refers to the taxonomy of the most closely related cultured relative, and may not refer to the exact genus of the clone sequence.

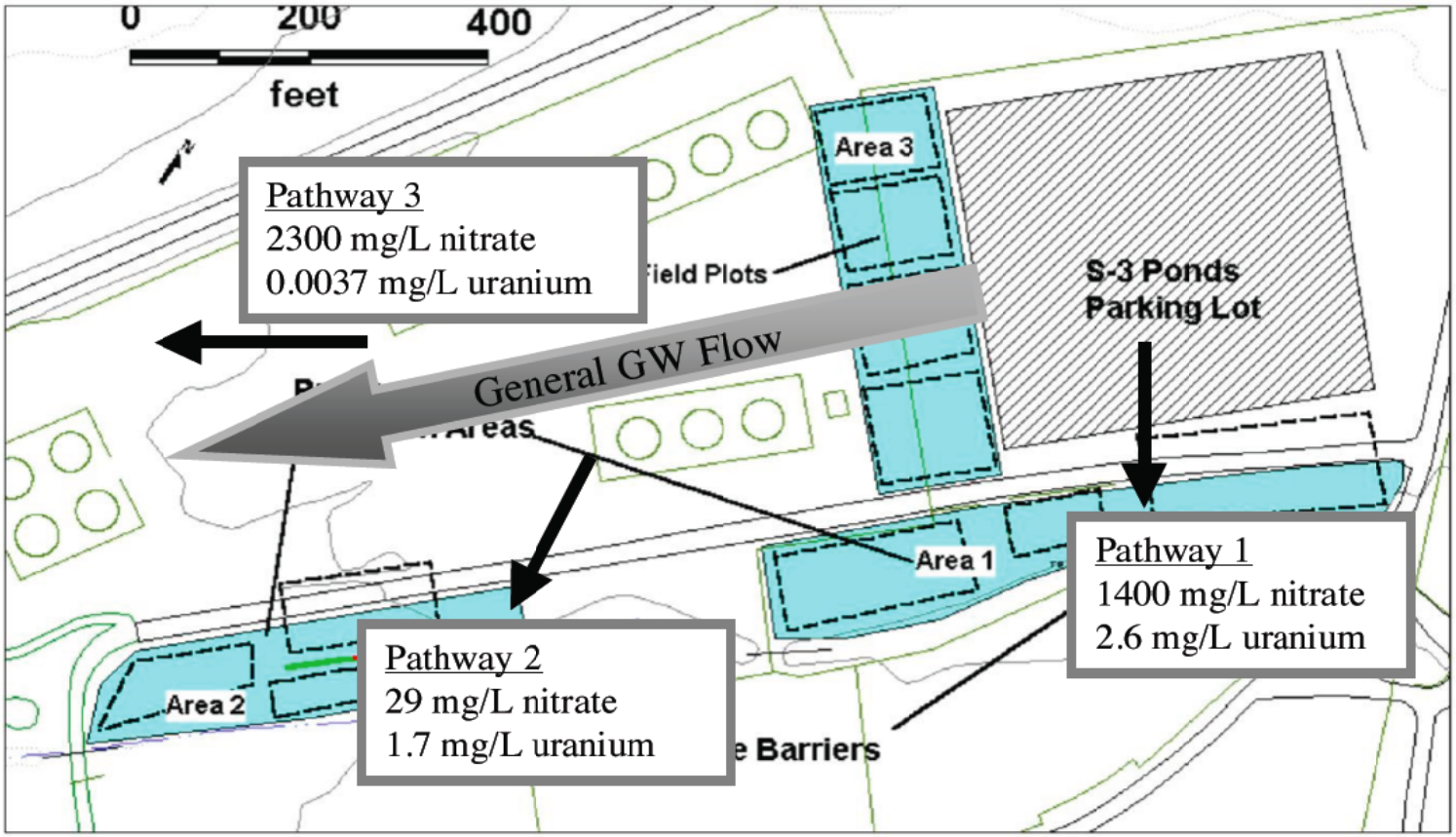

Figure 1. Diagram of S-3 ponds at the OR-IFC, Areas 1, 2, and 3 surrounding the field site, and typical groundwater concentrations of nitrate and uranium in the three general differential flow paths of contaminant migration. Contaminant migration pathways were simplified from http://public.ornl.gov/orifc/images/fig04.jpg. Diagram of S-3 ponds and Areas 1, 2 and 3 was originally located at: http://public.ornl.gov/orifc/images/FigureA1.jpg

CHAPTER 2

Preliminary Microcosm Studies: Uranium Reduction by Microbial Subsurface Communities Stimulated Under Iron-Reducing, Sulfate-Reducing, and Methanogenic Conditions

Abstract

Addition of an electron donor, such as ethanol, glucose, or acetate, to the subsurface in order to stimulate biological reduction of soluble U(VI) to insoluble U(IV) is an important strategy for uranium immobilization in contaminated aquifers. Electron donor addition typically results in anaerobic conditions and the respiratory process (sulfate-reducing, iron-reducing, and/or methanogenic) will depend on site geochemistry. While previous studies have found that U(VI) reduction is likely to occur under any of these conditions, the goal of this study was to examine the extent of U(VI) reduction under different geochemical conditions and the influence of the different microbial populations on the reduction process. Sulfate-reducing, iron-reducing, and methanogenic conditions were stimulated in sediment batch microcosms, and upon depletion of alternate electron acceptors, 100μM U(VI) was added. Within seven days, 89, 93, and 66 % of U(VI) was lost by reduction and/or precipitation from sulfate-reducing, iron-reducing, and methanogenic bottles, respectively. After 26 days, however, bicarbonate and nitric acid extractions of solid-associated U(VI) and total U showed that (i) the amount of reduced U(IV) was not affected by terminal electron accepting condition and that (ii) molybdate negatively

affected U(VI) reduction under all terminal electron accepting conditions. PLFA analysis of sediments showed different PLFAs associated with each treatment group with different groups of fatty acids correlated with the amount of U(IV) (per g dry sediment) under each terminal electron accepting condition stimulated during pre-incubations. These data support previous findings that organisms capable of U(VI) reduction are ubiquitous in the subsurface and demonstrate that extent of U(VI) reduction does not differ whether sediments are sulfate-reducing, iron-reducing, or methanogenic.

Introduction

Mining and enrichment of uranium for nuclear weapons and energy in the United States has resulted in the release of uranium into the environment and created a groundwater uranium contamination problem. Biological remediation of soluble U(VI) in groundwater can occur through microbial enzymatic reduction to insoluble and immobile U(IV) (28). Recent field experiments have shown that the addition of electron donors (glucose, ethanol, or acetate) into injection wells successfully results in the stimulation of endogenous subsurface microorganisms to grow and reduce U(VI) (3, 4, 11, 18, 21, 22, 41, 49, 55). This process has been demonstrated in a wide physiological range of pure cultures, including species within the genera *Geobacter* (24, 29, 43, 45), *Shewanella* (24), *Anaeromyxobacter* (54), *Desulfovibrio* (27, 28, 30), *Clostridium* (16, 17), *Thermoterrabacterium* (23), and *Salmonella* (42).

While studying pure cultures has aided in elucidating pathways of bacterial U(VI) reduction [for a review, see ((50)], it is also important to identify which microbial populations are responsible for U(VI) reduction *in situ*. Identifying U(VI) reducing communities has been attempted from both *in situ* and microcosm experiments utilizing a number of approaches based on 16S rRNA gene surveys (e.g. DGGE, clone libraries, stable isotope probing, microarrays, qPCR) and phospholipid fatty acid (PLFA) analysis. The most commonly detected species thought to contribute to U(VI) reduction in environmental samples include species of Fe(III)-reducing *Geobacteraceae* (2, 3, 10, 11, 20, 31-33, 35, 38, 46, 49, 51, 55) and *Anaeromyxobacter* (10, 35, 39, 48), and sulfate-reducing bacteria (SRB) species of *Desulfovibrionaceae* (1, 10, 31, 33, 36, 46) and *Desulfosporosinus* (10, 32, 34, 47).

Thus, multiple studies have suggested that the presence of these iron-reducing and sulfate-reducing species are important in U(VI) bioremediation.

However, problems arise in associating microbial populations with a specific function when multiple terminal electron accepting processes (TEAPs) occur simultaneously. Since U(VI) reduction often occurs during Fe(III)- or sulfate-reducing conditions, when Fe(III) or sulfate are present at far higher concentrations than U(VI), it is difficult to determine whether organisms detected in clone libraries are reducing U(VI), are involved in the aforementioned TEAPs, or are both reducing U(VI) and respiring other electron acceptors. For example, it has been observed that members of *Geobacteraceae* were associated with U(VI) reduction during Fe(III)-reducing conditions, but that U(VI) reduction ceased upon transition to sulfate-reducing conditions (3, 33). These studies and others have raised questions of whether (a) U(VI)-reducing bacteria comprise a separate population in sediments that reduce (VI) concurrently with other TEAPs, or (b) whether certain microbial populations are more efficient in U(VI) reduction (i.e. SRB vs. iron-reducers) than others in sediments.

To address these questions, and to examine sediment microbial populations involved in U(VI) reduction, different terminal electron accepting (TEA) conditions (sulfate reduction, iron reduction, and methanogenesis) were stimulated in sediment batch "pre-incubations," and after TEAs were depleted, U(VI) was added so that U(VI) reduction would be the predominant TEAP occurring under the different conditions stimulated. It was found that U(VI) reduction occurred under sulfate-reducing, iron-reducing, and methanogenic conditions. Correlation analysis of different PLFAs and the amount of uranium reduced suggest that there was not one

U(VI)-reducing population present in sediments; rather, different populations were correlated with U(VI) reduction under each TEA condition. The results of this study confirm that U(VI) reducing bacteria are ubiquitous in subsurface environments and suggest that the geochemistry and the predominant TEAPs of a site will primarily affect subsurface microbial community composition, thus possible affecting what microorganisms will be responsible for U(VI) reduction. Furthermore, the microbial populations involved in U(VI) reduction may not affect the extent to which this process occurs.

Materials and Methods

Site Description and groundwater and sediment collection.

Sediment and groundwater (GW) used in this study were sampled adjacent to a closed municipal landfill (LF), the Norman Landfill Environmental Site, operated by US Geological Survey Toxic Substances Hydrology Program. This site has been characterized geochemically and microbiologically (7, 12); briefly, biogeochemical processes important in the aquifer underlying the LF associated with the leachate plume include Fe(III) reduction, SO_4^{2-} reduction, and methanogenesis (12). Also, both *in situ* and lab-scale experiments have shown sediments to be capable of U(VI) reduction (41).

GW was collected using a peristaltic pump from a multilevel sampler well (MLS 35) located near the southwest edge of the west cell of the LF, downgradient of the leachate plume. In the laboratory, GW was prepared anaerobically by boiling for 5 minutes, sparging with N_2:CO_2 (80:20) for 30 min in 2-L bottles and then sealed with rubber stoppers. GW was sterilized by autoclaving at 121°C for 20 minutes. Prepared in this manner, GW from MLS 35 has been found to have a pH of 6.9-7.2 (41). A previous study found this area of the LF aquifer to be circumneutral (pH 6.2-7.5) and predominantly sulfate-reducing (with sulfate concentrations ranging from 0.5 to 5 mM year round, averaging ~2 mM), with acetate-utilizing SRB and methanogens present (7). The sulfate concentration in GW collected from MLS 35 at the time of sampling (July, 2002) was approx. 300 μM. Sediment was collected using a hand auger from nearby MLS 35 from below the water table, approx. 2 m below the surface (in the anoxic zone, as indicated by the transition from brown to blackened sediments).

Sediments were transported to the laboratory in sterile jars, which were flushed with N_2 for several minutes and stored at 4°C.

Initial Microcosm Study: U reduction by Norman landfill sediments in microcosms in the presence of nitrate, sulfate, and Fe(III).

To determine the sequence of TEAPs (NO_3^- reduction, Fe(III) reduction, SO_4^{2-} reduction, U(VI) reduction, and methanogenesis) in LF sediments, sediment slurry microcosms were set up with 25 (±0.5) g landfill sediment and 50 ml sterile groundwater in sterile 120-ml serum bottles inside an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, Michigan). Bottles were sealed with butyl rubber stoppers and aluminum seals and headspace was exchanged aseptically with N_2:CO_2 (80:20). Bottles were amended with sterile, anoxic stock solutions of sodium acetate, Na_2SO_4, $NaNO_3$, and uranyl acetate to reach final concentrations of 15 mM acetate, 5 mM NO_3^-, 5 mM SO_4^{2-}, and 25 μM U(VI). Autoclaved sediment microcosms with the same amendments (20 min, 120°C), served as heat-killed abiotic controls. All microcosms were incubated in the dark at room temperature. Samples (0.3 ml) were removed via syringe periodically in an anaerobic chamber and centrifuged to remove biomass; supernatant was used for anion and U(VI) analyses. For soluble Fe(II) analysis, 50 μl samples were diluted in 50 μl 0.5 N HCl inside an anaerobic chamber. Headspace was also sampled periodically to measure methane.

U(VI) reduction by LF sediments in microcosms pre-incubated to stimulate sulfate-reducing (SR), Fe(III)-reducing (FeR), and methanogenic (Meth) geochemical conditions and populations.

(i) Preincubations to stimulate different TEA conditions. Sediment microcosms were set up in a similar manner to those described above to establish sulfate-reducing (SR), Fe(III)-reducing (FeR), and methanogenic (Meth) conditions. All microcosms were amended with 2.5 mM ethanol to serve as an electron donor and different electron acceptors (or none) to stimulate the desired TEAP (See Table 1 for complete description of amendments made to each treatment group). U(VI) was not added at this point. Some FeR and Meth microcosms were amended with 0.5 mM sodium molybdate, a competitive inhibitor of SO_4^{2-} reduction (9, 37), to prevent reduction of SO_4^{2-} naturally present in groundwater (approx. 300 μM) and stimulation of SRB populations. All amendments were added to bottles via syringe from sterile anoxic stock solutions. Preliminary experiments had shown that LF sediments required approx. 25 and 15 days to deplete added amounts of SO_4^{2-} and Fe(III), respectively, and approx. 25 days to produce CH_4 when no other electron acceptors were added; thus SR and Meth microcosms were pre-incubated for 25 days, and FeR bottles for 15 days, prior to U(VI) addition (in the dark, room temp). During the pre-incubation period, samples were removed periodically to monitor anions, soluble Fe(II), and CH_4.

(ii) Analysis of U(VI) reduction under different TEA conditions. After pre-incubations (at "T_0"), ethanol and U(VI) as uranyl hydroxide were added to each bottle from sterile stock solutions to reach final concentrations 1 mM and 100 μM, respectively (Table 1). Bottles from each pre-incubation group were split into subgroups to

determine the extent of abiotic U(VI) reduction (by adding formaldehyde) and any effect of molybdate on U(VI) reduction. In addition, 1.0 mM SO_4^{2-} was added to some SR bottles [SR (+SO4)] to determine whether SRB stimulated in SR pre-incubations required sulfate reduction activity in order to reduce U(VI). In all, U(VI) reduction was analyzed in four SR subgroups, five FeR subgroups, and five Meth subgroups (for complete descriptions of each treatment group and amendments, see Table 1). Bottles were incubated at room temperature in the dark for seven days, during which subsamples were taken for soluble U(VI), anion, and Fe(II) analysis. Bottles were sacrificed from each subgroup for sediment-associated U analysis (Days 7 and 26) and polar lipid fatty acid (PLFA) extraction and analysis (Day 26).

Polar lipid fatty acid (PLFA) extraction and analysis

Frozen sediment samples from Day 26 live bottles (approx. 20 g sediment per treatment group) were sent on dry ice to the Univ. of Tennessee Center for Biomarker Analysis (Knoxville, TN) for PLFA extraction and analysis. PLFAs were extracted with the single-phase chloroform-methanol-buffer system and as previously described (8, 52), fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography (19), and analyzed according to standard protocols (40). Lastly, sediment biomass (cells/g sediment) was estimated from total PLFA/g sediment using the conversion $2.5x10^4$ cells per pmol PLFA (5).

Analytical methods

Anions and acetate were measured from microcosm subsamples by ion chromatography (Dionex, model DX500 fitted with the AS-4A and AS11 columns for anions and acetate analyses, respectively; Dionex Corporation, Sunnyvale, CA).

Soluble Fe(II) was measured by the ferrozine assay (26). Methane was measured by gas chromatography (Shimadzu GC14A fitted with a Chemipak C-18 column and a flame ionization detector). Total U(VI) (soluble and solids-associated) and reduced U(IV) were extracted using sodium bicarbonate and nitric acid, respectively, from duplicate sediment samples (0.5 g) of each live treatment subgroup (under SR, FeR, and Meth conditions) on Day 7 and from each treatment group (live and formaldehyde-killed) on Day 26 (15). Soluble U(VI) from GW samples and U from sediment extractions was measured by kinetic phosphorescence analysis (KPA-11; Chemcheck Instruments, Richland, WA).

Results and Discussion

Succession of TEAPs, including U(VI) reduction, in LF sediment microcosms. In acetate-amended batch LF sediment microcosms, U(VI) reduction was concomitant with SO_4^{2-} reduction, following NO_3^- and Fe(III) reduction (Fig 1). By 70 days of incubation, >99% of the SO_4^{2-} had been reduced and 81.8% of the U(VI) had been removed. After the onset of methanogenic conditions (at Day 80), U(VI) loss was slow, with 49% of the remaining U(VI) lost after Day 80. The observation of concomitant U(VI) and sulfate-reduction has previously been documented from the Norman LF in sediment slurries (41), and sulfate-reducing communities have been associated with U(VI) reduction in other sites as well (14). Overall, U(VI) levels corresponded to both acetate and SO_4^{2-} consumption (Fig 1), suggesting the possible role of acetate-utilizing SRB in U(VI) reduction in LF sediments; however, whether this role is direct or indirect (i.e. acetate-utilizing SRB providing a substrate, such as H_2, for U(VI)-reducing bacteria) is unclear.

The slowing of U(VI) reduction upon the onset of methanogenesis suggested U(VI)-reducing bacteria may require concomitant SO_4^{2-} or Fe(III) reduction in these LF sediments. It is also possible that methanogens may have out-competed U(VI)-reducing bacteria in the sediment for remaining electron donor. Similarly, it has been observed in both a microcosm study (33) and an *in situ* study (3) with U-contaminated sediments, that when U(VI)-reduction was concomitant with Fe(III) reduction, a change in geochemical conditions (to SO_4^{2-} reduction) resulted in the loss of U(VI)-reducing activity. Thus, populations actively involved in U(VI) reduction (e.g. SRB,

Fe(III)-reducing bacteria) at different sites may depend on natural geochemical conditions and microbial populations present at the onset of U(VI) reduction.

U(VI) reduction by LF sediments in microcosms under sulfate-reducing, iron-reducing, and methanogenic communites

(i) *Preincubations to stimulate different TEAPs*. LF sediments were pre-incubated with ethanol and electron acceptors (Table 1) to stimulate sulfate-reducing (SR), Fe(III)-reducing (FeR), and methanogenic (Meth) geochemical conditions and microbial populations. Sulfate loss from each treatment group indicated that SR conditions were established in SR bottles, and that the addition of 0.5 mM molybdate was successful in inhibiting SO_4^{2-} loss in FeR and Meth bottles (Fig 2A). Fe(II) was produced in FeR bottles (with and without molybdate) to much higher concentrations than in SR and Meth bottles (Fig 2B). As well, more methane accumulated in Meth bottles than in other treatment groups (Fig 2C). The addition of molybdate resulted in more methane production compared to Meth bottles without added molybdate. In all, geochemical data suggests that the desired TEA conditions were established in SR, FeR, and Meth bottles during pre-incubations. However, because some methane was produced in even SR and FeR bottles by the end of pre-incubations, geochemical conditions of sediments may have slightly shifted though the dominant TEAP occurring in SR and FeR bottles during preincubations were sulfate reduction and Fe(III) reduction, respectively.

(ii) U(VI) immobilization and reduction under different TEA conditions. Soluble U(VI) loss was monitored under SR, FeR, and Meth conditions. Within 7 days, the majority of U(VI) was removed from SR, FeR, and Meth bottles (89, 93, and 66%

loss, respectively) (Figs 3A-C). In SR (+SO4) bottles, the addition of 1 mM SO_4^{2-} had little effect on the amount of soluble U(VI) removed within seven days (81.4%), but the addition of molybdate to SR bottles after pre-incubation resulted in complete inhibition of soluble U(VI) (Fig 3A) removal. However, the addition of molybdate (added either during or after pre-incubation period), had little effect of U(VI) loss under FeR and Meth conditions (Fig 3B, C). Experimental controls verified that molybdate concentration did not affect U(VI) measurement by kinetic phosphorescence analysis (KPA) (data not shown); thus, molybdate did have a negative effect on initial U(VI) immobilization by SR communities, compared to FeR and Meth communities. Differential extractions of sediment-associated U after seven days from each treatment group, however, showed that U speciation in all treatment groups remained largely U(VI) (data not shown), indicating that loss of soluble U(VI) during this time period was due to sorption or precipitation. Formaldehyde inhibited measurement of U(VI) by KPA in liquid samples and therefore the extent to which abiotic factors contributed to initial U(VI) immobilization could not be determined.

After 26 days of incubation, bicarbonate and nitric acid extractions of sediments revealed that U(VI) reduction to U(IV) had occurred to an equal extent in SR, FeR, and Meth control bottles (no significant difference between the amount of U(IV)/g sediment in each treatment group). Abiotic reduction highest in FeR bottles (Fig 4), suggesting that reduced Fe(II) minerals formed during iron reduction may contribute significantly to U(VI) reduction. In SR bottles, the addition of sulfate to SR (+SO4) bottles did not significantly increase the amount of U(IV) associated per gram

sediment compared to the no-sulfate control (SR) (Fig 4), suggesting that concomintant SO_4^{2-} reduction was not required for U(VI) reduction in these sediments.

Interestingly, though molybdate had no effect on initial U(VI) immobilization in FeR and Meth bottles (Figs 3B and C), the presence of 0.5 mM molybdate significantly negatively affected U(VI) reduction under all (SR, FeR, and Meth) conditions (Fig 4). Although molybdate is a "specific" competitive inhibitor of sulfate reduction, the use of this inhibitor has also been found to inhibit reductive dechlorination in sediments under methanogenic conditions, regardless of sulfate reduction (6, 13). Also, it has been found that H_2 uptake by sediments is inhibited by 20 mM molybdate (37). Because H_2 is an important intermediate in anaerobic sediments, it is possible that the addition of molybdate may inhibit TEAPs other than sulfate reduction, such as U(VI) reduction and reductive dechlorination, if H_2, rather than ethanol or acetate, is being used as an electron donor by bacteria involved in the reductive processes.

(iii) PLFA microbial community analysis of U(VI)-reducing sediments under different TEA conditions. PLFAs were extracted and analyzed from sediments from each treatment group frozen after 26 days of incubation with U(VI). Based on amount of total PLFA/g sediment, all samples contained within one order of magnitude of 10^6 cells/g sediment (Fig 5A). No correlation was seen between biomass and sediment U(IV) concentration, suggesting that U(VI) reduction is not a function of general microbial biomass. The addition of SO_4^{2-} to SR (+SO4) bottles resulted in an increase in biomass compared to the control SR group (Fig 5A), and a decrease in the 16Carbon trans/cis ratio (Fig 5B), which indicates the stress level of organisms in an

environment. Though SO_4^{2-} addition had a positive impact on biomass and stress levels in SR bottles, there was little significant increase in sediment U(IV) concentration in SR bottles, indicating that U(VI) reduction by these SO_4^{2-} reducing sediments is neither inhibited nor stimulated by sulfate addition.

The major groups of PLFAs of each treatment group were normal saturates (NSats), monounsaturates (Monos), and terminal-branched saturates (TBSats) (Fig 5C), which are generally ubiquitous among all bacteria (NSats), gram-negative bacteria (Monos), and gram-postive bacteria (TBSats) (53). Specific dominant PLFAs included 16:1w7c (25-36% of total PLFAs in all bottles), 16:0 (18-31%), and 18:1w7c (5.5-17%) (Table 2). These three fatty acids were also found in the C^{13}-labelled PLFA fraction from ^{13C}ethanol-amended sediment slurries undergoing U(VI) reduction in a stable isotope probing study (33) and were also found to be enriched from ethanol addition during an *in situ* U(VI) bioremediation study in which nitrate was also present (44); thus these PLFAs may represent sediment microorganisms that generally respond to ethanol addition.

Though group PLFA profiles appeared similar in all treatment groups (Fig 5C), PLFA profiles revealed different microbial community compositions under each TEA condition stimulated during pre-incubations in LF sediments (Fig 6), as there were differences in individual PLFAs among each treatment group (Table 2). For example, 16:0 (NSat), 16:1w7c (Mono), cy17:0 (Mono), and 10Me16:0 (MBSat) were all found in greater proportions of total PLFA in FeR bottles than SR and Meth bottles (Table 2), and redundancy analysis revealed these were more associated with FeR bottles than others (Fig 6A). Interestingly, cy17:0 and 10Me16:0 are PLFAs associated with the

sulfate-reducing genus *Desulfobacter* (53), and not particularly with any known dissimilatory Fe(III)-reducing bacteria. PLFAs associated with SR bottles included Monos 18:1w7c, 18:1w7t, and 17:1w8, and those associated with Meth bottles included Monos 17:1, 15:1, and 16:1w9c, the NSat 15:0, and the MBSat br16:0 (Table 2, Fig. 6A).

Molybdate affected microbial community composition in all TEA treatment groups. In SR bottles, molybdate addition (SR (+M) bottle) resulted in a microbial community structure more similar to Meth bottles than SR and SR (+SO4) bottles (Figs 6A and B). Similarly, in Meth bottles without added molybdate (where some sulfate reduction did occur during pre-incubations—Fig. 2A), community composition based on PLFAs was similar to SR bottles (Figs 6A and B). As well, FeR bottles without molybdate grouped as closely with SR bottles as FeR bottles with molydate (Figs 6A and B). In SR bottles, the molybdate addition resulted in the decrease of the *Desulfobacter*-asociated MBSat 10Me16:0, as well as a decrease in 18:0, i16:0, and 18:1w9c, compared to SR bottles without molybdate (Table 2). Overall, MBSats were 40% lower in proportion to total PLFA in SR (+M) compared to SR. In Meth bottles with molybdate, there was a decrease in the TBSat a15:0 compared to the control Meth bottle without molybdate (Table 2). Overall, there was 29% less TBSats in Meth (+M) than in Meth bottles. Though TBSats are generally indicative of gram-positive bacteria, they are also common in some SRB as well (53). Also observed in Meth bottles were decreases in the proportion of 18:1w9c (common in both bacteria and microeucaryotes), 18:0, and polysaturates 18:2w6 and 18:3w3 (typical of microeukaryotes and some deep-sea microorganisms) in bottles with molybdate added

compared to the control (Table 2). Branched monounsaturates (BMonos), which are indicative of Fe(III)- and/or sulfate-reducing bacteria (particularly *Desulfovibrio*) (53), were not very abundant in any of the treatment groups, but the addition of molybdate in FeR(+M) bottles resulted in a 73% decrease in BMonos, with nearly all BMonos found less in FeR bottles containing molybdate (Table 2) compared to the FeR control; this effect of molybdate on BMonos was not seen in SR or Meth conditions (Table 2). Thus, molybadate affected microbial populations differently, depending on the TEA conditions stimulated during pre-incubations. It is also possible that molybdate affected different groups of SRB under each condition.

Lastly, molybdate contributed to a small decrease in 18:0 in all treatment groups (Table 2). This fatty acid was also found in the C^{13}-labelled PLFA fraction from ^{13C}ethanol-amended sediment slurries undergoing U(VI) reduction (33) and is common in microeukaryotes and methylotrophs (53). Any potential role of these subsurface microorganisms in U(VI) reduction is currently unknown. This fatty acid was also positively correlated with U(IV) concentrations in sediments ($r = +0.605$, $p<0.05$).

Among all treatment groups, none of the major PLFA groups were significantly positively correlated with μmol U(IV)/g sediment (Table 3), suggesting that there is not one group of U(VI)-reducing population present under all geochemical conditions. Different groups of PLFA were positively associated with U(IV) under SR, FeR, and Meth conditions. There were positive correlations between Monos and U(IV) ($r=0.926$) in SR bottles, between BMonos and U(IV) in FeR bottles ($r=0.886$), and between TBSats and U(IV) in Meth bottles ($r=0.999$) (Table 3).

Though only the latter correlation coefficient is significant ($p<0.05$), the different associations of different PLFA groups with U(IV) in SR, FeR, and Meth bottles suggests that different populations are associated U(VI) reduction under different geochemical conditions. Because different microbial populations are likely involved in U(VI) reduction under different terminal electron accepting conditions, it may prove useful to understand the biogeochemical processes and microbial populations that dominate a field site when engineering an efficient U(VI) bioremediation strategy.

References

1. **Abdelouas, A., W. Lutze, W. Gong, E. H. Nuttall, B. A. Strietelmeier, and B. J. Travis.** 2000. Biological reduction of uranium in groundwater and subsurface soil. Sci Total Environ **250:**21-35.

2. **Akob, D. M., H. J. Mills, T. M. Gihring, L. Kerkhof, J. W. Stucki, A. S. Anastacio, K. J. Chin, K. Kusel, A. V. Palumbo, D. B. Watson, and J. E. Kostka.** 2008. Functional diversity and electron donor dependence of microbial populations capable of U(VI) reduction in radionuclide-contaminated subsurface sediments. Appl Environ Microbiol **74:**3159-70.

3. **Anderson, R. T., H. A. Vrionis, I. Ortiz-Bernad, C. T. Resch, P. E. Long, R. Dayvault, K. Karp, S. Marutzky, D. R. Metzler, A. Peacock, D. C. White, M. Lowe, and D. R. Lovley.** 2003. Stimulating the in situ activity of *Geobacter* species to remove uranium from the groundwater of a uranium-contaminated aquifer. Appl Environ Microbiol **69:**5884-91.

4. **Baldwin, B. R., A. D. Peacock, M. Park, D. M. Ogles, J. D. Istok, J. P. McKinley, C. T. Resch, and D. C. White.** 2008. Multilevel samplers as microcosms to assess microbial response to biostimulation. Ground Water **46:**295-304.

5. **Balkwill, D. L., F. R. Leach, J. T. Wilson, J. F. McNabb, and D. C. White.** 1988. Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface aquifer sediments. Microb Ecol **16:**73-84.

6. **Basu, S. K., J. A. Oleszkiewicz, and R. Sparling.** 2005. Effect of sulfidogenic and methanogenic inhibitors on reductive dehalogenation of 2-chlorophenol. Environ Technol **26:**1383-91.

7. **Beeman, R. E., and J. M. Suflita.** 1987. Microbial ecology of a shallow unconfined ground water aquifer polluted by municipal landfill leachate. Microb Ecol **14:**39-54.

8. **Bligh, E. G., and W. J. Dryer.** 1954. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology **37:**911-917.

9. **Cappenberg, T. E.** 1974. Interrelations between sulfate-reducing and methane-producing bacteria in bottom deposits of a fresh-water lake. II. Inhibition experiments. Antonie Van Leeuwenhoek **40:**297-306.

10. **Cardenas, E., W. M. Wu, M. B. Leigh, J. Carley, S. Carroll, T. Gentry, J. Luo, D. Watson, B. Gu, M. Ginder-Vogel, P. K. Kitanidis, P. M. Jardine, J. Zhou, C. S. Criddle, T. L. Marsh, and J. M. Tiedje.** 2008. Microbial

communities in contaminated sediments, associated with bioremediation of uranium to submicromolar levels. Appl Environ Microbiol **74:**3718-29.

11. **Chang, Y. J., P. E. Long, R. Geyer, A. D. Peacock, C. T. Resch, K. Sublette, S. Pfiffner, A. Smithgall, R. T. Anderson, H. A. Vrionis, J. R. Stephen, R. Dayvault, I. Ortiz-Bernad, D. R. Lovley, and D. C. White.** 2005. Microbial incorporation of 13C-labeled acetate at the field scale: detection of microbes responsible for reduction of U(VI). Environ Sci Technol **39:**9039-48.

12. **Cozzarelli, I. M., J. M. Suflita, G. A. Ulrich, S. H. Harris, M. A. Scholl, J. L. Schlottmann, and S. Christenson.** 2000. Geochemical and microbiological methods for evaluating anaerobic processes in an aquifer contaminated by landfill leachate. Environ Sci Technol **34:**4025-4033.

13. **El Fantroussi, S., E. Ntibahezwa, S. Thomas, H. Naveau, and S. N. Agathos.** 1998. Effect of specific inhibitors on the anaerobic reductive dechlorination of 2,4,6-trichlorophenol by a stable methanogenic consortium. Anaerobe **4:**197-203.

14. **Elias, D. A., L. R. Krumholz, D. Wong, P. E. Long, and J. M. Suflita.** 2003. Characterization of microbial activities and U reduction in a shallow aquifer contaminated by uranium mill tailings. Microb Ecol **46:**83-91.

15. **Elias, D. A., J. M. Senko, and L. R. Krumholz.** 2003. A procedure for quantitation of total oxidized uranium for bioremediation studies. J Microbiol Methods **53:**343-53.

16. **Francis, A. J., C. J. Dodge, F. Lu, G. P. Halada, and C. R. Clayton.** 1994. XPS and XANES Studies of Uranium Reduction by *Clostridium* sp. . Environmental Science and Technology **28:**636-639.

17. **Gao, W., and A. J. Francis.** 2008. Reduction of uranium(VI) to uranium(IV) by clostridia. Appl Environ Microbiol **74:**4580-4.

18. **Gu, B., H. Yan, P. Zhou, D. B. Watson, M. Park, and J. Istok.** 2005. Natural humics impact uranium bioreduction and oxidation. Environ Sci Technol **39:**5268-75.

19. **Guckert, J. B., C. P. Antworth, P. D. Nichols, and D. C. White.** 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediment. FEMS Microbial Ecology **31:**147-158.

20. **Holmes, D. E., K. T. Finneran, R. A. O'Neil, and D. R. Lovley.** 2002. Enrichment of members of the family *Geobacteraceae* associated with

stimulation of dissimilatory metal reduction in uranium-contaminated aquifer sediments. Appl Environ Microbiol **68:**2300-6.

21. **Hwang, C., W. Wu, T. J. Gentry, J. Carley, G. A. Corbin, S. L. Carroll, D. B. Watson, P. M. Jardine, J. Zhou, C. S. Criddle, and M. W. Fields.** 2009. Bacterial community succession during in situ uranium bioremediation: spatial similarities along controlled flow paths. ISME J **3:**47-64.

22. **Istok, J. D., J. M. Senko, L. R. Krumholz, D. Watson, M. A. Bogle, A. Peacock, Y. J. Chang, and D. C. White.** 2004. In situ bioreduction of technetium and uranium in a nitrate-contaminated aquifer. Environ Sci Technol **38:**468-75.

23. **Khijniak, T. V., A. I. Slobodkin, V. Coker, J. C. Renshaw, F. R. Livens, E. A. Bonch-Osmolovskaya, N. K. Birkeland, N. N. Medvedeva-Lyalikova, and J. R. Lloyd.** 2005. Reduction of uranium(VI) phosphate during growth of the thermophilic bacterium *Thermoterrabacterium ferrireducens*. Appl Environ Microbiol **71:**6423-6.

24. **Liu, C., Y. A. Gorby, J. M. Zachara, J. K. Fredrickson, and C. F. Brown.** 2002. Reduction kinetics of Fe(III), Co(III), U(VI), Cr(VI), and Tc(VII) in cultures of dissimilatory metal-reducing bacteria. Biotechnol Bioeng **80:**637-49.

25. **Lovley, D. R., and E. J. Phillips.** 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl Environ Microbiol **51:**683-689.

26. **Lovley, D. R., and E. J. Phillips.** 1987. Rapid assay for microbially reducible ferric iron in aquatic sediments. Appl Environ Microbiol **53:**1536-1540.

27. **Lovley, D. R., and E. J. Phillips.** 1992. Reduction of uranium by *Desulfovibrio desulfuricans*. Appl Environ Microbiol **58:**850-6.

28. **Lovley, D. R., and E. J. P. Phillips.** 1992. Bioremediation of uranium contamination with enzymatic uranium reduction. . Environmental Science and Technology **26:**2228-2234.

29. **Lovley, D. R., E. J. P. Phillips, Y. A. Gorby, and E. R. Landa.** 1991. Microbial reduction of uranium. Nature **350**: 413-416

30. **Lovley, D. R., E. E. Roden, E. J. P. Phillips, and J. C. Woodward.** 1993. Enzymic iron and uranium reduction by sulfate-reducing bacteria. . Marine Geology **113:**41-53.

31. **Luo, W., W. M. Wu, T. Yan, C. S. Criddle, P. M. Jardine, J. Zhou, and B. Gu.** 2007. Influence of bicarbonate, sulfate, and electron donors on biological

reduction of uranium and microbial community composition. Appl Microbiol Biotechnol **77:**713-21.

32. **Madden, A. S., A. V. Palumbo, B. Ravel, T. A. Vishnivetskaya, T. J. Phelps, C. W. Schadt, and C. C. Brandt.** 2009. Donor-dependent extent of uranium reduction for bioremediation of contaminated sediment microcosms. J Environ Qual **38:**53-60.

33. **Mohanty, S. R., B. Kollah, D. B. Hedrick, A. D. Peacock, R. K. Kukkadapu, and E. E. Roden.** 2008. Biogeochemical processes in ethanol stimulated uranium-contaminated subsurface sediments. Environ Sci Technol **42:**4384-90.

34. **Nevin, K. P., K. T. Finneran, and D. R. Lovley.** 2003. Microorganisms associated with uranium bioremediation in a high-salinity subsurface sediment. Appl Environ Microbiol **69:**3672-5.

35. **North, N. N., S. L. Dollhopf, L. Petrie, J. D. Istok, D. L. Balkwill, and J. E. Kostka.** 2004. Change in bacterial community structure during in situ biostimulation of subsurface sediment cocontaminated with uranium and nitrate. Appl Environ Microbiol **70:**4911-20.

36. **Nyman, J. L., T. L. Marsh, M. A. Ginder-Vogel, M. Gentile, S. Fendorf, and C. Criddle.** 2006. Heterogeneous response to biostimulation for U(VI) reduction in replicated sediment microcosms. Biodegradation **17:**303-16.

37. **Oremland, R. S., and D. G. Capone.** 1988. Use of specific inhibitors in biogeochemistry and microbial ecology. Adv Microb Ecol **10:**285-383.

38. **Peacock, A. D., Y. J. Chang, J. D. Istok, L. Krumholz, R. Geyer, B. Kinsall, D. Watson, K. L. Sublette, and D. C. White.** 2004. Utilization of microbial biofilms as monitors of bioremediation. Microb Ecol **47:**284-92.

39. **Petrie, L., N. N. North, S. L. Dollhopf, D. L. Balkwill, and J. E. Kostka.** 2003. Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). Appl Environ Microbiol **69:**7467-79.

40. **Pinkart, H. C., D. B. Ringelberg, Y. M. Piceno, S. J. MacNaughton, and D. C. White.** 2002. Biochemical approaches to biomass measurements and community structure analysis, p. 101-113. *In* C. J. Hurst, R. L. Crawford, G. R. Knudsen, M. J. McInerney, and L. D. Stetzenbach (ed.), Manual of Environmental Microbiology Second Edition. ASM Press, Washington D.C.

41. **Senko, J. M., J. D. Istok, J. M. Suflita, and L. R. Krumholz.** 2002. In-situ evidence for uranium immobilization and remobilization. Environ Sci Technol **36:**1491-6.

42. **Shelobolina, E. S., S. A. Sullivan, K. R. O'Neill, K. P. Nevin, and D. R. Lovley.** 2004. Isolation, characterization, and U(VI)-reducing potential of a facultatively anaerobic, acid-resistant bacterium from low-pH, nitrate- and U(VI)-contaminated subsurface sediment and description of *Salmonella subterranea* sp. nov. Appl Environ Microbiol **70:**2959-65.

43. **Shelobolina, E. S., H. A. Vrionis, R. H. Findlay, and D. R. Lovley.** 2008. *Geobacter uraniireducens* sp. nov., isolated from subsurface sediment undergoing uranium bioremediation. Int J Syst Evol Microbiol **58:**1075-8.

44. **Spain, A. M., A. D. Peacock, J. D. Istok, M. S. Elshahed, F. Z. Najar, B. A. Roe, D. C. White, and L. R. Krumholz.** 2007. Identification and isolation of a *Castellaniella* species important during biostimulation of an acidic nitrate- and uranium-contaminated aquifer. Appl Environ Microbiol **73:**4892-904.

45. **Sung, Y., K. E. Fletcher, K. M. Ritalahti, R. P. Apkarian, N. Ramos-Hernandez, R. A. Sanford, N. M. Mesbah, and F. E. Loffler.** 2006. *Geobacter lovleyi* sp. nov. strain SZ, a novel metal-reducing and tetrachloroethene-dechlorinating bacterium. Appl Environ Microbiol **72:**2775-82.

46. **Suzuki, Y., S. D. Kelly, K. M. Kemner, and J. F. Banfield.** 2005. Direct microbial reduction and subsequent preservation of uranium in natural near-surface sediment. Appl Environ Microbiol **71:**1790-7.

47. **Suzuki, Y., S. D. Kelly, K. M. Kemner, and J. F. Banfield.** 2003. Microbial populations stimulated for hexavalent uranium reduction in uranium mine sediment. Appl Environ Microbiol **69:**1337-46.

48. **Thomas, S. H., E. Padilla-Crespo, P. M. Jardine, R. A. Sanford, and F. E. Loffler.** 2009. Diversity and distribution of *Anaeromyxobacter* strains in a uranium-contaminated subsurface environment with a nonuniform groundwater flow. Appl Environ Microbiol **75:**3679-87.

49. **Vrionis, H. A., R. T. Anderson, I. Ortiz-Bernad, K. R. O'Neill, C. T. Resch, A. D. Peacock, R. Dayvault, D. C. White, P. E. Long, and D. R. Lovley.** 2005. Microbiological and geochemical heterogeneity in an in situ uranium bioremediation field site. Appl Environ Microbiol **71:**6308-18.

50. **Wall, J. D., and L. R. Krumholz.** 2006. Uranium reduction. Annu Rev Microbiol **60:**149-66.

51. **Wan, J., T. K. Tokunaga, Y. Kim, E. Brodie, R. Daly, T. C. Hazen, and M. K. Firestone.** 2008. Effects of organic carbon supply rates on uranium mobility in a previously bioreduced contaminated sediment. Environ Sci Technol **42:**7573-9.

52. **White, D., W. M. Davis, J. S. Nickels, J. D. King, and R. J. Bobbie.** 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia **40:**51-62.

53. **White, D. C., J. O. Stair, and D. B. Ringelberg.** 1996. Quantitative comparisons of in situ microbial biodiversity by signature biomarker analysis. Journal of Industrial Microbiology **17:**185-196.

54. **Wu, Q., R. A. Sanford, and F. E. Loffler.** 2006. Uranium(VI) reduction by *Anaeromyxobacter dehalogenans* strain 2CP-C. Appl Environ Microbiol **72:**3608-14.

55. **Wu, W. M., J. Carley, J. Luo, M. A. Ginder-Vogel, E. Cardenas, M. B. Leigh, C. Hwang, S. D. Kelly, C. Ruan, L. Wu, J. Van Nostrand, T. Gentry, K. Lowe, T. Mehlhorn, S. Carroll, W. Luo, M. W. Fields, B. Gu, D. Watson, K. M. Kemner, T. Marsh, J. Tiedje, J. Zhou, S. Fendorf, P. K. Kitanidis, P. M. Jardine, and C. S. Criddle.** 2007. In situ bioreduction of uranium (VI) to submicromolar levels and reoxidation by dissolved oxygen. Environ Sci Technol **41:**5716-23.

Table 1. Summary of amendments made to each treatment groups set up to examine U(VI) reduction under different terminal electron accepting conditions.

Pre-incubation treatment groups and amendments				Treatment groups during U(VI) reduction		
TEA Conditions	Amendments (final concentrations)	Days of pre-incubation	No. of replicates	Subgroup	Amendments (final concentrations).	No. of replicates
Sulfate-Reducing (SR)	2.5mM SO_4^{2-} 2.5 mM ethanol	25	14	SR (+F)	2 mM ethanol, 100 µM U(VI)[2] 2% formaldehyde	2
				SR	2 mM ethanol, 100 µM U(VI)[2]	4
				SR (+SO4)	2 mM ethanol, 100 µM U(VI)[2] 1.0 mM SO_4^{2-}	4
				SR (+M)	2 mM ethanol, 100 µM U(VI)[2] 0.5 mM molybdate	4
Fe(III)-Reducing (FeR)	10 mM Fe(III)[1] 2.5 mM ethanol	15	10	FeR (+F)	2 mM ethanol, 100 µM U(VI)[2] 2% formaldehyde	2
				FeR	2 mM ethanol, 100 µM U(VI)[2]	4
				FeR (+M)	2 mM ethanol, 100 µM U(VI)[2] 0.5 mM molybdate	4
Fe(III)-Reducing + Molybdate (FeR+M)	10 mM Fe(III)[1] 0.5 mM molybdate 2.5 mM ethanol	15	6	FeR+M (+F)	2 mM ethanol, 100 µM U(VI)[2] 2% formaldehyde	2
				FeR+M	2 mM ethanol, 100 µM U(VI)[2]	4
Methanogenic (Meth)	2.5 mM ethanol	25	10	Meth (+F)	2 mM ethanol, 100 µM U(VI)[2] 2% formaldehyde	2
				Meth	2 mM ethanol, 100 µM U(VI)[2]	4
				Meth (+M)	2 mM ethanol, 100 µM U(VI)[2] 0.5 mM molybdate	4
Methanogenic + Molybdate (Meth+M)	2.5 mM ethanol 0.5 mM molybdate	25	6	Meth+M (+F)	2 mM ethanol, 100 µM U(VI)[2] 2% formaldehyde	2
				Meth+M	2 mM ethanol, 100 µM U(VI)[2]	4

[1]Fe(III) was added as an amorphous iron oxyhydroxide gel, prepared as previously described (25).
[2]U(VI) was added from an anoxic uranyl hydroxide solution dissolved in bicabonate, prepared as previously described (15)

Table 2. PLFA distributions among different treatment groups undergoing U(VI) reduction (Day 26).

	% of total PLFA in each treatment group								
	SR	SR (+SO4)	SR (+M)	FeR	FeR (+M)	FeR+M	Meth	Meth (+M)	Meth +M
NSats									
13:0	0.00	0.00	0.00	0.00	0.00	0.00	0.28	0.00	0.00
14:0	2.05	1.58	2.95	3.02	2.06	3.56	3.13	3.81	3.17
15:0	1.26	0.89	0.93	0.60	0.45	0.45	1.15	1.50	2.37
16:0	24.22	20.79	19.60	26.01	28.14	30.74	18.31	27.81	25.42
17:0	0.82	2.30	0.47	0.78	0.32	0.30	0.63	0.90	1.16
18:0	1.53	2.92	0.59	1.07	0.78	0.64	2.39	1.98	1.33
20:0	0.49	0.00	0.00	0.00	0.00	0.00	0.31	0.00	0.00
MB Sats									
br15:0	0.00	0.00	0.00	0.11	0.29	0.27	0.00	0.00	0.00
br16:0	0.35	0.21	0.18	0.00	0.22	0.10	0.21	0.30	0.31
10Me16:0	1.67	1.35	1.05	2.35	3.42	2.27	1.16	1.80	1.54
12Me16:0	0.33	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.28
br18:0	0.21	0.11	0.00	0.00	0.13	0.00	0.11	0.00	0.00
i10Me16:0	0.10	0.17	0.31	0.06	0.07	0.00	0.17	0.00	0.19
10Me18:0	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2Me18:0	0.00	0.00	0.00	0.00	0.22	0.00	0.64	0.00	0.00
10Me19:0	0.51	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TB Sats									
i14:0	0.57	0.25	0.32	0.30	0.09	0.18	1.11	0.34	0.41
i15:0	4.04	5.20	7.92	6.49	9.99	5.50	5.05	4.39	5.15
a15:0	3.82	4.34	8.76	5.38	1.37	1.85	5.50	3.57	2.80
i16:0	1.84	0.67	0.52	1.16	0.37	0.46	1.45	1.11	0.81
BMonos									
i16:1	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
br16:1	0.00	0.14	0.00	0.22	0.16	0.00	0.00	0.00	0.00
i17:1	0.00	0.96	2.22	1.94	0.20	0.24	0.77	1.41	0.63
a17:1	0.00	0.00	0.00	0.42	0.00	0.00	0.00	0.00	0.00
i17:0	0.88	0.65	0.73	0.92	0.56	0.62	0.72	0.73	0.81
a17:0	0.76	0.49	0.82	1.69	0.37	0.65	0.82	0.59	0.54
Monos									
14:1	0.00	0.17	0.00	0.20	0.00	0.06	0.51	0.00	0.00
15:1	0.00	0.00	0.48	0.00	0.00	0.00	0.00	0.00	2.45
16:1w9c	0.88	0.65	5.55	0.17	0.21	1.08	0.48	2.08	7.46
16:1w7c	29.99	26.25	33.13	31.89	33.52	36.23	25.73	31.46	24.50
16:1w7t	1.70	1.14	1.43	1.16	1.35	1.17	1.03	1.18	0.99
16:1w5c	2.13	2.47	1.28	1.52	1.87	1.68	1.56	2.82	2.07
17:1	0.78	0.26	0.56	0.00	0.12	0.00	0.29	0.62	4.95
17:1w8	0.26	0.17	0.00	0.24	0.00	0.00	0.00	0.00	0.00
cy17:0	3.45	1.83	2.76	4.92	7.02	4.72	2.02	3.47	2.85
18:1w9c	2.68	5.15	1.09	1.17	0.89	1.01	11.91	2.14	2.31
18:1w7c	10.81	16.61	6.08	6.18	5.62	5.90	5.85	5.99	5.48
18:1w7t	0.43	0.43	0.27	0.00	0.00	0.00	0.16	0.00	0.00
18:1w5c	0.00	1.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
19:1	0.00	0.47	0.00	0.00	0.00	0.00	0.00	0.00	0.00
cy19:0	1.07	0.16	0.00	0.00	0.00	0.33	0.81	0.00	0.00
Polys									
18:2w6	0.36	0.00	0.00	0.00	0.00	0.00	3.72	0.00	0.00
18:3w3	0.00	0.00	0.00	0.00	0.00	0.00	0.86	0.00	0.00

Table 3. Pearson correlation coefficients between major PLFA groups and μmol U(IV)/ g sediment in LF sediment microcosms.

PLFA Group	Pearson correlation coffiecient (r)			
	All treatment groups (n=9)	SR subgroups (n=3)	FeR subgroups (n=3)	Meth subgroups (n=3)
NSats	+0.089	+0.826*	-0.047	-0.964**
TBSats	-0.276	-0.956**	+0.267	+0.999***
Monos	-0.326*	+0.926*	-0.646	-0.494
MBSats	+0.348*	+0.466	-0.893*	+0.367
BMonos	-0.098	-0.820*	+0.886*	+0.044

*0.1<p<0.2
**0.05<p<0.1
***p<0.05

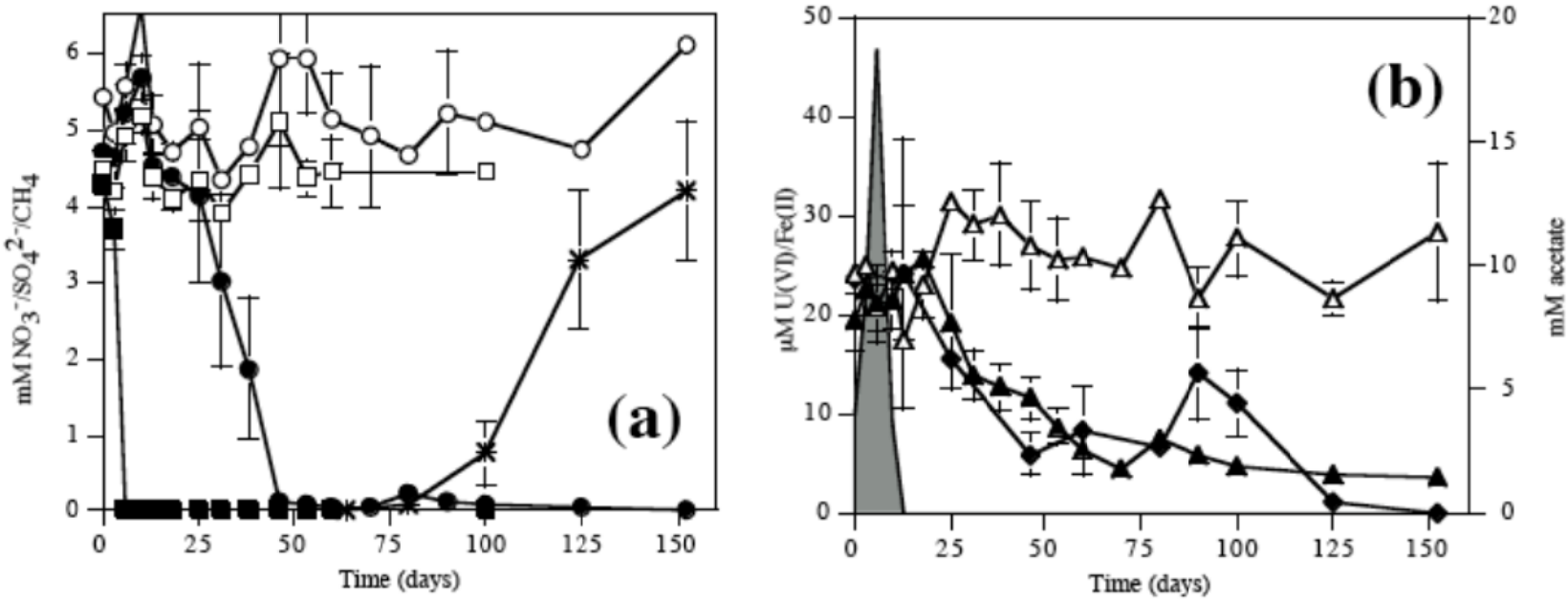

Figure 1. Succession of TEAPs in LF Aquifer sediments amended with acetate. **(a)** Loss of NO_3^- (squares) and SO_4^{2-} (circles) in live (closed symbols) vs. heat-killed (open symbols) sediments and CH_4 production from live sediments (✱). No CH_4 production was observed in heat-killed controls. **(b)** Loss of U(VI) (triangles) in live (closed symbols) vs. heat-killed (open symbols) sediments, Fe(II) accumulation (shaded) and acetate loss (diamonds) in live sediments. Fe(II) production and acetate loss were not observed in heat-killed controls.

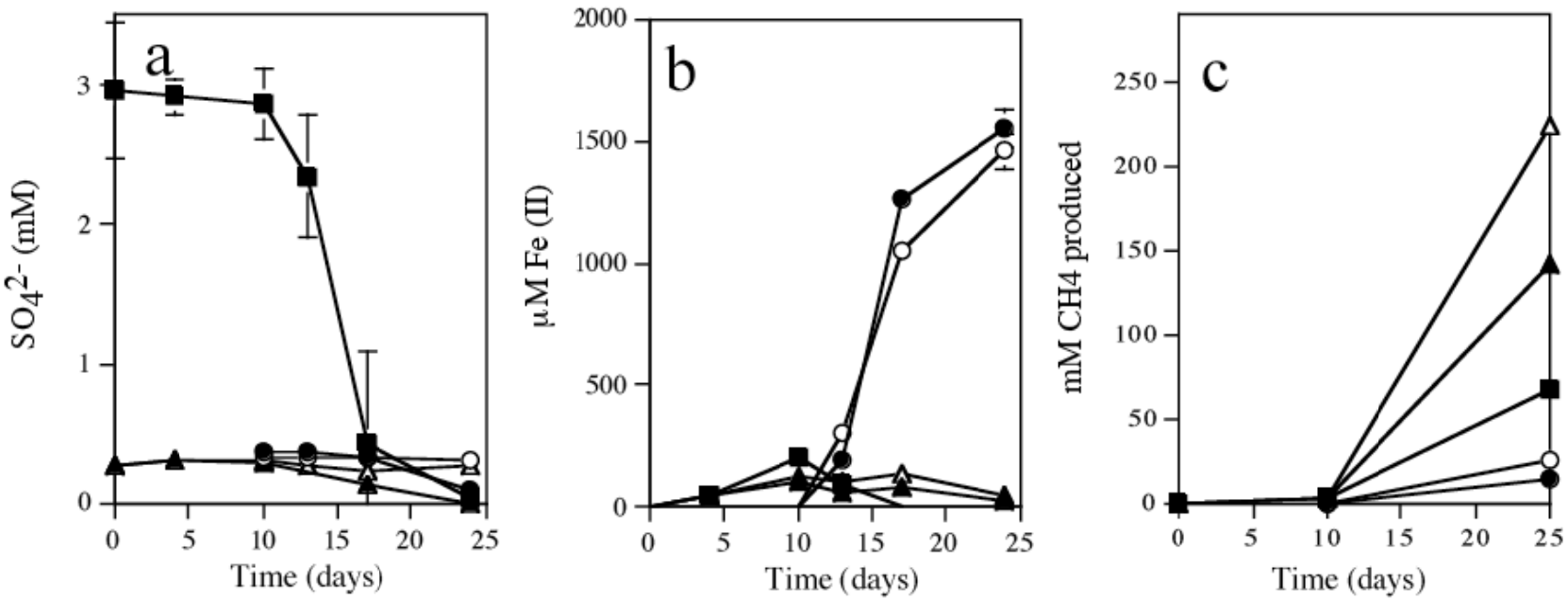

Figure 2. Sulfate (a), Fe(II) (b), and methane (c) data from pre-incubations stimulating sulfate-reducing (squares), iron-reducing (circles), and methanogenic (triangles) conditions. Samples with added molybdate to inhibit growth of sulfate-reducing bacteria are represented by open symbols. Error bars represent standard deviations of triplicate samples at each timepoint.

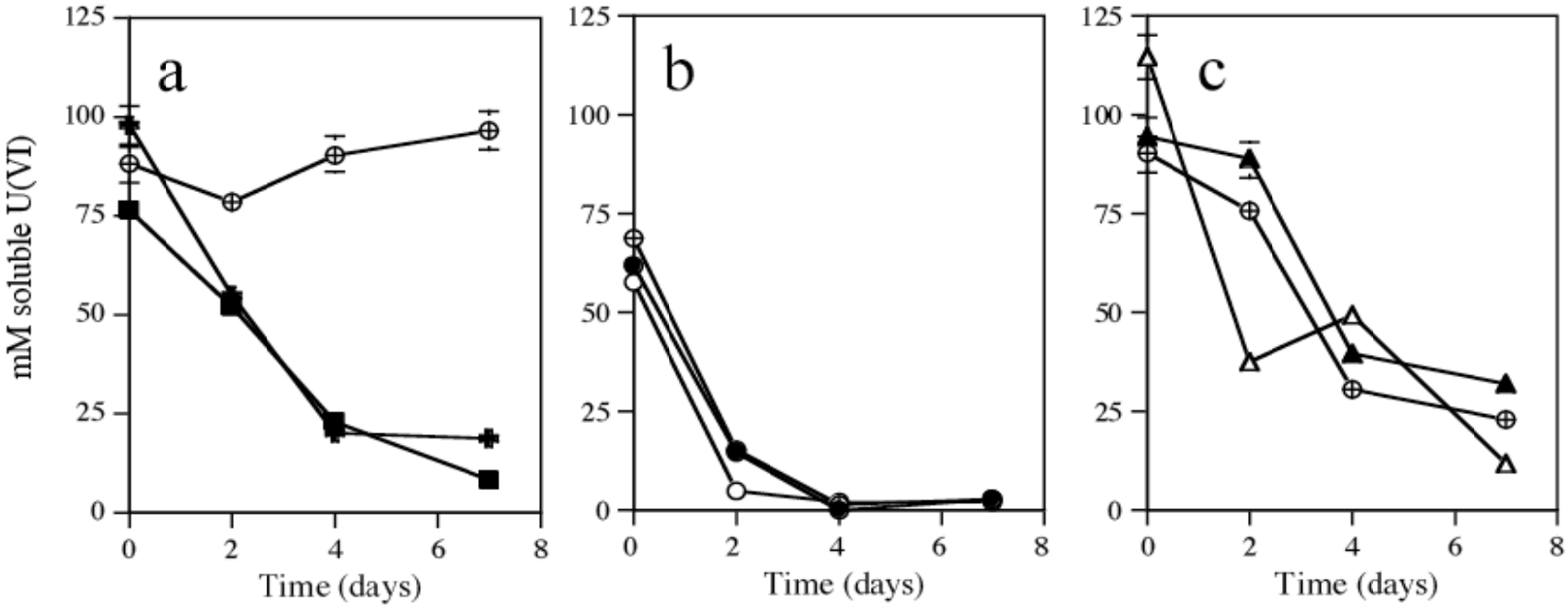

Figure 3 Loss of soluble U(VI) from groundwater in slurries set up in pre-incubations to stimulate sulfate-reducing (a), iron-reducing (b), and methanognic (c) conditions. Sulfate-reducing bottles (a) were divided into three treatment groups upon U(VI) addition: SR [sulfate-reducing, no added sulfate (■)], SR(+SO4) [sulfate-reducing, 1 mM added sulfate (✚)], and SR+(M) [sulfate-reducing, 0.5 mM molybdate ⊕)]. Iron-reducing bottles (b) consisted of treatment groups FeR [iron-reducing (●)], FeR+M [iron-reducing, 0.5 mM molybdate added during pre-incubation (○)], and FeR(+M) [iron-reducing, 0.5 mM molybdate added after pre-incubation (⊕)]. Similarly methanogenic bottles (c) consisted of treatment groups Meth [methanogenic (▲)], Meth+M [methanogenic, 0.5 mM molybdate added during pre-incubation (△)], and Meth(+M) [methanogenic, 0.5 mM molybdate added after pre-incubation (⊕)]. Formaldehyde from killed controls inhibited soluble U(VI) measurments, thus killed controls are not shown for Days 0-7.

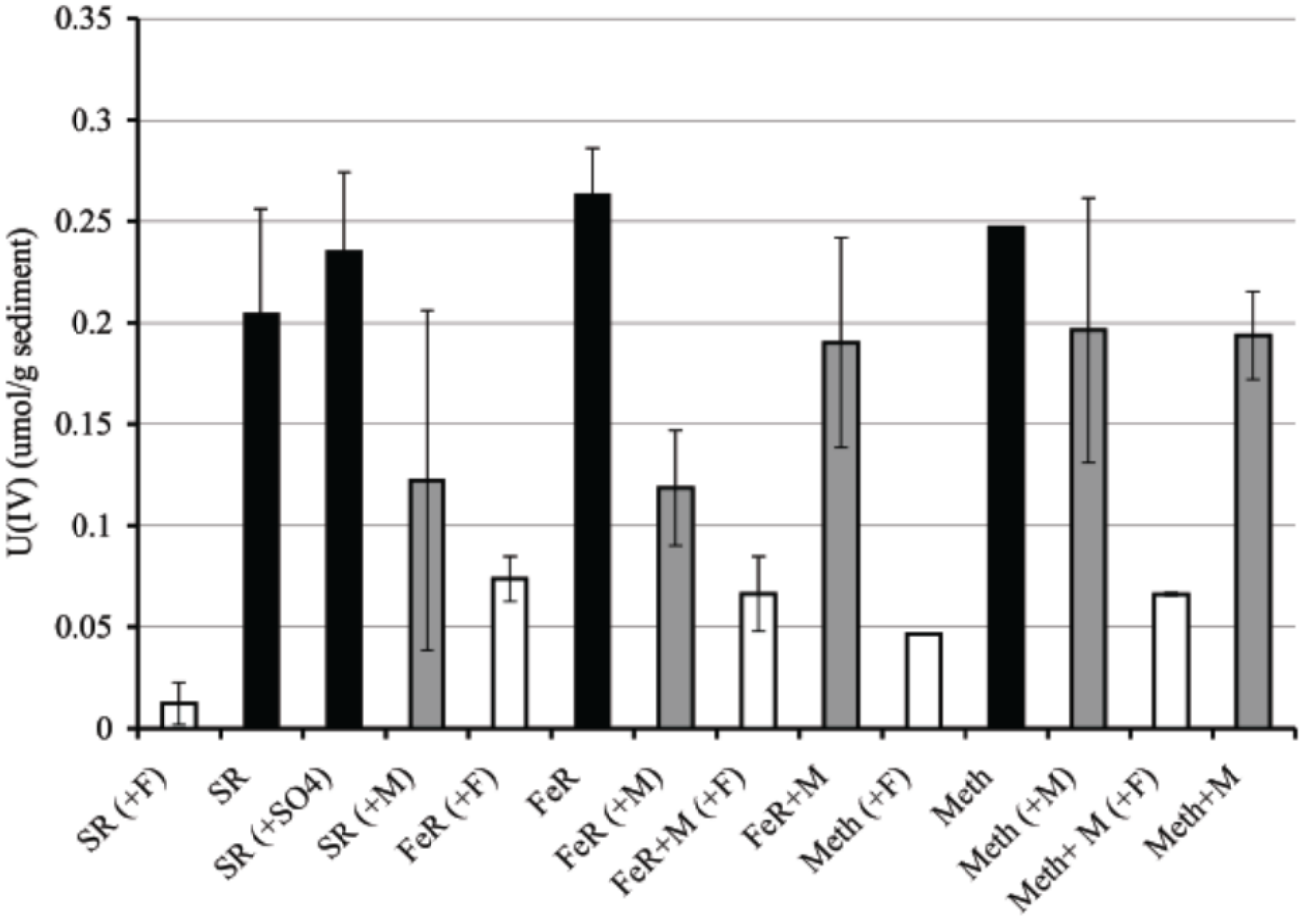

Figure 4. Amount of reduced U(IV) in sediment for each treatment group. White bars indicate formaldehyde-killed controls. Gray bars indicate treatment groups amended with 0.5 mM molybdate. Black bars represent live treatment groups with no added formaldehyde or molybdate.

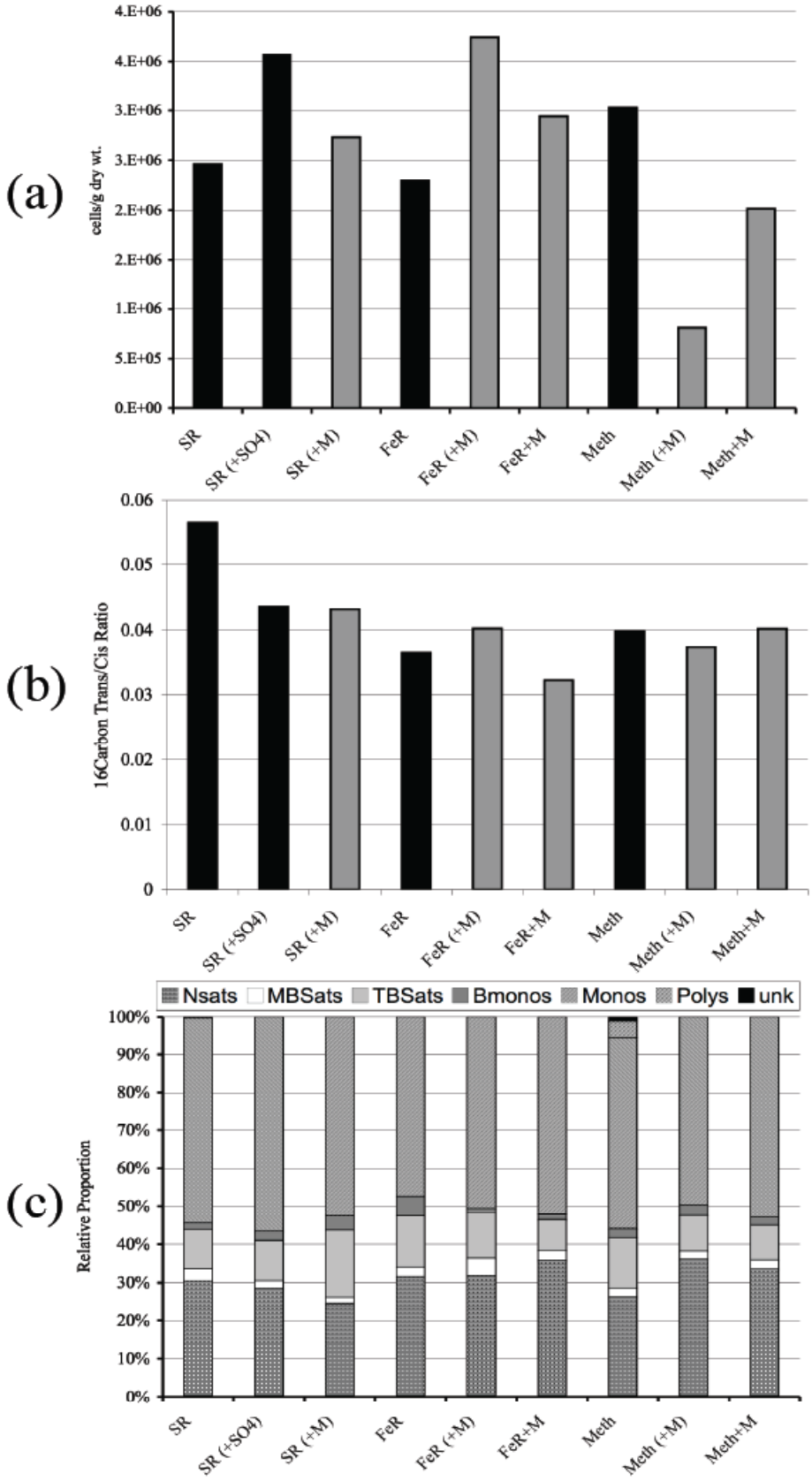
(a)
cells/g dry wt.
4.E+06
4.E+06
3.E+06
3.E+06
2.E+06
2.E+06
1.E+06
5.E+05
0.E+00
SR
SR (+SO4)
SR (+M)
FeR
FeR (+M)
FeR+M
Meth
Meth (+M)
Meth+M
(b)
16Carbon Trans/Cis Ratio
0.06
0.05
0.04
0.03
0.02
0.01
0
SR
SR (+SO4)
SR (+M)
FeR
FeR (+M)
FeR+M
Meth
Meth (+M)
Meth+M
(c)
Nsats
MBSats
TBSats
Bmonos
Monos
Polys
unk
Relative Proportion
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
SR
SR (+SO4)
SR (+M)
FeR
FeR (+M)
FeR+M
Meth
Meth (+M)
Meth+M

Figure 5 (previous page). PLFA data from sediment samples in live treatment groups after 26 days incubation with U(VI). (a) Viable biomass of each treatment group, based on amount of total PLFA/g dry sediment. (b) 16 Carbon Trans/Cis Ratio of each treatment group, an indicator of the stress of organisms in an environment. Ratios <0.05 indicate conditions of non-stress whereas ratios > 0.1 indicate starvation or exposure to toxins. (c) Microbial community profile of each treatment group, based on lipid composition. Nsats = normal saturates (ubiquitous); MBSats = mid-chain branched saturates (sulfate-reducers); TBSats = terminally branched saturates (gram-positives); Bmonos = branched monounsaturates (iron/sulfate reducers); Monos = monounsaturates (gram-negatives); Polys = polyunsaturates (eukaryotes).

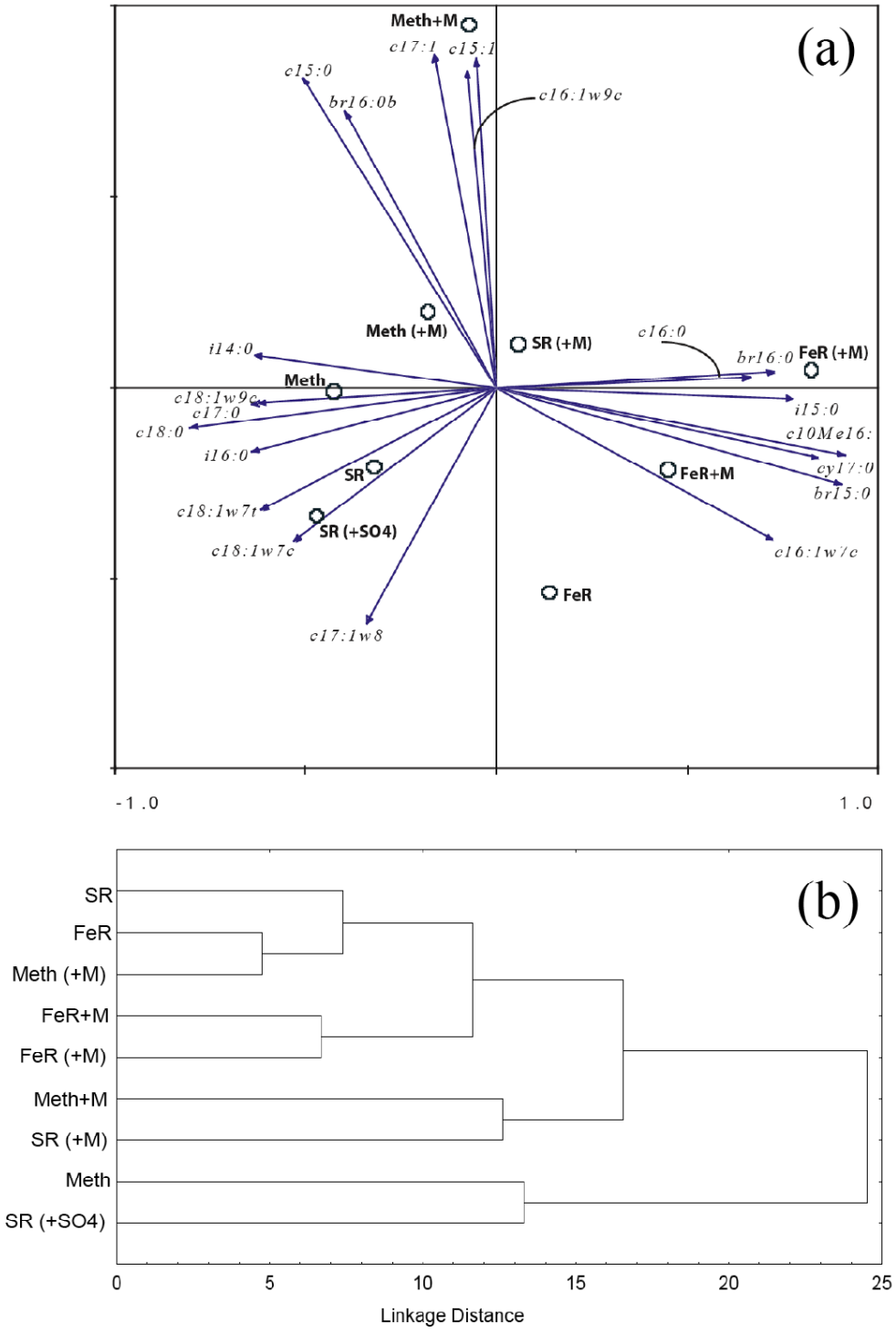

Figure 6. Microbial community PLFA profiles from treatment groups. (a) Redundancy analysis plot, showing the association of different fatty acids among treatment groups. (b) Tree diagram displaying Euclidean distances between each treatment group, showing the microbial community relatedness of the different treatment groups.

CHAPTER 3

Identification and Isolation of a *Castellaniella* Species Important During Biostimulation of an Acidic Nitrate- and Uranium-Contaminated Aquifer

Abstract

Immobilization of uranium in groundwater can be achieved through microbial reduction of U(VI) to U(IV) upon electron donor addition. Microbial community structure was analyzed in ethanol-biostimulated and control sediments from a high-nitrate (>130 mM), low pH, uranium-contaminated site in Oak Ridge, TN. Analysis of SSU rRNA gene clone libraries and polar lipid fatty acids (PLFAs) from sediments revealed that biostimulation resulted in a general decrease in bacterial diversity. Specifically, biostimulation resulted in an increase in the proportion of β-*Proteobacteria* (10% of total clones in the control sediment vs. 50 and 79% in biostimulated sediments), and a decrease in the proportion of γ-*Proteobacteria* and *Acidobacteria*. Clone libraries derived from dissimilatory nitrite reductase genes (*nir*K and *nir*S) were also dominated by clones related to β-*Proteobacteria* (98% and 85% of total *nir*K and *nir*S clones, respectively). Within the *nir*K libraries, one clone sequence made up 59 and 76% of sequences from biostimulated sediments but only made up 10% of the control *nir*K library. Phylogenetic analysis of SSU rRNA and *nir*K gene sequences from denitrifying pure cultures isolated from the site indicate that

all belong to a *Castellaniella* species; nearly identical sequences also constituted the majority of biostimulated SSU rRNA and *nir*K clone libraries. Thus, by combining culture-independent with culture-dependent techniques, we were able to link SSU rRNA clone library information with *nir*K sequence data and conclude that a potentially novel *Castellaniella* species is important for *in situ* nitrate removal at this site.

Introduction

Due to the Cold War Legacy, uranium has become an important groundwater contaminant in the United States, thus mandating remediation by the US Department of Energy (DOE). Soluble U(VI) can be biologically reduced to U(IV), which is insoluble, thus immobilizing the radionuclide and posing less of a threat to drinking water wells located near sources of contamination (24, 44). It has been suggested that bacteria capable of U(VI) reduction are ubiquitous in the environment (1), and recent field experiments have shown that the addition of electron donors (glucose, ethanol, or acetate) into injection wells will result in the stimulation of endogenous microorganisms in the subsurface to grow and reduce U(VI) (3, 12, 36, 54, 60, 64).

Microbial communities stimulated to reduce U(VI) via electron donor addition have been studied using both *in situ* and microcosm experiments. Members of *Geobacteraceae* family have been stimulated during uranium-reduction in contaminated sediments from ShipRock, NM (33), Rifle, CO (3, 13), and Oak Ridge, TN (51, 54). From studies done with sediment from Oak Ridge, *Anaeromyxobacter* was also stimulated under metal-reducing conditions (51, 55). In other studies, sulfate-reducing bacteria have been linked to uranium reduction (1, 13, 49, 52, 61). Of these, two studies have also found *Clostridium* to also be associated with U(VI) reduction (52, 61), and another found that *Pseudomonas* was also stimulated upon uranium removal in high salinity sediment (49).

At the DOE Field Research Center (FRC) in Oak Ridge, TN, where groundwater contains >130 mM nitrate and micromolar concentrations of uranium, addition of a biodegradable electron donor results in denitrification as the primary

terminal electron-accepting process (36). Because nitrate serves as a more energetically favorable electron acceptor, uranium reduction has been shown to occur only after nitrate has been depleted to low levels (17, 23, 36, 48, 60). Thus, at sites such as the FRC, denitrifying bacteria are likely to play a critical role in uranium bioremediation. A recent phylogenetic survery of sediment from the FRC revealed several potential nitrate-reducing bacteria (2), but it remains unclear what species are involved in nitrate removal upon biostimulation.

The goal of this study was to characterize changes in the *in-situ* microbial community structure of uranium- and nitrate-contaminated subsurface sediments upon stimulation with ethanol, and to identify denitrifying bacteria that may be important during the *in-situ* removal of nitrate. While other molecular studies have identified mainly sulfate- and metal-reducers in uranium-contaminated sediments, it was hypothesized in this study that electron donor addition to high-nitrate subsurface sediments co-contaminated with low levels of uranium would result mainly in the stimulation of denitrifying bacteria. Because denitrification is not a phylogenetically conservered function, numerous methods were used to analyze the microbial community structure of biostimulated and control sediments, including functional gene (*nir*K and *nir*S) clone libraries, SSU rRNA gene clone libraries, polar lipid fatty acid analysis, and cultivation of nitrate-reducing bacteria from FRC sediments. Results of this study show that biostimulation of high-nitrate subsurface sediments with ethanol results in a decrease in bacterial diversity and enriches for members of the class β-*Proteobacteria*, namely members of the newly-described genus *Castellaniella (*formerly *Alcaligenes defragrans*), capable of nitrate reduction.

Materials and Methods

Field Site Description.

The field site in this study is the U.S. Department of Energy's (DOE's) Environmental Remediation Sciences Program Field Research Center (FRC), which is located near the western edge of the Y-12 national security complex at the Oak Ridge Reservation (Oak Ridge, TN). The source of the contamination plume in the shallow unconfined aquifer at the FRC comes from the former S-3 waste disposal ponds. These ponds received acidic (pH<2) liquid waste containing nitric acid, uranium, technetium, other dissolved metals, and organic contaminants from 1951 to 1983; the ponds were neutralized in 1984 and capped in 1988. Several monitoring wells have been installed within the Area 1 field plot (just south of the former S-3 ponds), and groundwater within Area 1 has been described as acidic (pH ranging from 3.0 to 6.8), with high concentrations of nitrate (up to 168 mM), U(VI) (up to 5.8 μM), Tc(VII) (up to 12,000 pCi/L), and < 1 mM sulfate (36). Table 1 shows nitrate, uranium, and pH data from four monitoring wells before push-pull experiments began. Other contaminants in Area 1 include aluminum, nickel, tetrachloroethylene, and other chlorinated hydrocarbons. A more detailed description of the site as well as groundwater and sediment geochemical data can be found at: http://www.esd.ornl.gov/nabirfrc/index.html.

In situ biostimulation of subsurface sediments.

Single-well, push-pull tests were done in wells FW028 and FW034 in Area 1 as previously described (36, 65, 66). Test solutions for push-pull tests were prepared using high-nitrate (>130 mM) Area 1 groundwater (from well FW021) amended with

300 mM ethanol, 50-100 mM sodium bicarbonate, and 1.25 mM Br^- as a conservative tracer. Sediment cores were sampled adjacent to wells FW028 and FW034 (cores FB064 and FB067, respectively) approximately one week following injection of test solutions; the injection phase lasted only a few hours for FW028 but lasted approximately three weeks for FW034, due to differences in well flow characteristics due to past push-pull experiments. Thus, FB064 and FB067 were sampled five and 31 days, respectively, after the beginning of the injection phase. One sediment core (FB066) was also taken adjacent to an Area 1 donor control well FW016, which has never been biostimulated in push-pull tests. Sediment sampling and handling procedures followed those previously described (65, 66) in order to keep sediment material anoxic. Core sizes were all approximately one meter in length and were sampled from the following depths below the surface: 6.1-7.0 m, 3.4-4.3 m, and 3.0-4.0 m for cores FB064, FB067, and FB066, respectively. Intact subsections of cores, approximately 10 cm in length, were frozen at -80°C and were later shipped on dry ice to the University of Oklahoma for molecular analysis. A subsection of another core from borehole FB064, taken from 5.2-5.7 m below the surface, was stored at 4°C and shipped to University of Oklahoma on ice for enrichment and isolation of denitrifying bacteria.

Enrichment and Isolation of Denitrifying Pure Cultures.

Media for enrichment of dissimilatory nitrate-reducing microorganisms was prepared anaerobically (5) with the following components (per liter): 10 ml vitamin solution (47); 5 ml metals solution (47); 0.1 g NaCl; 0.1 g NH_4Cl; 10 mg KCl; 3 mg KH_2PO_4; 40 mg $MgCl_2 \cdot 6H_20$; 40 mg $CaCl_2 \cdot 2H_20$; 11.9 g HEPES; 11.7 g MES; and 8.5 g

$NaNO_3$. The pH of the media was adjusted to either 4.5 or 7.5 using HCl or NaOH, and dispensed into serum tubes under a N_2 headspace. Ethanol was added from a sterile, anoxic stock solution to reach a final concentration of 100 mM.

Anaerobic nitrate-reducing enrichment cultures were set up in an anaerobic glovebag by adding 1 gram of homogenized biostimulated sediment from borehole FB064 to 10 ml nitrate-reducing liquid medium at both pH 4.5 and 7.5. Headspace of enrichment cultures was exchanged three times with N_2. Enrichment cultures were incubated in the dark at room temperature. Upon observable growth and removal of nitrate, enrichments were serially diluted and plated onto solid nitrate-reducing media both with and without ethanol at either pH 4.5 or 7.5, depending on the pH of the enrichment culture. Nitrate-reducing solid media had the same composition as the liquid media except contained 1.5% agar and 1.7 g/L $NaNO_3$. After autoclaving, media was dispensed into plates and dried overnight. Plates were placed in an anaerobic glovebag (Coy Instruments) overnight. Subsequently, a piece of sterile filter paper was placed in the lid of each Petri dish and saturated with 500 μl of a sterile, anoxic 1 M ethanol solution. All plates were incubated at room temperature in an anaerobic glove bag. Colonies from plates containing ethanol that differed in morphology from colonies on ethanol-free plates were further re-isolated and transferred to nitrate-reducing liquid media at pH 4.5 or 7.5. In total, 24 colonies were obtained from pH 7.5 enrichment cultures and 22 from pH 4.5 enrichment cultures.

DNA extraction. DNA was extracted from frozen soil cores from boreholes FB064, FB067, and FB066 (from depths of 6.4, 4.6, and 3.6 m below the surface, respectively) using the FastDNA® SPIN Kit for Soil (QBiogene, Irvine, CA), which involves a

silica and ceramic bead-beating method to achieve cell lysis. Manufacturer's instructions were followed except nuclease-free water was used as the eluent. In order to increase DNA yield and to account for heterogeneity of the cores, 10 DNA extractions using 0.3 g sediment were done from each core. The 10 DNA samples were then pooled and concentrated by centrifugation at 13,000 rpm at 45°C. Samples were stored at -20°C.

DNA was extracted from pure cultures by boiling late-log phase washed cells for five minutes; samples were centrifuged to remove cell debris, and supernatants were transferred to clean, sterile 1.5 ml microcentrifuge tubes and stored at -20°C for use as DNA template for PCR reactions.

PCR, cloning, and sequencing.

Partial SSU rRNA genes from sediment community DNA and denitrifying isolates were amplified using 2 μl of DNA template in a 50 μl PCR mixture (< 100 ng/μl final concentration) containing the components (final concentrations): 1X PCR buffer (Invitrogen Corp., Carlsbad, CA), 2.5 mM $MgCl_2$, 100 μM each deoxynucleoside triphosphate, 10 pmol/ml each primer (uni8f and eubac805r) (19), and 1.5 U of Platinum® *Taq* DNA polymerase (Invitrogen). Amplification of partial SSU rRNA genes was carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) using the following parameters: initial denaturation at 94°C for 5 min; 35 cycles of 95°C for 30 sec, 50°C for 60 sec, and 72°C for 90 sec; and a final extension step at 72°C for 20 min. Near complete SSU rRNA genes of two denitrifying isolates

(4.5A2 and 7.5A2) were amplified in the same manner, only using universal primers 27F and 1492R and an annealing temperature of 45°C.

Amplification of *nir*K and *nir*S genes from sediment community DNA and denitrifying isolates used the same PCR reaction mixture as described above, except that primer concentrations were 12.5 pmol/ml; *nir*K primers were nirK1F and nirK5R, and *nir*S primers were nirS1F and nirS6R (9). PCR parameters were as follows: 94°C for 5 minutes; 35 cycles of 94°C for 30 sec, 54°C for 45 sec, and 72°C for 45 sec; and a final extension at 72°C for 20 min.

PCR products were cloned using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA) either directly from PCR product or after a gel-purification step using a commercially available kit (QBioGene). Sequencing of inserts was performed in the Advanced Center for Genome Technology at the University of Oklahoma (Norman, OK) or at the Oklahoma Medical Research Foundation (Oklahoma City, OK).

Phylogenetic Anaylsis.

SSU rRNA gene sequences were aligned using ClustalX (62). Sequences with similarities ≥ 97% were placed into the same operational taxonomic unit (OTU); also, sequences with ≥ 93% similarity were placed into the same genus-level taxonomic group (GLTG). Possible chimera within our libraries were identified using Bellerophon (34) and by manual inspection. Chimeric sequences made up approximately 10% of total sequences and were removed from further phylogenetic analyses. Initial phylogenetic placement of each SSU rRNA gene OTU was determined using the Ribosomal Database's (RDPs) Classifier program (14). Closely related sequences and sequences identified from this site in previous studies were

downloaded from GenBank and aligned with our sequences using ClustalX; the multiple alignment was imported into PAUP 4.01b10 for final phylogenetic analysis. Evolutionary distance-based trees were generated using the neighbor-joining algorithm and Jukes-Cantor corrections. Bootstrap values were determined using 1000 replicates.

Shannon-Weiner diversity index, Simpson's dominance index, and species evenness were calculated as previously described (57). A limitation of these indices is that each OTU is considered equivalent, regardless of the degree of sequence divergence (46). To ameliorate this bias, diversity indices were calculated at both the OTU level as well as the GLTG level; also, average nucleotide divergence was calculated for each clone library (46). Calculations of % coverage were done as described (58) at both the OTU and GLTG level.

A chi-square test for an r x k contingency table was done to determine whether population distribution in biostimulated samples differed from the unstimulated sample. Rows (r) were phylum affiliation and columns (k) were different samples (biostimulated and unstimulated). Expected frequencies for each phylum in each sample (E) were calculated by E = (Row Total) x (Column Total) / Grand Total. Chi-squared value was determined by $\chi^2 = \Sigma (O-E)^2 / E$. The critical χ^2 value was chosen with nine degrees of freedom and with a p-value of 0.05.

Phylogenetic analysis of *nir*K and *nir*S genes was done similarly to that of SSU rRNA gene described above. Sequences were grouped into OTUs based on ≥98% nucleotide sequence similarity, and the closest relatives were identified and downloaded using BLAST. Other reference *nir*K and *nir*S sequences were

downloaded from the Functional Gene Pipeline/Repository (http://flyingcloud.cme.msu.edu/fungene/). Neighbor-joining trees were constructed from translated amino acid sequences. Similarity values reported in the results are based on amino acid similarity.

PLFA extraction and analysis.

Lyophilized sediment from each core was extracted with the single-phase chloroform-methanol-buffer system (8), as later modified (67). The total lipid extract was fractionated into neutral lipids, glycolipids, and polar lipids by silicic acid column chromatography (29). Methods of PLFA analysis were conducted as previously described (56). Biomass (cells/g sediment) was calculated from total PLFA/g sediment using the conversion $2.5x10^4$ cells per pmol PLFA (6). Shannon-Weiner diversity indices for sediment sample were calculated based on PLFA (31).

Analytical Methods.

Uranium speciation from sediment cores FB064, FB067, and FB066 was determined by sequential extractions of total U(VI) (soluble and solids-associated) and U(IV) from triplicate 0.5 g sediment subsamples using sodium bicarbonate and nitric acid, respectively (18). Uranium from each extraction was measured by kinetic phosphorescence analysis (KPA-11; Chemcheck Instruments, Richland, WA). Nitrate and nitrite from nitrate-reducing enrichments and sediment-associated porewater were measured by ion chromatography (Dionex, model DX500 fitted with the AS-4A column; Dionex Corporation, Sunnyvale, CA). Push-pull groundwater analysis was done at Oregon State University as previously described (36).

GenBank accession numbers. SSU rRNA, *nir*K, and *nir*S sequences from this study were deposited to GenBank and can be retrieved from accession numbers EF175318 to EF175380 and EF177768 to EF177803.

Results

Isolation and Phylogenetic Analysis of Denitrifying Pure Cultures. From nitrate-reducing enrichments using biostimulated sediment as inoculum, all 46 pure cultures, once re-streaked for purity, shared the same colony morphology on nitrate-reducing media; colonies were convex, round, small (<1mm in diameter) and white, with smooth margins. Upon inoculation into liquid media at pH 4.5 and pH 7.5, all pure cultures were capable of growth (to a final O.D. of approximately 0.4 at 600 nm) using nitrate and ethanol as the sole electron acceptor and donor, respectively; gas production indicated that the organisms coupled growth to denitrification rather than reduction of nitrate to nitrite or ammonium. Because of the similar colony morphologies and growth characteristics, 10 of the pure cultures were chosen at random for phylogenetic analysis; SSU rRNA gene sequences of these isolates were 97.6-100% similar to each other with an average similarity of 99.4%, suggesting these isolates belong same species within the family *Alcaligenaceae* and the class β-*Proteobacteria*.

Two strains, 4.5A2 and 7.5A2 (isolated at pH 4.5 and 7.5, respectively), which had 99.9% SSU rRNA gene sequence similarity, were chosen for further phylogenetic analysis. Isolates 4.5A2 and 7.5A2 were 99.4 and 99.7% similar to clone FB46-16, which was identified from biostimulated FRC sediments in a previous study (51). The closest cultured relative was *Alcaligenes* sp. AMS10, which was isolated from a PAH-

degrading consortium (GenBank accession no. AY635901). The closest validly described relatives belong to the genus *Castellaniella*, which consists of two described species, *C. defragrans* and *C. denitrificans*, both of which were previously identified as *Alcaligenes defragrans* (40). Isolates 4.5A2 and 7.5A2 were 98.3 and 98.5% similar to *C. defragrans* 54Pin, which was isolated from activated sludge on nitrate and α-pinene (25), and 98.4% similar to *C. denitrificans* TJ4, a phenol-degrading, denitrifying bacterium (4). Neighbor joining analysis and bootstrap values supported that FRC isolates 4.5A2 and 7.5A2 may not belong to either of the previously described species of *Castellaniella* and could represent a novel species within the genus *Castellaniella* (Fig. 1). However, further physiological tests are needed to prove this.

While the *nir*S gene was not detected by PCR in any of the ten isolates, all contained a *nir*K gene, which provides evidence that these strains are denitrifying bacteria. All *nir*K partial gene sequences from these isolates were 99.0-100% similar to each other, reaffirming that these isolates are likely different strains among the same species. Furthermore, translated amino acid sequences of NirK from isolates 4.5A2 and 7.5A2 were 100% identical to each other, 84.8% identical to NirK of a clone identified from acetate-fed activated sludge (clone NR2-819K1, GenBank accession no. BAD36891), and 81.8% identical to NirK from *Alcaligenes* sp. N., isolated from a denitrifying reactor (20).

***In situ* biostimulation of contaminated subsurface sediments and reduction of U(VI).** Push-pull tests were done with ethanol-amended, high-nitrate (142.3 mM) FW021 groundwater (neutralized with bicarbonate) in two wells, FW028 and FW034.

Prior to biostimulation, the groundwater from FW028 contained high levels of nitrate (167.2 mM) and uranium (2.2 μM) and was more acidic than FW034, which contained < 1mM nitrate and 0.475 μM uranium (Table 1). The control well, FW016, was also acidic but contained 11.4 mM nitrate (Table 1). Following injection of ethanol-amended FW021 groundwater into FW028 and FW034, push-pull data showed nitrate and ethanol loss in both test wells by the time of sediment sampling, and U(VI) accumulation in FW028, suggesting U(IV) oxidation may have occurred in this well (Table 1). However, analysis of uranium from bicarbonate- and nitric-acid-extractable fractions from sediment cores showed that the majority of the uranium in both cores adjacent to ethanol-stimulated wells (FB064 and FB067, corresponding to wells FW028 and FW034, respectively) was reduced whereas only 4.6% of the total uranium from the control core FB066 (adjacent to FW016) was reduced (Table 1), suggesting that the U in stimulated cores remained fairly reduced, compared to the control, which has never been biostimulated. Some of the U(IV) in biostimulated cores may have been due to previous push-pull tests performed in adjacent wells (36). Biomass estimates based on total PLFA from sediment cores following *in situ* biostimulation showed that FB064 and FB067 had approximately 37- and three-fold higher biomass than the control core, FB066 (Table 1). Porewater nitrate concentrations from the three cores varied, which can be explained by the differences in initial nitrate concentrations of the three sites, while nitrite was present at high concentrations (≥ 10 mM) in all three (Table 1). Nitrate and nitrite data, however, indicate that nitrate reduction was not complete in these sediment cores.

Differences in bacterial community structure between ethanol-stimulated and un-stimulated sediment samples.

(i) Diversity statistics. According to all diversity indices calculated from SSU rRNA gene clone library data (at both OTU and GLTG level), both biostimulated sediments, FB064 and FB067, were less diverse than the control sediment, FB066 (Table 2). The percent coverage was 64, 78, and 71% (at the OTU level) and 83, 83, and 80% (at the GLTG level) for sediment samples FB064, FB067, and FB066, respectively. There was a significant negative linear correlation between log biomass of the sediments and average nucleotide divergence (AND) ($r = -0.999$, $p = 0.01$), indicating that genetic diversity decreased with increasing biomass. Similarly, when diversity indices were calculated based on GLTGs, there were negative correlations between log biomass vs. Shannon-Weiner diversity index ($r = -0.992$, $p < 0.05$) and log biomass vs. evenness ($r = -0.999$, $p = 0.01$). In addition, there was a positive correlation between log biomass and Simpson's Dominance index at both the OTU level ($r = 0.977$, $p < 0.1$) and the GLTG level ($r = 0.993$, $p < 0.04$), indicating that increasing biomass resulted in the selection of one dominant species or genus. Correlations between log biomass and diversity indices were more significant when using GLTGs rather than OTUs; this is due to the high number of OTUs in sample FB064 that belonged to the same GLTG. Taking all diversity indices into account, biostimulation may have led to an overall decrease in bacterial diversity and increase in dominance of one species or genus. Past push-pull biostimulation experiments performed in injection wells FW028 and FW034 (36) may have also contributed to this effect.

(ii) Community composition. The majority of clones from SSU rRNA gene clone libraries from biostimulated sediment cores, FB064 and FB067, belonged to β, δ, and γ subdivisions of *Proteobacteria* (88.5%); the remaining clones belonged to *Bacteroidetes, Firmicutes, Actinobacteria, Acidobacteria,* and candidate divisions TM7, ZB1, Termite Group I, and WD272_C2 (Table 3, Figs. 2 and 3). In the SSU rRNA gene clone library from the un-stimulated sediment core (FB066), *Proteobacteria* (β and γ subdivisions) made up only 56.9% of the total clones, while other clones were affiliated with *Acidobacteria* (27.5% of total clones), *Bacteroidetes, Firmicutes*, and candidate division WD272_C2 (Table 3, Figs. 2 and 3). By performing chi-square tests based on r x k contingency tables of frequencies of each phylum, it was found that the community structures of the two biostimulated samples (FB064 and FB067) did not differ significantly ($p > 0.2$) whereas community structures of the biostimulated vs. un-stimulated samples were significantly different ($p < 0.001$). Thus, biostimulation of subsurface sediments with ethanol-amended groundwater significantly impacted the subsurface microbial community structure at the phylum/division level. Most noticeably, these differences may have been due to the frequencies of *Proteobacteria* and *Acidobacteria* OTUs in the biostimulated vs. the control clone libraries (Table 3).

Biostimulation resulted in an increase in the proportion of β-*Proteobacteria* sequences in the SSU rRNA gene clone libraries (9.8% of total clones in FB066 compared to 79.3% and 50.0% in FB064 and FB067, respectively) (Table 3). As biomass of the samples increased (Table 1), so did the percent of clones that belong to β-*Proteobacteria* (Table 3). Of the β-*Proteobacteria* clones from the core with the

highest biomass, FB064, 69.6% belonged to OTU 34 and 10.7% belonged to OTU 35. Both OTUs 34 and 35 grouped with members of the genus *Castellaniella* (Fig. 2) and were 100% and 97.6% similar to FRC isolate 7.5A2, respectively. Only one clone from FB067 belonged to OTU 34; rather, 87.5% of β-*Proteobacteria* clones from FB067 belonged to OTU 45, whose closest relative was clone BIsii8 (97.8% similarity), which was identified from an industrial waste gas biofilter (26); its two closest cultured relatives were *Burkholderia brasilensis*, a N_2-fixing bacterium (GenBank accession no. AJ238360), and *Burkholderia kururiensis*, a TCE-degrading bacterium isolated from a TCE-contaminated aquifer (72).

Unlike the effect observed on the class β-*Proteobacteria*, biostimulation resulted in a decrease in the proportion of γ-*Proteobacteria* sequences in the SSU rRNA gene clone libraries (Table 3). In the control clone library (FB066), 47.1% of total clones were affiliated with γ-*Proteobacteria*, and of these, the majority (70.8%) belonged to the family *Xanthomonadaceae* while others are affiliated with *Pseudomonadaceae*. The dominant γ-*Proteobacteria* OTU from the control FB066 (OTU 100) belonged to the genus *Rhodanobacter* and was closely related to other sequences identified from unstimulated contaminated sites, including groundwater from the FRC (Fig. 2).

Similarly, biostimulated sediments contained a decreased proportion of *Acidobacteria* clones compared to the control sediment (Table 3). The dominant OTU from the control sediment sample FB066 (OTU 128) belonged to *Acidobacteria* and clustered with other environmental *Acidobacteria* clones (Fig. 3); however, only one

Acidobacteria-affiliated sequence was detected in the biostimulated libraries (Table 3).

(iii) Novel bacterial diversity identified in SSU rRNA gene clone libraries. From the three SSU rRNA gene clone libraries generated in this study, 7.5% of all clones belonged to divisions with no cultivated representatives. Three clones belonged candidate divisions TM7, Termite Group I, and ZB1 (Table 3, Fig. 3). Nine clones from FB066 and FB067 (belonging to OTUs 131, 132, 133, 134, and 164) clustered with each other and with other soil clones from the FRC (Fig. 3). The closest non-FRC relatives of these clones came from volcanic ash and PCB-polluted soil; bootstrap values from Fig. 3 support that these clones likely belong to the same division as these novel FRC sequences. This candidate division is within the lineage of WD272_C2, based on Hugenholtz taxonomy (16), and these sequences may either represent a novel division or a novel lineage within the *Firmicutes* (Fig. 3).

(iv) PLFA analysis of sediment samples. In accordance with clone library data, PLFA data (Table 4) shows that community structure was more diverse and evenly distributed in the unstimulated sample (FB066) compared to the two biostimulated sediment samples (FB064 and FB067). Shannon-Weiner (H) indices calculated from PLFA data further confirm that the unstimulated sediment was less diverse ($H = 2.774$) than the stimulated sediments, FB064 ($H = 1.908$) and FB067 ($H = 2.461$). As with clone library data, there was a significant negative linear correlation between log biomass and Shannon-Weiner diversity indices based on PLFA data ($r = -0.992$, $p < 0.05$).

As biomass of the samples increased (Table 1), so did the percentage of monounsaturates (Table 4), which are generally indicative of gram-negative bacteria (68). Furthermore, biostimulated samples contained a smaller percentage of terminal-branched saturates (TBSats) compared to the control (Table 4). TBSats are generally indicative of gram-positive bacteria; however, other microorganisms may contain these fatty acids as well (68).

Table 4 shows that the dominant PLFAs from the genus *Castellaniella* ($C_{16:0}$, $C_{16:1}\omega7c$, $C_{17:0}$ cyclo, and $C_{18:1}\omega7c$) (40) were higher in the biostimulated samples compared to the control. Although other microorganisms can contain these particular PLFAs, it is likely that some or most of these fatty acids that increased with biomass were derived from *Castellaniella* species, given that species of this genus were dominant in biostimulated clone libraries.

Denitrifying community composition based on *nir*K and *nir*S clone libraries.

From the three *nir*K clone libraries, 67 clones were sequenced and 10 OTUs were identified. From all three *nir*K libraries, 98.5% of clones had closest cultured relatives that are β-*Proteobacteria* (Table 5). Ethanol stimulation resulted in an increase in proportion of total sequences within *nir*K clone libraries that belong to OTU1K (Table 5, Fig. 4). Clones belonging to OTU1K made up 76 and 59.4% of total clones from libraries derived from biostimulated cores FB064 and FB067, respectively, but only 20% of the total clones from the control clone library from FB066. Also, OTU1K was 100% similar to the *nir*K sequences from isolate 4.5A2 and 7.5A2, indicating that these genes may belong to the same *Castellaniella* species dominant in nitrate-reducing enrichments and in SSUrRNA gene clone libraries from biostimulated

sediment (Fig. 4), although it is possible some of these genes belong to other species as horizontal transfer of *nir*K genes within a site has previously been implicated (32). Seventy percent of clones from the control *nir*K clone library from FB066 belonged to OTU7K, whose closest relative was the *nir*K gene product from *Alcaligenes* sp. DSM30128 (81.7% similarity). Amino acid sequences derived from OTU1K and OTU7K, however, were only 77.8% similar to each other.

Clone libraries from *nir*S genes were constructed from biostimulated samples FB064 and FB067 but not from the control core, FB066, since *nir*S PCR product could not be obtained from this sample. While the overwhelming majority of *nir*K clones seemed to belong to *Castellaniella*, *nir*S clone libraries were more diverse than *nir*K clone libraries (Table 5); although the reason for this difference in diversity is unknown, the inverse relationship between *nir*S and *nir*K diversity in groundwater at the FRC has previously been observed (69). From the *nir*S clone libraries constructed from the two biostimulated samples, FB064 and FB067, 136 clones were sequenced and 26 OTUs were identified. In accordance with *nir*K libraries, the majority of clones from the *nir*S libraries had closest cultured relatives that are β-*Proteobacteria* (84.6% of total clones); these clones, however, were related to families other than *Alcaligenaceae* (Table 5). The dominant OTU from FB064 was OTU1S (57.6% of total clones), which was closely related to the *nir*S gene product from the anaerobic benzene-degrading *Dechloromonas aromatica* (90.5% similarity) (Fig. 5). OTU20S made up 16.7% of the *nir*S clone library from FB064 (Table 5), and it's closest relative was clone R2-s02 (77.6% similarity), identified from a metallurgic wastewater treatment system (71) (Fig. 5). NirS sequences from FB067 were more diverse (Table

5), and the most abundant OTUs clustered with *D. aromatica* (OTUs 38S and 39S), *Ralstonia metallidurans* and *R. eutropha* (OTUs 14S, 18S, and 19S), and *Magnetospirillum magnetotacticum* (OTUs 27S, 28S, and 34S) (Fig. 5).

Discussion

By using a combination of PLFA analysis, SSU rRNA and functional gene (*nir*K and *nir*S) clone libraries, and a cultivation approach, we were able to examine the effect of biostimulation on microbial community structure and identify and isolate a microorganism that likely plays a role in nitrate removal in an acidic aquifer co-contaminated with nitrate and uranium. The use of PCR and cloning methods for microbial community analysis is qualitative or "semi-quantitative" due to several well-recognized limitations (30). In this study, PCR and cloning biases may have affected the frequency in which some OTUs and GLTGs in clone libraries were detected. Also, the limited number of clones analyzed may have led to underestimated levels of diversity and detection of only the most abundant species and genera. The percent coverage in each library ranged from 64-78% at the OTU level and 80-83% at the GLTG level. The use of PLFA analysis, however, as a quantitative measure helped demonstrate the inverse relationship between biomass and diversity, while the cultivation approach confirmed the dominance of *Castellaniella* in sediment from FB064 and its ability to grow on ethanol and nitrate. However, variations in numbers of specific organisms or groups were only semi-quantitative as they were based on

clone library data; a quantitative approach, such as real-time PCR or fluorescent *in situ* hybridization using group-specific primers/probes would help determine whether the numbers of organisms within these samples were different.

Several studies have documented impacts of radionuclide, heavy metal, and hydrocarbon contamination on microbial community structure, and the general consensus is that pollution decreases microbial diversity (22, 28, 39, 43, 45, 57). Two previous studies done on microbial community structures of pristine vs. contaminated areas of the aquifer at the FRC have found that contamination resulted in a decrease in microbial diversity and selected for β-*Proteobacteria* species related to or belonging to *Azoarcus* (22) and *Alcaligenaceae* (57). Furthermore, β-*Proteobacteria* were found to be abundant in other contaminated environments, including PCB-contaminated soil (50), a waste-gas biofilter (26, 27), metal- and petroleum-contaminated soil (39), heavy metal-amended soil microcosms (45), and metallurgic wastewater (70). Similarly, our results show that β-*Proteobacteria* SSU rRNA clones, primarily those affiliated with *Alcaligenaceae* and *Burkholderiaceae*, are present in contaminated sediment samples from the FRC (Fig. 2). Also, the majority of *nir*K and *nir*S clones in this study shared similarity to *nir*K and *nir*S gene products from cultured β-*Proteobacteria* belonging to the families *Alcaligenaceae*, *Burkholderiaceae*, as well as *Rhodocyclaceae* (Table 5, Figs. 4 and 5), suggesting that several of the β-*Proteobacteria* genera detected in SSU clone libraries may also be capable of denitrification at this site. In a recent phylogenetic survey of bacterial populations from FRC sediment, SSU rRNA clones belonging to *Alcaligenaceae* and *Burkholderiaceae* were found to be dominant as well as metabolically active (2).

These results, along with the results of this study suggest that the enrichment of β-*Proteobacteria* in sediments observed in this study could be due to growth of β-*Proteobacteria* already widespread and/or active in the aquifer prior to biostimulation that have adapted to the groundwater contaminants at the FRC, which include nitrate, heavy metals, radionuclides, and hydrocarbons.

While our SSU rRNA gene clone libraries showed an abundance of β-*Proteobacteria* clones in biostimulated sediments, multiple lines of evidence suggest the dominance of a *Castellaniella* species in biostimulated sediments and their role in nitrate removal *in situ*. While several studies have proven successful in using molecular approaches to identify bacteria important in bioremediation (12, 33, 61), very few studies have both identified and isolated microorganisms responsible for *in situ* bioremediation. In one study, organisms were cultivated that had been identified by DGGE from 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading enrichments; these isolates were capable of 2,4-D degradation, suggesting their importance in bioremediation in contaminated environments (42). Another study used stable isotope probing (SIP) of RNA to show that *Azoarcus* was involved in benzene degradation in groundwater incubations under denitrifying-conditions, and further isolated organisms belonging to the same phylotype showing that they could oxidize benzene to CO_2 (41). These two studies, however, do not prove the importance of the isolated organisms for *in situ* bioremediation. In a different study, however, SIP was used to identify *in situ* naphthalene degraders; one dominant clone was identified, and an isolate matching this clone (belonging to the genus *Polaromonas*) was cultivated and shown to also contain a naphthalene dioxygenase gene also detected in the site sediment (38).

Similarly, in this study, isolates belonging to the genus *Castellaniella* were cultivated that matched dominant clones from both SSU rRNA gene and *nir*K clone libraries generated from biostimulated sediment where nitrate reduction was occurring. Furthermore, PLFA analysis from sediment samples showed an increase in fatty acids common to the genus *Castellaniella* associated with biomass increase. Both *Castellaniella* sp. 4.5A2 and 7.5A2 contained *nir*K and were capable of growth on nitrate as the sole electron acceptor and producing gaseous end-product, indicating these organisms are capable of denitrification; if the *Castellaniella* identified *in situ* through SSU rRNA and nirK clone libraries share similar physiology to these isolates, then *Castellaniella* may play an active role in denitrification at this site upon biostimulation with ethanol. Along with the *Polaromonas* study (38), this paper shows a relationship between microbial community structure and function through the isolation of a microorganism dominant in clone libraries while also using functional gene sequences to suggest that microorganism is involved in the process of interest *in situ*.

The *Castellaniella* species identified in this studied may represent a novel species (Fig. 1). Other *Castellaniella* isolates have been isolated from activated sludge and are capable of denitrification coupled to the oxidation of monoterpenes (25), taurine (15), and phenol (4). Furthermore, other *Alcaligenaceae* isolates have been implicated in the degradation of xenobiotic compounds (10) as well as in nitrate removal systems (53). FRC *Castellaniella* isolates 4.5A2 and 7.5A2 are pH-tolerant and were isolated at both low and neutral pHs; thus, they may have been able to out-compete other denitrifiers for nitrate in the acidic groundwater found in Area 1.

A similar molecular ecology study at the FRC found that electron donor addition resulted in an increase in δ-*Proteobacteria*, such as *Geobacter* and *Anaeromyxobacter*, in contaminated FRC Area 1 sediments (51). However, push-pull tests in those experiments were done with low-nitrate groundwater from well GW835 (36), and samples were taken at the end of the extraction phase. Those experiments point to an important role for Fe(III)-reducing bacteria during biostimulation. In this study, groundwater wells were injected with high-nitrate (>130 mM)) groundwater from FW021 and sediment samples were taken one week following injection of ethanol-amended groundwater (at the beginning of the extraction phase), at which point denitrification was likely occurring (Table 1). The differences in nitrate concentrations of the injection solutions as well as the time at which sediment samples were taken could reflect the differences in community compositions based on SSU rRNA gene clone libraries. Since several terminal electron accepting processes sequentially occur during biostimulation (36), it is likely that the results from our study provide a snapshot of the microbial community structure during the denitrification phase, while the previous study (51) provides a snapshot of the microbial community structure from when geochemical conditions were more reduced. This would reflect observations in other studies that shifts in microbial community structure occur during different stages of bioremediation processes (35, 37, 73).

In this study descriptive diversity statistics are provided to describe the effect of biostimulation on *in situ* diversity of microbial populations. A recent study has shown that bioremediation in a fluidized bed reactor treating nitrate- and uranium-

contaminated groundwater resulted in an initial decrease in bacterial diversity followed by an increase in diversity (35). In accordance with this finding, other molecular studies have also shown that biostimulation of hydrocarbon-contaminated sediments results in an initial decrease in species diversity followed by an increase in diversity (37, 59). Our results also support that biostimulation resulted in a decrease in bacterial diversity; however, it is possible that biodiversity could later increase, as observed in above studies. The effects of fluctuations in species diversity on ecosystem function (in this case, nitrate and uranium removal from groundwater at the FRC) are unclear. While many ecological studies have linked species richness or high species diversity in natural systems or microcosms with an increase in ecosystem function and/or stability (7, 11, 63), few studies have examined the effect of bacterial species diversity on ecosystem function in engineered systems, where often, one substrate is available for consumption as opposed to natural ecosystems where increased species richness might aid in a more productive consumption of all available resources. For example, in glucose-fed methanogenic bioreactors, it was found that a bioreactor with lower bacterial diversity, or more "flexible" microbial communities, was more functionally stable than a more species-rich bioreactor (21). Similarly, at the FRC, the desired ecosystem function (i.e. nitrate and uranium reduction) may likely be unaffected by lower diversity when a simple substrate such as ethanol is used as an electron donor.

In summary, we have employed multiple approaches to determine the effect of biostimulation on the microbial community structure of an acidic nitrate- and uranium-contaminated aquifer. We have identified and isolated a *Castellaniella* species that is

important in *in situ* nitrate removal. Furthermore, we have found that biostimulation results in a decrease in bacterial diversity; however, the effect of this reduction in diversity on bioremediation strategies remains to be investigated.

Acknowledgements

We thank Dave Watson and Maryanna Bogle for field sampling and providing sediment core material, Melora Park for assistance with groundwater data analysis, and Mandy Michalsen for helpful discussions. This work was supported by the Office of Biological and Environmental Research (OBER) of the Office of Science, U.S. Department of Energy (DOE), Environmental Remediation Sciences Program (ERSP) (FG03-02ER63443, DE-FC02-96ER62278, and FG02-00ER62986).

References

1. **Abdelouas, A., W. Lutze, W. Gong, E. H. Nuttall, B. A. Strietelmeier, and B. J. Travis.** 2000. Biological reduction of uranium in groundwater and subsurface soil. Sci Total Environ **250:**21-35.

2. **Akob, D. M., H. J. Mills, and J. E. Kostka.** 2007. Metabolically active microbial communities in uranium-contaminated subsurface sediments. FEMS Microbiol Ecol **59:**95-107.

3. **Anderson, R. T., H. A. Vrionis, I. Ortiz-Bernad, C. T. Resch, P. E. Long, R. Dayvault, K. Karp, S. Marutzky, D. R. Metzler, A. Peacock, D. C. White, M. Lowe, and D. R. Lovley.** 2003. Stimulating the in situ activity of *Geobacter* species to remove uranium from the groundwater of a uranium-contaminated aquifer. Appl Environ Microbiol **69:**5884-91.

4. **Baek, S. H., K. H. Kim, C. R. Yin, C. O. Jeon, W. T. Im, K. K. Kim, and S. T. Lee.** 2003. Isolation and characterization of bacteria capable of degrading phenol and reducing nitrate under low-oxygen conditions. Curr Microbiol **47:**462-6.

5. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressureized atmosphere. Appl Environ Microbiol **32:**781-91.

6. **Balkwill, D. L., F. R. Leach, J. T. Wilson, J. F. McNabb, and D. C. White.** 1988. Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface aquifer sediments. Microb Ecol **16:**73-84.

7. **Bell, T., J. A. Newman, B. W. Silverman, S. L. Turner, and A. K. Lilley.** 2005. The contribution of species richness and composition to bacterial services. Nature **436:**1157-60.

8. **Bligh, E. G., and W. J. Dryer.** 1954. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology **37:**911-917.

9. **Braker, G., J. Zhou, L. Wu, A. H. Devol, and J. M. Tiedje.** 2000. Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in pacific northwest marine sediment communities. Appl Environ Microbiol **66:**2096-104.

10. **Busse, H. J., T. el-Banna, H. Oyaizu, and G. Auling.** 1992. Identification of xenobiotic-degrading isolates from the beta subclass of the *Proteobacteria* by a

polyphasic approach including 16S rRNA partial sequencing. Int J Syst Bacteriol **42:**19-26.

11. **Cardinale, B. J., M. A. Palmer, and S. L. Collins.** 2002. Species diversity enhances ecosystem functioning through interspecific facilitation. Nature **415:**426-9.

12. **Chang, Y. J., P. E. Long, R. Geyer, A. D. Peacock, C. T. Resch, K. Sublette, S. Pfiffner, A. Smithgall, R. T. Anderson, H. A. Vrionis, J. R. Stephen, R. Dayvault, I. Ortiz-Bernad, D. R. Lovley, and D. C. White.** 2005. Microbial incorporation of 13C-labeled acetate at the field scale: detection of microbes responsible for reduction of U(VI). Environ Sci Technol **39:**9039-48.

13. **Chang, Y. J., A. D. Peacock, P. E. Long, J. R. Stephen, J. P. McKinley, S. J. Macnaughton, A. K. Hussain, A. M. Saxton, and D. C. White.** 2001. Diversity and characterization of sulfate-reducing bacteria in groundwater at a uranium mill tailings site. Appl Environ Microbiol **67:**3149-60.

14. **Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje.** 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res **33:**D294-6.

15. **Denger, K., H. Laue, and A. M. Cook.** 1997. Anaerobic taurine oxidation: a novel reaction by a nitrate-reducing *Alcaligenes* sp. Microbiology **143 (Pt 6):**1919-24.

16. **DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen.** 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol **72:**5069-72.

17. **Elias, D. A., L. R. Krumholz, D. Wong, P. E. Long, and J. M. Suflita.** 2003. Characterization of microbial activities and U reduction in a shallow aquifer contaminated by uranium mill tailings. Microb Ecol **46:**83-91.

18. **Elias, D. A., J. M. Senko, and L. R. Krumholz.** 2003. A procedure for quantitation of total oxidized uranium for bioremediation studies. J Microbiol Methods **53:**343-53.

19. **Elshahed, M. S., J. M. Senko, F. Z. Najar, S. M. Kenton, B. A. Roe, T. A. Dewers, J. R. Spear, and L. R. Krumholz.** 2003. Bacterial diversity and sulfur cycling in a mesophilic sulfide-rich spring. Appl Environ Microbiol **69:**5609-21.

20. **Etchebehere, C., I. Errazquin, E. Barrandeguy, P. Dabert, R. Moletta, and L. Muxi.** 2001. Evaluation of the denitrifying microbiota of anoxic reactors. FEMS Microbiol Ecol **35:**259-265.

21. **Fernandez, A. S., S. A. Hashsham, S. L. Dollhopf, L. Raskin, O. Glagoleva, F. B. Dazzo, R. F. Hickey, C. S. Criddle, and J. M. Tiedje.** 2000. Flexible community structure correlates with stable community function in methanogenic bioreactor communities perturbed by glucose. Appl Environ Microbiol **66:**4058-67.

22. **Fields, M. W., T. Yan, S. K. Rhee, S. L. Carroll, P. M. Jardine, D. B. Watson, C. S. Criddle, and J. Zhou.** 2005. Impacts on microbial communities and cultivable isolates from groundwater contaminated with high levels of nitric acid-uranium waste. FEMS Microbiol Ecol **53:**417-28.

23. **Finneran, K. T., M. E. Housewright, and D. R. Lovley.** 2002. Multiple influences of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediments. Environ Microbiol **4:**510-6.

24. **Finneran, K. T. A., Robert T.; Nevin, Kelly P.; Lovley, Derek R. .** 2002. Potential for bioremediation of uranium-contaminated aquifers with microbial U(VI) reduction. Soil & Sediment Contamination **11:**339-357.

25. **Foss, S., U. Heyen, and J. Harder.** 1998. *Alcaligenes defragrans* sp. nov., description of four strains isolated on alkenoic monoterpenes ((+)-menthene, alpha-pinene, 2-carene, and alpha-phellandrene) and nitrate. Syst Appl Microbiol **21:**237-44.

26. **Friedrich, U., K. Prior, K. Altendorf, and A. Lipski.** 2002. High bacterial diversity of a waste gas-degrading community in an industrial biofilter as shown by a 16S rDNA clone library. Environ Microbiol **4:**721-34.

27. **Friedrich, U., H. Van Langenhove, K. Altendorf, and A. Lipski.** 2003. Microbial community and physicochemical analysis of an industrial waste gas biofilter and design of 16S rRNA-targeting oligonucleotide probes. Environ Microbiol **5:**183-201.

28. **Frostegard, A., A. Tunlid, and E. Baath.** 1993. Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Appl Environ Microbiol **59:**3605-3617.

29. **Guckert, J. B., C. P. Antworth, P. D. Nichols, and D. C. White.** 1985. Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediment. FEMS Microbial Ecology **31:**147-158.

30. **Head, I. M., J. R. Saunders, and R. W. Pickup.** 1998. Microbial evolution, diversity, and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. Microb Ecol **35:**1-21.

31. **Hedrick, D. B., A. Peacock, J. R. Stephen, S. J. Macnaughton, J. Bruggemann, and D. C. White.** 2000. Measuring soil microbial community diversity using polar lipid fatty acid and denaturing gradient gel electrophoresis data. J Microbiol Methods **41:**235-48.

32. **Heylen, K., D. Gevers, B. Vanparys, L. Wittebolle, J. Geets, N. Boon, and P. De Vos.** 2006. The incidence of *nirS* and *nirK* and their genetic heterogeneity in cultivated denitrifiers. Environ Microbiol **8:**2012-21.

33. **Holmes, D. E., K. T. Finneran, R. A. O'Neil, and D. R. Lovley.** 2002. Enrichment of members of the family *Geobacteraceae* associated with stimulation of dissimilatory metal reduction in uranium-contaminated aquifer sediments. Appl Environ Microbiol **68:**2300-6.

34. **Huber, T., G. Faulkner, and P. Hugenholtz.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics **20:**2317-9.

35. **Hwang, C., W. M. Wu, T. J. Gentry, J. Carley, S. L. Carroll, C. Schadt, D. Watson, P. M. Jardine, J. Zhou, R. F. Hickey, C. S. Criddle, and M. W. Fields.** 2005. Changes in bacterial community structure correlate with initial operating conditions of a field-scale denitrifying fluidized bed reactor. Appl Microbiol Biotechnol**:**1-13.

36. **Istok, J. D., J. M. Senko, L. R. Krumholz, D. Watson, M. A. Bogle, A. Peacock, Y. J. Chang, and D. C. White.** 2004. In situ bioreduction of technetium and uranium in a nitrate-contaminated aquifer. Environ Sci Technol **38:**468-75.

37. **Iwamoto, T., K. Tani, K. Nakamura, Y. Suzuki, M. Kitagawa, M. Eguchi, and M. Nasu.** 2000. Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. FEMS Microbiol Ecol **32:**129-141.

38. **Jeon, C. O., W. Park, P. Padmanabhan, C. DeRito, J. R. Snape, and E. L. Madsen.** 2003. Discovery of a bacterium, with distinctive dioxygenase, that is responsible for in situ biodegradation in contaminated sediment. Proc Natl Acad Sci U S A **100:**13591-6.

39. **Joynt, J., M. Bischoff, R. Turco, A. Konopka, and C. H. Nakatsu.** 2006. Microbial community analysis of soils contaminated with lead, chromium and petroleum hydrocarbons. Microb Ecol **51:**209-19.

40. **Kampfer, P., K. Denger, A. M. Cook, S. T. Lee, U. Jackel, E. B. Denner, and H. J. Busse.** 2006. *Castellaniella* gen. nov., to accommodate the phylogenetic lineage of Alcaligenes defragrans, and proposal of *Castellaniella defragrans* gen. nov., comb. nov. and *Castellaniella denitrificans* sp. nov. Int J Syst Evol Microbiol **56:**815-9.

41. **Kasai, Y., Y. Takahata, M. Manefield, and K. Watanabe.** 2006. RNA-based stable isotope probing and isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater. Appl Environ Microbiol **72:**3586-92.

42. **Lee, T. H., S. Kurata, C. H. Nakatsu, and Y. Kamagata.** 2005. Molecular analysis of bacterial community based on 16S rDNA and functional genes in activated sludge enriched with 2,4-dichlorophenoxyacetic acid (2,4-D) under different cultural conditions. Microb Ecol **49:**151-62.

43. **Liao, M., C. L. Chen, and C. Y. Huang.** 2005. Effect of heavy metals on soil microbial activity and diversity in a reclaimed mining wasteland of red soil area. J Environ Sci (China) **17:**832-7.

44. **Lovley, D. R., and E. J. P. Phillips.** 1992. Bioremediation of uranium contamination with enzymatic uranium reduction. . Environmental Science and Technology **26:**2228-2234.

45. **Macnaughton, S., J. R. Stephen, Y. J. Chang, A. Peacock, C. A. Flemming, K. T. Leung, and D. C. White.** 1999. Characterization of metal-resistant soil eubacteria by polymerase chain reaction--denaturing gradient gel electrophoresis with isolation of resistant strains. Can J Microbiol **45:**116-24.

46. **Martin, A. P.** 2002. Phylogenetic approaches for describing and comparing the diversity of microbial communities. Appl Environ Microbiol **68:**3673-82.

47. **McInerney, M. J., M. P. Bryant, and N. Pfenning.** 1979. Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol. **122:**129-135.

48. **Michalsen, M. M., B. A. Goodman, S. D. Kelly, K. M. Kemner, J. P. McKinley, J. W. Stucki, and J. D. Istok.** 2006. Uranium and technetium bio-immobilization in intermediate-scale physical models of an in situ bio-barrier. Environ Sci Technol **40:**7048-53.

49. **Nevin, K. P., K. T. Finneran, and D. R. Lovley.** 2003. Microorganisms associated with uranium bioremediation in a high-salinity subsurface sediment. Appl Environ Microbiol **69:**3672-5.

50. **Nogales, B., E. R. Moore, E. Llobet-Brossa, R. Rossello-Mora, R. Amann, and K. N. Timmis.** 2001. Combined use of 16S ribosomal DNA and 16S

rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. Appl Environ Microbiol **67:**1874-84.

51. **North, N. N., S. L. Dollhopf, L. Petrie, J. D. Istok, D. L. Balkwill, and J. E. Kostka.** 2004. Change in bacterial community structure during in situ biostimulation of subsurface sediment cocontaminated with uranium and nitrate. Appl Environ Microbiol **70:**4911-20.

52. **Nyman, J. L., T. L. Marsh, M. A. Ginder-Vogel, M. Gentile, S. Fendorf, and C. Criddle.** 2006. Heterogeneous response to biostimulation for U(VI) reduction in replicated sediment microcosms. Biodegradation **17:**303-16.

53. **Ozeki, S., I. Baba, N. Takaya, and H. Shoun.** 2001. A novel C1-using denitrifier *Alcaligenes* sp. STC1 and its genes for copper-containing nitrite reductase and azurin. Biosci Biotechnol Biochem **65:**1206-10.

54. **Peacock, A. D., Y. J. Chang, J. D. Istok, L. Krumholz, R. Geyer, B. Kinsall, D. Watson, K. L. Sublette, and D. C. White.** 2004. Utilization of microbial biofilms as monitors of bioremediation. Microb Ecol **47:**284-92.

55. **Petrie, L., N. N. North, S. L. Dollhopf, D. L. Balkwill, and J. E. Kostka.** 2003. Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). Appl Environ Microbiol **69:**7467-79.

56. **Pinkart, H. C., D. B. Ringelberg, Y. M. Piceno, S. J. MacNaughton, and D. C. White.** 2002. Manual of Environmental Microbiology Second Edition. ASM Press, Washington D.C.

57. **Reardon, C. L., D. E. Cummings, L. M. Petzke, B. L. Kinsall, D. B. Watson, B. M. Peyton, and G. G. Geesey.** 2004. Composition and diversity of microbial communities recovered from surrogate minerals incubated in an acidic uranium-contaminated aquifer. Appl Environ Microbiol **70:**6037-46.

58. **Rogers, D. R., C. M. Santelli, and K. J. Edwards.** 2003. Geomicrobiology of deep-sea deposits: estimating community diversity from low-temperature seafloor rocks and minerals. Geobiology **1:**109-117.

59. **Roling, W. F., M. G. Milner, D. M. Jones, K. Lee, F. Daniel, R. J. Swannell, and I. M. Head.** 2002. Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. Appl Environ Microbiol **68:**5537-48.

60. **Senko, J. M., J. D. Istok, J. M. Suflita, and L. R. Krumholz.** 2002. In-situ evidence for uranium immobilization and remobilization. Environ Sci Technol **36:**1491-6.

61. **Suzuki, Y., S. D. Kelly, K. M. Kemner, and J. F. Banfield.** 2003. Microbial populations stimulated for hexavalent uranium reduction in uranium mine sediment. Appl Environ Microbiol **69:**1337-46.

62. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **25:**4876-82.

63. **Tilman, D., P. B. Reich, and J. M. Knops.** 2006. Biodiversity and ecosystem stability in a decade-long grassland experiment. Nature **441:**629-32.

64. **Vrionis, H. A., R. T. Anderson, I. Ortiz-Bernad, K. R. O'Neill, C. T. Resch, A. D. Peacock, R. Dayvault, D. C. White, P. E. Long, and D. R. Lovley.** 2005. Microbiological and geochemical heterogeneity in an in situ uranium bioremediation field site. Appl Environ Microbiol **71:**6308-18.

65. **Watson, D. B., and Y. Roh** March, 2004, posting date. Natural and Accelerated Bioremediation Research (NABIR) Field Research Center (FRC) Site Characterization Plan Addendum 1. [Online.]

66. **Watson, D. B., G.R. Moline, P.M., Jardine, S.K., Holladay, T.L. Melhorn, B. Gu, A.V. Palumbo,, and C. C. B. B.P. Spalding, and W.E. Doll.** 2001, posting date. Natural and Accelerated Bioremediation Research (NABIR) Field Research Center (FRC) Site Characterization Plan. [Online.]

67. **White, D., W. M. Davis, J. S. Nickels, J. D. King, and R. J. Bobbie.** 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia **40:**51-62.

68. **White, D. C., J. O. Stair, and D. B. Ringelberg.** 1996. Quantitative comparisons of in situ microbial biodiversity by signature biomarker analysis. Journal of Industrial Microbiology **17:**185-196.

69. **Yan, T., M. W. Fields, L. Wu, Y. Zu, J. M. Tiedje, and J. Zhou.** 2003. Molecular diversity and characterization of nitrite reductase gene fragments (*nirK* and *nirS*) from nitrate- and uranium-contaminated groundwater. Environ Microbiol **5:**13-24.

70. **Yoshie, S., N. Noda, T. Miyano, S. Tsuneda, A. Hirata, and Y. Inamori.** 2002. Characterization of microbial community in nitrogen removal process of metallurgic wastewater by PCR-DGGE. Water Sci Technol **46:**93-8.

71. **Yoshie, S., N. Noda, S. Tsuneda, A. Hirata, and Y. Inamori.** 2004. Salinity decreases nitrite reductase gene diversity in denitrifying bacteria of wastewater treatment systems. Appl Environ Microbiol **70:**3152-7.

72. **Zhang, H., S. Hanada, T. Shigematsu, K. Shibuya, Y. Kamagata, T. Kanagawa, and R. Kurane.** 2000. *Burkholderia kururiensis* sp. nov., a trichloroethylene (TCE)-degrading bacterium isolated from an aquifer polluted with TCE. Int J Syst Evol Microbiol **50 Pt 2:**743-9.

73. **Zucchi, M., L. Angiolini, S. Borin, L. Brusetti, N. Dietrich, C. Gigliotti, P. Barbieri, C. Sorlini, and D. Daffonchio.** 2003. Response of bacterial community during bioremediation of an oil-polluted soil. J Appl Microbiol **94:**248-57.

Table 1. Summary of initial groundwater chemistry, push-pull test results, and sediment core characteristics.

Groundwater well (corresponding sediment core)	Initial groundwater chemistry[C]			Push-pull test results[B]			Sediment core data (following biostimulation)[E]			
	NO_3^- (mM)	U(VI) (μM)	pH	NO_3^- C/C_0[D]	EtOH C/C_0[D]	U(VI) C/C_0[D]	NO_3^- (mM)	NO_2^- (mM)	Biomass (x10^6 cells/g)	% U as U(IV)
FW028 (FB064)	167.2	2.22	4.4	0.327	0.358	15.2	126.5	17.7	204	67.1
FW034 (FB067)	0.769	0.475	6.79	0.640	0.528	0.924	39.77	14.8	18.5	75.7
FW016 (FB066)[A]	11.4	2.58	3.92				2.612	9.98	5.58	4.6
FW021[B]	142.3	5.80	3.05							

[A]FW016 served as a control well.
[B]FW021 groundwater was used for the injection solution for push-pull tests in FW028 and FW034.
[C]Data publicly available at http://public.ornl.gov/nabirfrc/frcsite3.cfm
[D]These data represent analyte concentrations (C) on first date of the extraction phase (on or near the date of sediment core sampling) compared to initial concentrations in the injection solution (C_0) and have been adjusted to account for loss due to dilution and dispersion, as determined by loss of bromide.
[E]Sediment core FB064 was obtained five days after the injection phase began and three days prior to the start of the extraction phase for well FW028; FB067 was sampled 31 days after the injection phase began and on the same day of the start of the extraction phase.

Table 2. Descriptive diversity statistics based on SSU rRNA gene clone library data from two ethanol-stimulated and one control sediments.

Sample	No. of clones	No. of OTUs[1]	No. of GLTGs[2]	Avg. Nucleotide Div.	Diversity indices based on OTUs[1]			Diversity indices based on GLTGs[2]		
					Shannon-Weiner	Simpson's	Evenness	Shannon-Weiner	Simpson's	Evenness
FB064 (stimulated)	58	23	13	0.0994	2.010	0.3181	0.641	1.234	0.5333	0.481
FB067 (stimulated)	64	21	18	0.1518	2.173	0.2266	0.714	2.012	0.2427	0.696
FB066 (control)	51	21	16	0.1767	2.512	0.1272	0.825	2.222	0.1572	0.801

[1]OTU = Operational taxonomic unit (97% similarity cutoff)
[2]GLTG = Genus-level taxonomic group (93% similarity cutoff)

Table 3. Summary of phylogenetic distribution of SSU rRNA clones from samples FB064, FB067, and FB066.

Phylum/Candidate Division	% of total clones		
	FB064 (stimulated)	FB067 (stimulated)	FB066 (control)
Proteobacteria	93.1	84.4	56.9
β	79.3	50.0	9.8
δ	0.0	6.3	0.0
γ	12.1	26.6	47.1
unclassified	1.7	1.6	0.0
Bacteroidetes	1.7	0.0	2.0
Firmicutes	3.4	3.1	2.0
Actinobacteria	0.0	1.6	2.0
Acidobacteria	0.0	1.6	27.5
Candidate Division WD272_C2	0.0	6.3	9.8
Candidate Division TM7	1.7	0.0	0.0
Candidate Division ZB1	0.0	1.6	0.0
Termite Group I	0.0	1.6	0.0

Table 4. PLFA analysis of samples FB064, FB067, and FB066. Columns shaded in gray indicate the dominant PLFAs from described species in the genus *Castellaniella* (40).

	% of Total PLFA		
	FB064 (stimulated)	FB067 (stimulated)	FB066 (control)
Total Normal Saturates	**27.30**	**34.39**	**28.92**
14:0	1.02	1.34	0.00
15:0	0.18	0.00	0.00
16:0	25.70	26.37	20.33
17:0	0.10	0.52	0.70
18:0	0.30	6.16	7.08
20:0	0.00	0.00	0.33
22:0	0.00	0.00	0.48
Total Mid-Chain Branched Saturates	**0.38**	**0.00**	**12.07**
i10me16	0.16	0.00	1.27
10Me16:0	0.18	0.00	3.84
12me16:0	0.04	0.00	0.65
i10me17:0	0.00	0.00	4.90
10Me18:0	0.00	0.00	1.40
Total Terminal-Branched Saturates	**3.67**	**11.18**	**22.59**
i14:0	0.12	0.17	0.00
i15:0	1.09	4.00	5.69
a15:0	1.05	1.45	4.44
i16:0	0.59	0.73	3.30
i17:0	0.66	3.35	6.51
a17:0	0.15	1.49	2.65
Total Branched Monounsaturates	**1.54**	**6.05**	**7.66**
br16:1a	0.02	0.00	0.00
br16:1b	0.04	0.00	0.00
i17:1a	0.41	1.48	1.97
i17:1b	0.00	0.00	0.00
br18:1	0.98	4.57	5.69
br19:1	0.09	0.00	0.00
Total Monounsaturates	**66.96**	**47.07**	**28.76**
16:1w9c	0.07	0.33	0.00
16:1w7c	10.21	4.56	1.83
16:1w7t	0.56	1.81	0.00
16:1w5c	0.22	0.76	0.00
cy17:0	31.79	7.40	4.96
17:1	0.17	0.00	0.00
18:1w9c	0.11	14.34	9.66
18:1w7c	7.44	8.25	5.79
18:1w7t	0.59	3.87	2.44
18:1w5c	0.22	0.00	0.00
cy19:0	15.52	5.75	4.09
19:1	0.07	0.00	0.00
Total Polysaturates	**0.00**	**1.31**	**0.00**

Table 5. Summary of distribution of *nirK* and *nirS* OTUs from samples FB064 and FB067 (stimulated) and FB066 (control).

OTU name	No. of clones per OTU			Closest Cultured relative**	% similarity ***
	FB064	FB067	FB066*		
***nirK* OTUs**					
1K	19	19	2	FRC isolates 4.5A2 and 7.5A2	100.0
2K	1	0	0	*Nitrosomonas* sp. TA92liNH$_4$	84.8
4K	0	0	1	*Ochromobactrum* sp. 4FB14	93.5
7K	1	11	7	*Alcaligenes* sp. DSM 30128	81.7
8K	0	1	0	FRC isolate 4.5A2	84.7
9K	0	1	0	FRC isolate 4.5A2	94.2
10K	1	0	0	FRC isolate 4.5A2	92.5
11K	1	0	0	FRC isolate 4.5A2	98.0
12K	1	0	0	FRC isolate 4.5A2	87.5
13K	1	0	0	FRC isolate 4.5A2	100.0
***nirS* OTUs**					
1S	38	0	--	*Dechloromonas aromatica*	90.5
9S	0	7	--	*Thiobacillus denitrificans*	75.9
10S	0	1	--	*Ralsonia eutropha*	75.1
11S	0	1	--	*Magnetospirillum magnetotacticum*	85.1
14S	0	10	--	*Ralstonia metallidurans*	78.5
18S	0	1	--	*Ralsonia eutropha*	82.2
19S	0	3	--	*Ralsonia eutropha*	82.7
20S	11	0	--	*Ralstonia metallidurans*	74.1
21S	6	0	--	*Azoarcus tolulyticus*	81.5
22S	1	0	--	*Azoarcus tolulyticus*	84.3
23S	1	0	--	*Azoarcus tolulyticus*	94.6
24S	1	0	--	*Azoarcus tolulyticus*	87.0
25S	1	0	--	*Azoarcus tolulyticus*	86.6
26S	0	2	--	*Ralstonia metallidurans*	84.4
27S	0	8	--	*Magnetospirillum magnetotacticum*	86.6
28S	0	3	--	*Magnetospirillum magnetotacticum*	82.8
34S	0	5	--	*Magnetospirillum magnetotacticum*	85.2
35S	4	0	--	*Magnetospirillum magnetotacticum*	87.9
36S	0	3	--	*Thauera aromatica*	80.4
37S	2	0	--	*Ralstonia metallidurans*	74.7
38S	0	10	--	*Dechloromonas aromatica*	88.8
39S	0	10	--	*Dechloromonas aromatica*	87.8
41S	0	3	--	*Dechloromonas aromatica*	89.8
42S	0	1	--	*Dechloromonas aromatica*	91.9
43S	1	0	--	*Dechloromonas aromatica*	96.6
44S	0	2	--	*Dechloromonas aromatica*	88.8

*Amplified PCR product using *nir*S primers was could not be obtained from this sample.
**GenBank accession numbers for closest cultured relatives are located next to corresponding genus and species names in Figures 4 and 5.
***Similarity values are based on pairwise distance values from multiple alignment files using translated amino acid sequences

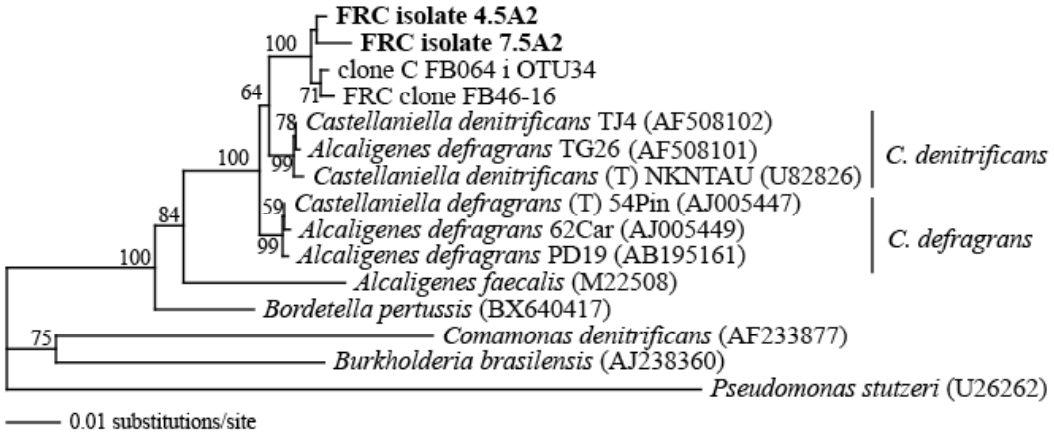

Figure 1. Distance phylogram based on near full-length SSU rRNA gene sequences (approx. 1490 base pairs) from FRC isolates (in bold), FRC sediment clone sequences (clone C FB064 I OTU34 was identified from FRC biostimulated sediment in this study), and other members of *Castellaniella* as well as related organisms in the order *Burkholderiales* (accession numbers are shown in parentheses). Bootstrap values are based on 1000 replicates and are shown for branches with bootstrap support >50%.

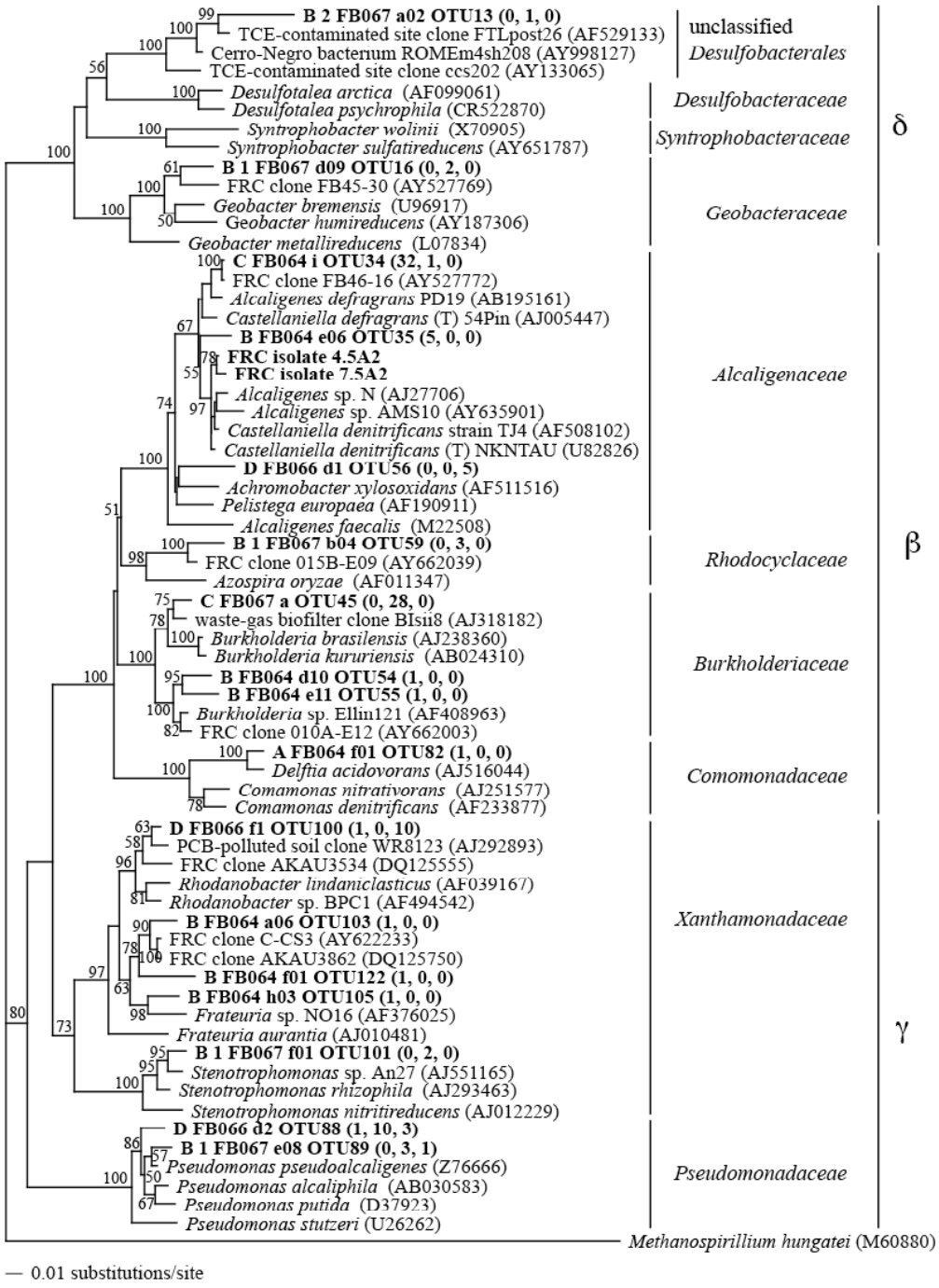

Figure 2. Distance phylogram of *Proteobacteria* partial SSU rRNA gene sequences (approx. 800 base pairs). Bootstrap values are based on 1000 replicates and are shown for branches with bootstrap support >50%. Selected OTUs from this study as well as FRC isolate sequences are in bold and numbers in parentheses indicate the number of clones belonging to that OTU from sediments FB064, FB067, and FB066, respectively. Accession numbers of sequences from GenBank are in parentheses.

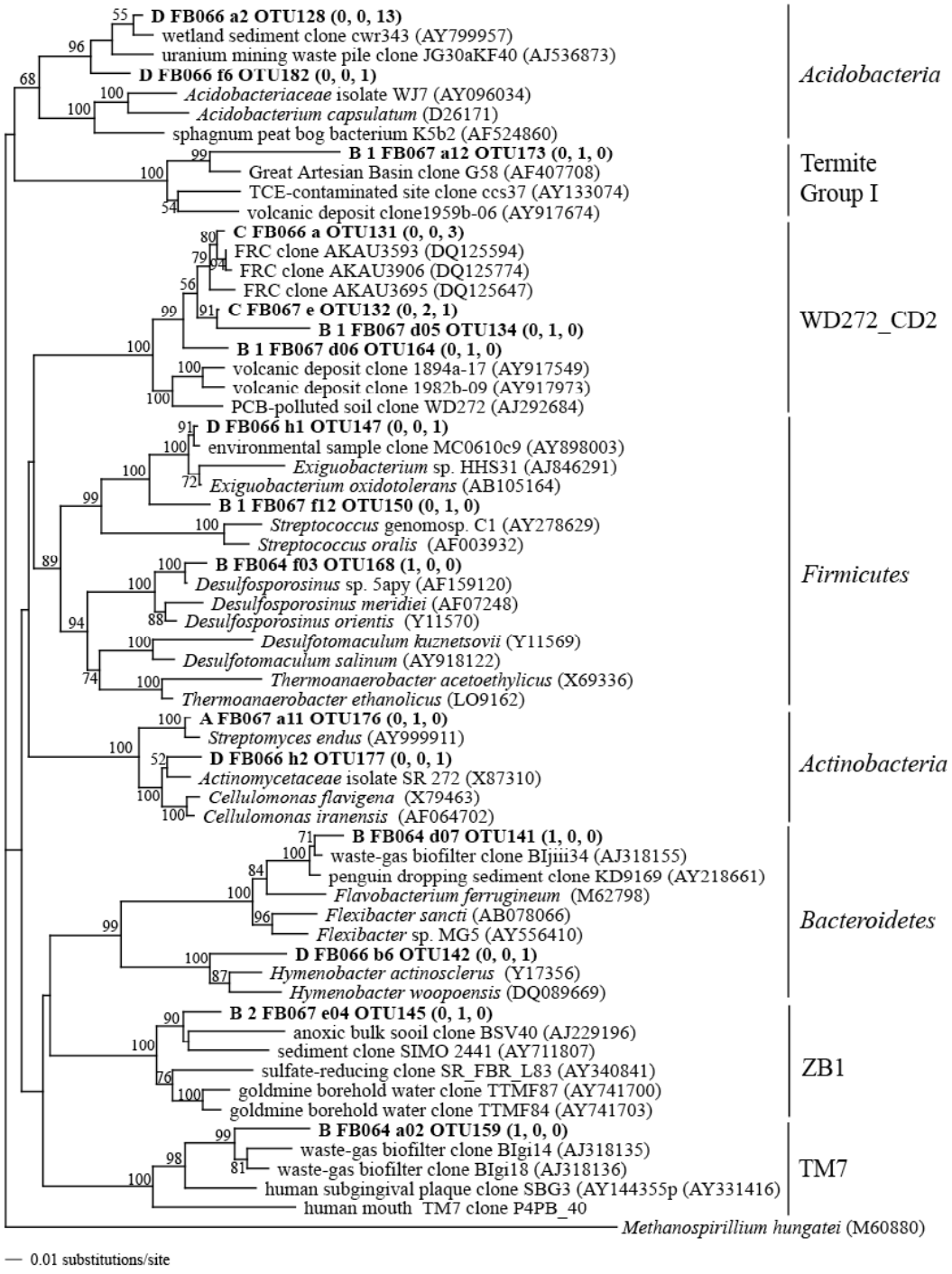

Figure 3. Distance phylogram of non-*Proteobacteria* partial SSU rRNA gene sequences (approx. 800 base pairs). Bootstrap values are based on 1000 replicates and are shown for branches with bootstrap support >50%. Selected OTUs from this study are in bold and numbers in parentheses indicate the number of clones belonging to that OTU from sediments FB064, FB067, and FB066, respectively. Accession numbers of sequences from GenBank are in parentheses.

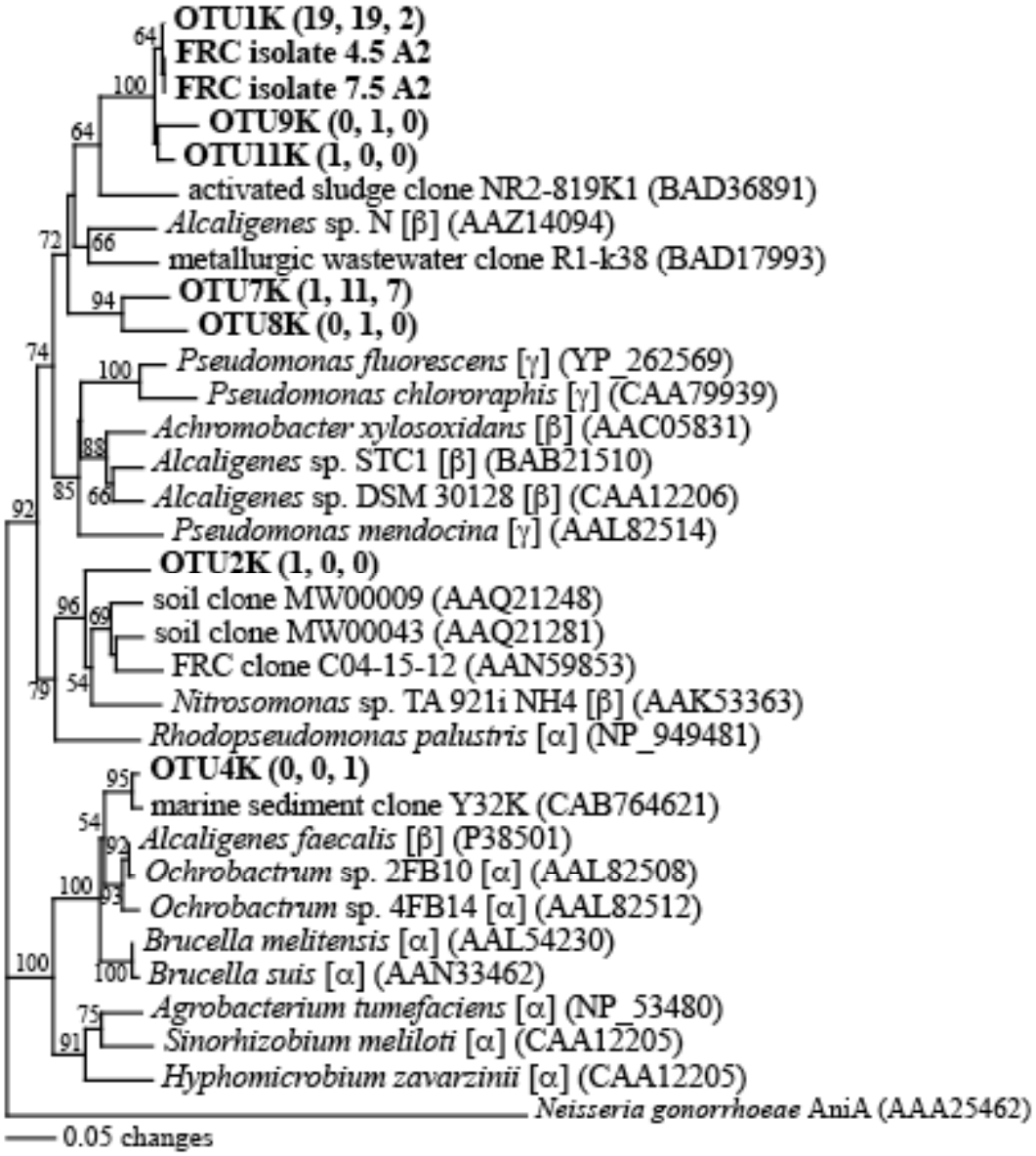

Figure 4. Distance phylogram of partial *nir*K gene product sequences. Bootstrap values are based on 1000 replicates and are shown for branches with bootstrap support >50%. Selected OTUs from this study are in bold and numbers in parentheses indicate the number of clones belonging to that OTU from sediments FB064, FB067, and FB066, respectively. Accession numbers of sequences downloaded from GenBank are in parentheses.

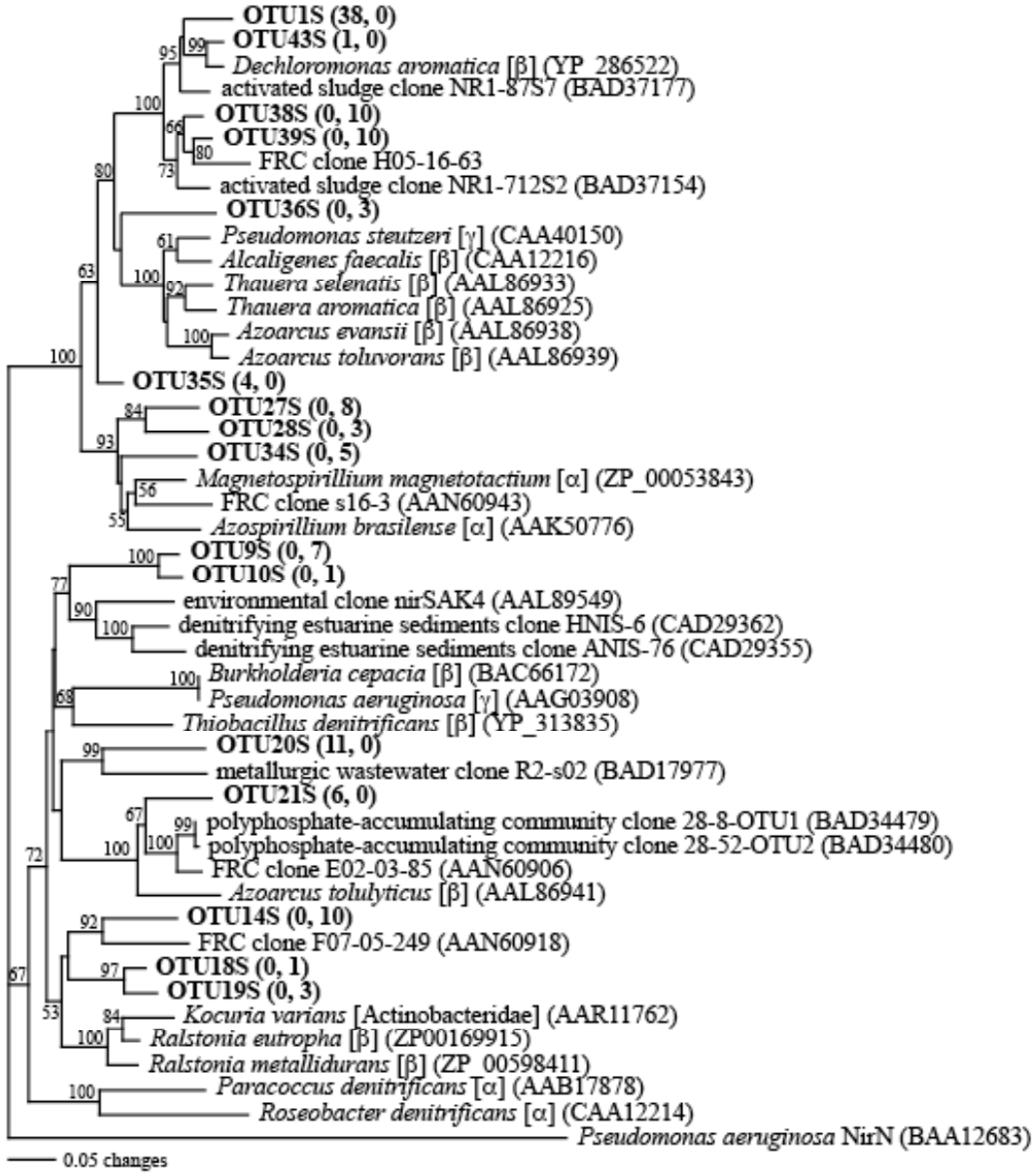

Figure 5. Distance phylogram of partial *nir*S gene product sequences. Bootstrap values are based on 1000 replicates and are shown for branches with bootstrap support >50%. Selected OTUs from this study are in bold and numbers in parentheses indicate the number of clones belonging to that OTU from sediments FB064 and FB067, respectively. Accession numbers of sequences downloaded from GenBank are in parentheses.

CHAPTER 4

Interactions Among Denitrifying Bacteria Growing in Acidic High Nitrate Groundwater

Abstract

Bioremediation strategies of nitrate- and uranium-contaminated sites require large additions of electron donor to the subsurface to stimulate denitrification and subsequently uranium reduction; thus, in contaminated aquifers with high concentrations of nitrate, denitrifiers play a critical role in bioremediation. Six strains of denitrifying bacteria belonging to the genera *Rhizobium*, *Pseudomonas*, and *Castellaniella* were isolated from bio-stimulated groundwater and sediment from the Oak Ridge Integrated Field Research Challenge Site (OR-IFRC), where biostimulation of low pH (3.5-6.5) and high nitrate (up to 140 mM) groundwater is occurring. In the experiments presented here, we characterized each isolate in regards to their growth rates, pH tolerance, nitrite tolerance, and growth on different denitrification intermediates. Furthermore, growth of three of these isolates were measured in tri-cultures and pure cultures incubated in OR-IFRC high-nitrate groundwater at pHs 5 and 7 to whether the best-adapted or most efficient denitrifying isolate alone would outperform a mixed assemblage in denitrification of high-nitrate groundwater or whether the denitrifying isolates interact within a mixed assemblage to achieve optimum rates of denitrification. Results from these experiments showed that *Castellaniella* str. 4.5A2, was the most efficient pure culture alone in groundwater,

reducing 56 and 84% of the nitrate at pH values 5 and 7, respectively. Mixed assemblages out-performed *Castellaniella* in groundwater, reducing 88 and 98% of nitrate with zero-order nitrate reduction rates of 1.3 and 2.6 mM NO_3^-/ day at pHs 5 and 7, respectively. Growth and kinetic experiments of each isolate clearly demonstrated each are better adapted to different stages of denitrification, explaining the ability of mixed assemblages to out-perform the best-fit pure culture. *Pseudomonas* str. GN33#1 had the fastest NO_3^- reduction rate in kinetic assays (V_{max}=15.8 μmol e^-•min^{-1}• mg $protein^{-1}$) and the fastest generation time on NO_3^- (2.6 hrs). *Castellaniella* str. 4.5A2 was the most NO_2^- tolerant (capable of growth in the presence of up to 100 mM NO_2^-), had the fastest growth rate on NO_2^- (4.0 hrs). *Rhizobium* str. GN32#2 had the fastest growth rate on nitrous oxide (3.4 hrs), and was the only isolate capable of growth during the later stages of denitrification in mixed assemblage groundwater experiments at pH 7. As data from groundwater experiments show that all three isolates grow together in OR-IFRC groundwater, we conclude that these isolates interact and function together within in a mixed community, rather than compete, in denitrification of acidic, high-nitrate groundwater.

Introduction

Biological U(VI) reduction, a bioremediation strategy designed to immobilize U in the subsurface, preventing U migration with contaminated groundwater, has been implemented in field scale studies through the injection of electron donor solutions (e.g. acetate, glucose, ethanol) into the subsurface (3, 8, 11, 27, 29, 31, 52, 65, 69). Nitrate as a co-contaminant, particularly in high concentrations, however, poses several problems in regards to this strategy. Namely, U(VI) reduction is not likely to occur until after nitrate, as a competitive electron acceptor, is removed, or reduced to low concentrations (8, 18, 31, 52). Also, denitrification intermediates, that often accumulate during bioremediation of high-nitrate contaminated aquifers (18, 31, 44), can lead to the re-oxidation and mobilization of previously reduced U(IV) (18, 52, 53). At the Oak Ridge Integrated Field Research Challenge site (OR-IFRC), nitrate concentrations often exceed 100 mM and have been typically dealt with by either an above-ground pump and treat approach to first remove nitrate prior to in situ U(VI) reduction (29, 30) or by the addition of larger quantities of electron donor to the subsurface, to reduce nitrate *in situ* (8, 31, 58). High nitrate concentrations such as those found at the OR-IFRC are also typical of other nitrate-impacted sites, including other subsurface environments (56), and industrial wastewater (16, 19, 22, 33, 47, 67). Biostimulation typically results in the stimulation of denitrifying bacteria, and community shifts are likely to occur as denitrification proceeds (e.g. 43, See Chapter 1 for detailed review).

The relationships between contaminant levels, pH, denitrifying community composition, and denitrification activity in any given nitrate-contaminated site are

intertwined and complex. High levels of contamination have been shown to have a negative effect on the diversity of both cultivable and non-cultivable bacterial populations in OR-IFRC groundwater (17). Also, acidity and pH-dependent nitrite accumulation can have effects on denitrification activity (e.g. 9, 15, 22, 23, 48, 66). The diversity of denitrifying assemblages and physiology of microorganisms in high-nitrate sites in turn also have important effects on the desired function (i.e. denitrification--including rates of nitrate reduction and levels of nitrite accumulation). Previous studies have suggested that diversity and composition of denitrifying communities in soils may directly affect the kinetics of denitrification in soils (10, 28, 49), and a handful of cultivation-independent studies of microbial communities involved in denitrification in bioreactors treating high-nitrate water have been performed to examine how diversity and composition are related to function and stability (20, 21, 30).

While many of the aforementioned studies have looked at relationships between environmental conditions, microbial community composition, and denitrification function, little is known about what factors contribute to the growth and survival of different denitrifying species in high-nitrate environments or how denitrifying species interact as a community to optimize rates of denitrification. Furthermore, questions remain regarding the roles different species of denitrifying bacteria have in the different stages of denitrification (i.e. nitrate, nitrite, and nitrous oxide reduction). The objectives of this study were to understand the growth, competition, and interactions of denitrifying species throughout the denitrification process, with the intentions of identifying characteristics that might contribute to

growth and survival in contaminated groundwater. From acidic high-nitrate OR-IFRC groundwater and sediment undergoing bioremediation, we cultivated and characterized six denitrifying isolates belonging to the genera *Castellaniella*, *Rhizobium*, and *Pseudomonas*, and tested the effects of pH on nitrate reduction and nitrite accumulation in both pure and mixed cultures. Results suggest that pH and nitrite tolerance give *Castellaniella* isolates a competitive advantage in acidic groundwater undergoing bioremediation and that each genus is better adapted to different stages of denitrification: *Pseudomonas* in nitrate reduction, *Castellaniella* in nitrite reduction, and *Rhizobium* in nitrous oxide reduction. Thus, these isolates may cooperate, rather than compete, as a denitrifying consortia in acidic high-nitrate groundwater undergoing bioremediation.

Materials and Methods

Site description and sample collection

The field research site from which groundwater and sediment samples were collected for cultivation is the U.S. Department of Energy's (DOE's) Integrated Field-scale Subsurface Research Challenge site in Oak Ridge, Tennessee (OR-IFRC), located near the western edge of the Y-12 national security complex. The contamination plume at the site contains various contaminants, including NO_3^-, uranium (U), technetium (Tc), other dissolved metals, and organic copounds. Further detailed groundwater (GW) and sediment geochemical data can be found at http://www.esd.ornl.gov/orifrc/

GW and sediment samples used for cultivation were collected from monitoring wells and cores adjacent to monitoring wells installed within the Area 1 field plot (Table 1). GW in Area 1 is characterized as acidic (pH 3.0-6.8), with high concentrations of NO_3^- (up to 168 mM) and low sulfate (< 1 mM). Radionuclides U and Tc are present (up to 5.8 μM and 19,000 pCi/L, respectively), as well as aluminum and nickel (See Chapter 1 for a detailed review). All groundwater samples were taken during *in situ* push pull tests in Area 1 (Table 1), designed to monitor bacterial NO_3^- and U reduction in response to electron donor addition (31, 58). GW biomass was collected from glucose-stimulated wells FW032 and FW033, as described (51, 53). Sediment cores were sampled adjacent to an ethanol-stimulated well, FW028, as described (58). Samples were shipped on ice to University of Oklahoma and stored at 4°C.

Groundwater from an Area 1 monitoring well FW021 was also sampled (Sept, 2003) and shipped to University of Oklahoma, where it was stored at 4°C until used

for growth and competition experiments. FW021 groundwater has been characterized as acidic (pH 3.3) containing 142 mM NO_3^-, 0.4 mM SO_4^{2-}, and 5.8 μM U(VI) (31).

Cultivation approach and growth medium composition.

Six strains of denitrifying bacteria were isolated from glucose-amended groundwater and ethanol-stimulated sediment (Table 1). Isolates GN32#1, GN33#2, GN33#1, and GN33#3 were cultivated from groundwater as previously described (51, 53). Briefly, these isolates were obtained by serial dilution and direct plating of glucose-stimulated groundwater onto HEPES-buffered minimal medium agar plates (50 mM HEPES, pH7), containing glucose (10 mM dextrose) and nitrate (20 mM $NaNO_3$) as the sole electron donor and acceptor, respectively. The isolation of strains 4.5A2 and 7.5A2 was described in a previous study (58). This involved developing denitrifying enrichments at pHs 4.5 and 7.5 using ethanol-stimulated sediment from Area 1 as an inoculum in a defined minimal medium (MM) containing ethanol (100 mM) and nitrate (100 mM $NaNO_3$). Isolates were obtained by serial dilution of active enrichments and plating onto media containing 50 mM ethanol and 20 mM $NaNO_3$. All plates were incubated under anoxic conditions in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI). All isolates were maintained by transferring liquid cultures via syringe every two months into 20 mM nitrate MM (pH 6.8) with either 10 mM glucose as a sole electron donor (for isolates GN32#1, GN32#2, GN33#1, and GN33#3) or 50 mM ethanol (for isolates 4.5A2 and 7.5A2).

MM used for growth experiments had the following composition (per liter): 0.1 g NaCl; 0.1 g NH_4Cl; 10 mg KCl; 3 mg KH_2PO_4; 40 mg $MgCl_2 \cdot 6H_20$; 40 mg $CaCl_2 \cdot 2H_20$; 11.9 g HEPES; 11.7 g MES; 10 ml vitamin solution (62); 5 ml trace

metals solution (62). The pH was adjusted to 6.8-7.0, unless otherwise noted (i.e. for pH-dependent growth experiments). Typically, either nitrate (10 or 20 mM $NaNO_3$) or O_2 (air) was added as an electron acceptor and glucose (5 mM dextrose) or ethanol (25 or 50 mM) as an electron donor. Nitrate MM was prepared anaerobically (7) in 18 mm serum tubes fitted with butyl rubber stoppers (N_2 headspace), and aerobic media in 16 or 20 mm capped test tubes. Electron donors were added aseptically from sterile stock solutions to media after sterilization by autoclaving at 120°C for 20 min. Unless otherwise noted, all cultures of denitrifying isolates were incubated in the dark at room temperature (approx 23°C).

16S rRNA gene analysis.

DNA extraction and PCR amplification of near full-length 16S rRNA genes was performed as previously described (58). Briefly, DNA was extracted by a boiling method from washed late-log phase cultures. Near full length 16S rRNA genes were amplified using 2 μl of isolate DNA template in a 50 μl PCR mixture, containing universal bacterial primers 27F and 1492R, using the following parameters: initial denaturation at 94°C for 5 min; 35 cycles of 95°C for 30 sec, 45°C °C for 60 sec, and 72°C for 90 sec; and a final extension step at 72°C for 20 min. PCR products were sequenced at the Oklahoma Medical Research Foundation (Oklahoma City, OK).

OR-IFRC isolate and closely related 16S rRNA gene sequences (identified by BLAST and Greengenes) were aligned using Greengenes' NAST alignment tool (13, 14) and initially classified using the Greengenes' Classifier tool as well as the Ribosomal Database Projects Classifier program (12). A distance phylogram of aligned sequences was constructed using the neighbor-joining algorithm and Jukes-

Cantor corrections using ARB software package (40) with the Greengenes May 2007 ARB database (13). Distance trees were also generated via the same methods using PAUP 4.0b10 software (Sinauer Associates, Sunderland, MA) to verify tree branching and generate bootstrap values, based on 1000 replicates.

Cell size, morphology, arrangement, and motility

Liquid cultures of each isolate were heat-fixed and gram-stained according to standard methods (57) to test for purity of isolates, as well as to observe cell morphologies and gram-reactions. Wet mounts of live and formaldehyde-fixed (~3% formaldehyde) cells from freshly grown cultures were also prepared to observe cell morphologies and arrangements, as well as motility. All cell preparations (live, heat-fixed, and formaldehyde-fixed) were viewed at 1000X total magnification under oil immersion using phase contrast microscopy.

Cell morphology, arrangement, size, and motility were also observed from liquid cultures of *Castellaniella* str. 4.5A2 growing in pH 5 and pH 7 nitrate MM. Samples were formaldehyde-fixed during various time points during growth at both pH 5 and 7. Cell sizes were counted from each sample from 100-150 random individual cells.

Growth experiments.

(i) pH and temperature ranges and optima. For all isolates, pH and temperature optima and ranges were determined in both aerobic and nitrate MM (Table 2). Isolates were transferred from mid- to late-log phase cultures and growth curves were carried out in triplicate for each isolate. All isolates were tested at pH values of 3.5-8.5, at 0.5 pH unit intervals (Experiments 1, 2, 5, and 6, Table 2) and at temperatures from 4-

42°C (Exps. 7-10, Table 2) using either glucose, ethanol, or acetate as an electron donor. Growth was measured by optical density (OD) at 600 nm using a Spectronic $20D^+$ (ThermoScientific, Waltham, MA). Generation times (hrs) were calculated from exponential curves fitted through OD_{600nm} values taken during exponential growth phase, at A_{600}<0.4. The pH ranges and optima for three isolates (4.5A2, GN32#2, and GN33#1) were also tested in anaerobic MM with different concentrations of nitrate (10 and 100 mM $NaNO_3$) and ethanol as an electron donor (Exps. 3 and 4, Table 2). During all anaerobic pH growth experiments (Exps. 1-4, Table 2), subsamples were collected from duplicate tubes periodically for nitrate and nitrite analysis. These anions were measured by ion chromatography (Dionex, model DX500 fitted with the AS-4A column; Dionex Corporation, Sunnyvale, CA).

(ii) Utilization of electron donors and electron acceptors. All isolates were also tested for growth in aerobic MM (pH 7, at 30°C) on the following organic substrates: L-arginine, glycine, L-glutamic acid, L-alanine, L-proline, aspartic acid (amino acids); acetate, lactate, pyruvate, malate, fumarate, succinate, and citrate (organic acids); glycerol, D-mannitol, D-sorbitol (sugar alcohols); D-ribose, D-xylose, D-fructose, dextrose, D-lactose (sugars); and ethanol, phenol, benzoate, starch, and yeast extract. Substrates were added to sterile aerobic MM from filter-sterilized stock solutions to reach a final concentration of 1 g/L organic substrate. As a control, each isolate was grown in aerobic MM containing no added organic substrate. Lastly, isolates 4.5A2, GN32#2, and GN33#1 were tested for chemolithotrophic growth in nitrate MM (N_2:CO_2 (80:20) headspace) with 4.3 mM biogenic UO_2 (a black insoluble mineral) as an inorganic electron donor. Utilization of U(IV) as an electron donor was determined

by visualization of dissolution of the black precipitate compared to an uninoculated control.

All isolates were tested for growth in anaerobic MM (pH 7, 50 mM ethanol) on the following electron acceptors: Sodium nitrate (20 mM), sodium nitrite, (20 mM), sodium fumarate (20 mM), glycine (20 mM), sodium sulfate (20 mM), sodium sulfite (20 mM), sodium thiosulfate (20 mM), dimethyl sulfoxide (20 mM), trimethylamine N-oxide (20 mM), amorphous ferric oxyhydroxide gel (20 mM), and Mn(IV)O_2 (15 mM). Electron acceptors were added from anoxic sterile stock solutions. Insoluble iron gel and MnO_2 were prepared from $FeCl_3$, and $MnCl_2 \bullet 4H_2O$, respectively, as previously described (38, 45). Utilization of these electron acceptors was determined by looking for dissolution of the insoluble minerals and detection of reduced products Fe(II) and Mn(II) by the ferrozine assay (39) and formaldoxime (4, 24) colorimetric assays, respectively. As a control, each isolate was grown in aerobic MM containing no added electron acceptor. Growth curves were also done for three isolates (*Castellaniella* str. 4.5A2, *Rhizobium* str. 4.5A2, and *Pseudomonas* str. GN33#1) in anaerobic MM to test growth on different concentrations of nitrate (10 and 100 mM Na NO_3), nitrite (5, 10, and 50 mM), and nitrous oxide (25 mM N_2O) at pH values of 5 and 7.

(iii). Growth on solid media. All isolates were tested for aerobic growth on Luria Bertani (LB) agar plates (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) and MM plates with yeast extract as a carbon source (1 g/L). Plates were incubated at 30°C and checked for colony morphology at 24, 48, and 72 hours. To determine a quantitative differential and selective plating method for the three isolates, *Castellaniella* str.

4.5A2, *Rhizobium* str. GN32#2, and *Pseudomonas* str. GN33#1, pure cultures of each strain as well as a mixed culture of all three isolates were serially diluted and spread onto LB plates (a selective and differential medium, to grow only and differentiate between 4.5A2 and GN33#1) and MM plates with sorbitol (1 g/L) (a selective medium, growing only GN32#2). Colonies were counted and morphologies observed at 24, 48, and 72 hours of incubation at 30°C.

Growth and nitrate reduction in contaminated groundwater by pure cultures and mixed assemblages at pHs 5 and 7.

Isolates *Castellaniella* str. 4.5A2, *Rhizobium* str. GN32#2, and *Pseudomonas* str. GN33#1 were used for growth and competition experiments in OR-IFRC FW021 GW ($[NO_3^-]$~120 mM). The goals of these experiments were to a) determine whether these isolates could grow in Area 1 GW, b) to determine NO_3^- reduction rates and NO_2^- accumulation levels at different initial pH values for of each isolate and c) to determine if a mixed assemblage of the three isolates "outperforms" (i.e. better growth, faster and more efficient nitrate reduction) the best individual isolate in GW.

(i) Groundwater preparation. The pH of FW021 GW, after being stored at 4°C for approx. three years was 4.7. MES (potassium salt) was added to FW021 GW to a final concentration of 50 mM, and GW was centrifuged in 500 ml Beckman centrifuge bottles at 8,000 rpm (using Beckman JLA 10.500 rotor) for 15 minutes to remove solids (e.g. aluminum precipitates, biomass). Then, vitamin and trace metal solutions (62) were added to GW (10 ml/L and 5 ml/L), respectively, and GW was boiled for 5 min and cooled by sparging with N_2:CO_2 (80:20) for 45 minutes, after which the pH of GW had increased to 6.2, and was adjusted (under N_2:CO_2 atmosphere) to either pH 5

(using 1N HCl) or pH 7 (using 3.3 g $NaHCO_3$ per 750 ml GW). Anoxic FW021 GW, prepared at pH 5 and 7, was dispensed into 120 ml serum bottles (48 ml per bottle); bottles were sealed with butyl rubber stoppers and headspace was exchanged with N_2:CO_2 for 5 minutes, after which all bottles were autoclaved. After autoclaving, yeast extract was added to each bottle from a sterile stock solution to reach a final concentration of 0.01%.

(ii). Experimental set up and sampling procedure. Castellaniella str. 4.5A2, *Rhizobium* str. GN32#2, and *Pseudomonas* str. GN33#1 were grown individually, in 100 ml 100 mM nitrate MM (pH7) for 12 days, and pure culture GW bottles amended with ethanol (100 mM) at pH 5 and pH 7 were inoculated with 1.5 ml culture per bottle. A mixed assemblage of all three isolates was inoculated into GW (0.5 ml of each culture) at both pH 5 and 7 and amended with 100 mM ethanol. Cultures containing no ethanol were included as controls. Each set of inoculations was replicated in duplicate bottles at both pH 5 (10 total bottles) and 7 (10 total bottles). All bottles were incubated in the dark at room temperature (approx 23°C), and subsamples were removed periodically for viable counts of each isolate, anion analysis, microscopy, and pH measurement.

Subsamples were removed from pH 7 bottles after 0, 2, 4, 6, 10, 15, 21, 30, and 44 days of incubation and from pH 5 bottles after 0, 3, 7, 11, 17, 25, 34, 46, 64, and 85 days of incubation. At each of these time points, 1.5 ml was removed from each bottle into a sterile 15 ml Falcon tube, flushed with N_2:CO_2. From each of these, 0.5 ml was removed into a 1.5 ml microcentrifuge tube, placed on ice, for viable counts and anion analysis, and 0.1 ml was removed into a separate microcentrifuge

tube containing 9 μl formaldehyde to fix cells. The pH was measured from the remaining sample in each falcon tube. For viable counts, samples were vortexed rigorously to evenly distribute cells and serially diluted (in duplicate from each bottle; four dilutions series per treatment group) in 1X phosphate-buffered saline. From mixed assemblages, each dilution was plated onto three plates of both LB and MM+sorbitol (1 g/L) to quantify all strains. From pure culture incubations of 4.5A2 and GN33#3 bottles, dilution series were plated onto LB plates, and from GN32#2 bottles, dilution series were plated onto MM+sorbitol plates. All plates were incubated aerobically at 30°C, and colonies were counted at 24, 48, and 72 hours of incubation. After samples were removed from microcentrifuge tubes for serial dilutions, tubes were centrifuged at 13,000 rpm to remove biomass; supernatant was removed, diluted 1:10 in nanopure H_2O, and used for anion analysis. Formaldehyde-fixed samples were stored at 4°C until they were counted.

Nitrite minimum inhibitory concentrations (MICs)

Nitrite tolerance was determined at pHs 5, 6, and 7 for isolates 4.5A2, GN33#1, and GN32#2, as well as *Cupriavidus* (formerly *Ralstonia*) *metallidurans str*. CH34 (a known metal-resistant organism (25), used as a comparison in this study, kindly provided by Dr. Daniel van der Lelie of Brookhaven National Laboratory) in both 10 mM nitrate MM and aerobic MM (where no nitrite would accumulate due to denitrification during growth). 2X medium at each pH was prepared and dispensed into 24-well polystyrene plates (1 ml per well); 1 ml of NO_2^- stock solutions was added to each well to reach final [NO_2^-] of 0, 1, 5, 10, 50, 100, and 250 mM at each pH (NO_2^- addition did not change the pH of media). Anaerobic plates were placed in

an anaerobic chamber at least 24 hours prior to inoculation. Ethanol was added to each well (25 mM final conc.), and cells were inoculated from freshly grown cultures (grown in 10 mM nitrate MM at pHs 5, 6, or 7). One well in each plate served as an uninoculated, or sterile control. Aerobic 24-well plates were incubated at room temperature, shaking slowly (80 rpm), and anaerobic plates were incubated in the anaerobic chamber. Growth was monitored ("+" or "-", based on visible turbidity) daily for 10 days; after 14 days, 1 ml aliquots were sampled into cuvettes, and $O.D._{600nm}$ was measured from each well (WPA Biowave, S2100 Diode Array spectrophotometer). The minimum inhibitory concentration (MIC) for each isolate at each pH was defined as the lowest [NO_2^-] tested that inhibited visible growth within 14 days of incubation.

Kinetic parameters of denitrification

Cultures of *Castellaniella* str. 4.5A2, *Rhizobium* str. GN32#2, and *Pseudomonas* str. GN33#1 were grown for enzyme assays to determine the kinetic parameters of nitrate-, nitrite- and nitrous oxide reduction. Typically, 1 L of each culture was grown in 20 mM nitrate MM, pH 7 (inside an anoxic glove chamber) until cells reached mid- to late- log phase for NO_3^- and NO_2^- reduction assays. Cultures were incubated for an additional 24 hours for N_2O reduction assays (upon observation of visible bubbling in culture media).

On the day of each assay, cells were transferred into 500 ml centrifuge bottles inside the anaerobic chamber. Cells were collected by centrifugation (18,000 *x g* for 15 minutes at 4°C using a JLA-10.500 rotor), resuspended in, and washed 3X with anoxic cell buffer (20 mM HEPES, 0.1% NaCl, pH 7). Whole cell suspensions were

prepared by resuspending cells in anoxic cell buffer. Cell-free extracts were prepared by lysing cells with B-PER protein extraction reagent (Pierce Protein Research Products, Rockford, IL), according to manufacturer's instructions, removing intact cells and cell debris by centrifugation. All culture manipulations, other than centrifugation, were done inside the anaerobic chamber, until the point at which whole cells or cell extracts were dispensed into 30-ml serum bottles and sealed. Headspace was flushed with N_2 for 10-15 minutes to remove any H_2 or O_2 that may have been present. Whole cell suspensions and cell extracts yielded protein concentrations ranging from 100-1000 μg/ml and were kept on ice for up to 12 hours to be used in kinetic assays. Protein concentrations were determined with the bicinchoninic acid (BCA) assay kit (Pierce Protein Research Products, Rockford, IL). Prior to protein quantification, whole cells suspensions were diluted 1:2 in 1N NaOH and boiled for 5 min to extract proteins.

Nitrate and NO_2^- kinetic assays were performed in serum tubes, sealed with butyl rubber stoppers; each containing 8 ml (total volume) reaction buffer (pH 7), containing the following: 20 mM HEPES, 1% NaCl, 5 ml/L trace metals, and 1 mM benzyl viologen (BV) dye (spectrophotometric reagent). Prior to each assay, BV was reduced by adding an anoxic solution of 5 mM sodium dithionite (prepared fresh the day of each assay) drop-wise until reaction buffer reached an $O.D._{600nm}$ 0.7-0.9 (measured using a Spec20D$^+$). Cell extracts were added (0.1-0.3 ml) to reaction buffer containing reduced BV (electron donor) and pre-incubated for five minutes prior to the start of each reaction. Reactions were started by the addition of 0.1 ml of anoxic nitrate or NO_2^- stock solutions, to reach concentrations ranging from 0.01 to 1 mM,

and Abs_{600nm} was recorded every 15 seconds. Rates of BV loss were calculated using the Beer Lambert Law equation $A = \varepsilon \bullet c \bullet l$ to solve for c (concentration of BV), where $A = Abs_{600\ nm}$, ε = extinction coefficent for BV, 10.4 $cm^{-1} \bullet mM^{-1}$ (35), and l = path length of the sample (1.5 cm diameter inside Balch tube). As the oxidation of reduced BV yields one electron, rates were expressed for both nitrate and NO_2^- assays as μmol $e^- \bullet min^{-1} \bullet$mg $protein^{-1}$. Rates were calculated for each electron acceptor in duplicate or triplicate at several different initial concentrations (0.01-1 mM), and kinetic parameters K_m, and V_{max} were calculated by non-linear regression curve fitting of rate data (using SigmaPlot® software, Systat Software, Inc.,) to the Michaelis Menten equation: $V = (V_{max} \bullet [S_0])/(K_m + [S_0])$, where V = rate at each initial electron acceptor concentration ($[S_0]$). Controls performed included cell-extract only controls for each isolate as well as nitrate- or NO_2^--only (no cell extract) controls. Rates based on cell extracts were also compared to those using whole cell suspensions of GN33#1. Finally, nitrate reduction rates based on cell extracts of 4.5A2 determined from BV assays were compared to nitrate reduction rates in whole cell assays using ethanol as an electron donor (where nitrate loss was monitored over time from sub-samples removed throughout the time course of the experiment).

Because a previous study had found that BV could act as an electron donor for N_2O reductase of *Paracoccus denitrificans* cell-free extracts, but not in whole cells (35), N_2O kinetic assays were performed in this study in a similar manner to nitrate and NO_2- kinetic assays, using cell-free extracts. However, assays were done with 25 ml of reaction buffer in serum tubes (no headspace, to eliminate or minimize the partitioning of N_2O into gas phase), and reactions were started by the addition of a

saturated solution of N_2O prepared in anoxic water, assumed to be 25 mM, based on the maximum solubility of this gas at room temperature (35). Minimum volume of N_2O required for saturation was determined using the Ostwald coefficient for this gas at room temperature (0.679 /L) (68).

Results

Phylogeny (16S rRNA gene analysis).

Greengenes and RDP Classifier programs found that, based on 16S rRNA gene sequences, the six isolates fell into three gram-negative genera: *Rhizobium* (of the *Alphaproteobacteria*), *Castellaniella* (*Betaproteobacteria*) and *Pseudomonas* (*Gammaproteobacteria*). Phylogenetic distance trees verified these results; also, these genera have been detected among various clone libraries constructed from OR-IFC site sediment (Fig 1). *Castellaniella* strains 4.5A2 and 7.5A2 formed a clade with environmental clone sequences from a PCE-contaminated aquifer (unpublished, Genbank accession number EF644519) and an ethanol-stimulated sediment coupon deployed in an OR-IFRC Area 1 multilevel sampler well (50), distinct from clades of known published species of *Castellaniella* (*C. defragrans*, *C. denitrificans*, and *C. canei*) (32, 37) (Fig 1), suggesting that these isolates may represent a novel species within the genus.

Rhizobium strains GN32#1 and GN32#2 are 99.9% similar to each other and are most closely related (>99% similarity) to a clone from NO_3^- and Tc-reducing OR-IFRC sediment microcosms (36) and are >98% similar to cultivated isolates *Agrobacterium tumefaciens* TG12 and TG14 (classified as *Rhizobium* according to the RDP taxonomy), nitrate-reducing isolates capable of phenol degradation under low O_2 conditions (5), and *Rhizobium* sp. 52W, isolated from a rice paddy field (unpublished, Genbank accession number AB262326). *Rhizobium* strain GN33#3 is 97.1% similar to the other two OR-IFRC *Rhizobium* strains, suggesting it may represent a separate *Rhizobium* species (or subspecies). It is most closely related (>99%) to a clone from

an arsenite-oxidizing denitrifying enrichment (60) and is also >98% similar to root-associated soil isolates *Amorphomonas oryzae* B46 and B47 (also classified as *Rhizobium* according to the RDP taxonomy) (unpublished, Genbank accession numbers AB233493 and AB233494).

Pseudomonas str. GN33#1 is >99% similar to a soil isolate, *Pseudomonas stutzeri* str. 24a36 (55) as well as several strains of *Pseudomonas stutzeri* (Fig 1), and thus GN33#1 is likely a strain of this species, which has been used as a model to study denitrification (34, 64, 70, 71) and hydrocarbon degradation (6, 54, 59).

Cell morphology, arrangement, and motility

All isolates appeared as single, motile rods and stained gram-negative. Cells from *Rhizobium* and *Pseudomonas* isolates were slightly larger (>2 μm in length) than cells from both *Castellaniella* cultures (approx 1.5 μM in length). Cells from *Castellaniella* cultures appeared to move much more quickly than other isolates. At pH 5, motility of *Castellaniella* str. 4.5A2 was however, decreased, and more aggregates were present at pH 5 than at pH 7 in nitrate MM. Cells observed at pH 5 were also longer (1.6 ± 0.33 μm in length) during mid-log phase than cells growing at pH 7 (1.45 ± 0.35 μm in length). At both pH 5 and 7, cells increased in length as growth entered stationary phase.

Growth experiments.

(i) pH and temperature ranges and optima. pH and temperature ranges and typical growth curves for isolates 4.5A2, GN32#2 and GN33#1 are shown in Table 2 and Figure 2, and unless otherwise stated, data presented for 4.5A2 is typical of both *Castellaniella* strains (4.5A2 and 7.5A2) and data presented for GN32#2 is typical of

all *Rhizobium* strains. Overall, results show that *Castellaniella* strains are the most tolerant of low pH and that electron donor and initial [NO_3^-] affect acidic pH tolerance.

In 20 mM nitrate MM with ethanol (Exp. 2), *Castellaniella* strains 4.5A2 and 7.5A2 grew from pHs 4.5-8.0, and optimally at pH 6.5 (Table 2); growth rates were nearly identical at pHs 5.5-7.5, and only at pH 4.5 did growth appear affected (Fig 2). Aerobically with acetate (Exp. 6), these isolates did not grow below pH 5.5, and grew optimally at pH 7.5 (Table 2). Additional experiments in nitrate MM also showed that str. 4.5A2 was capable of growth with ethanol, but not acetate, as an electron donor at pH 5 (data not shown), suggesting that electron donor used for growth is critical in regards to pH tolerance. In 10 mM nitrate MM, strain 4.5A2 grew from pHs 4.0-8.5, whereas in 100 mM nitrate MM (Exp. 4), this strain did not grow below pH 4.5 (Table 2), suggesting that initial [NO_3^-] (or levels of denitrification intermediates that may accumulate, depending on initial [NO_3^-]), may also affect pH range for growth.

Rhizobium str. GN32#2 also grew at pHs 4.5-8.0, and optimally at pH 6.5 in 10 mM nitrate MM (Table 2, Exp 1), though growth was slower at acidic pHs (4.5 and 5.5) than *Castellaniella* strains (Fig 2). However, the other strains of *Rhizobium* (GN32#1 and GN33#3) were only capable of growth at pHs 5.5-8.0 (data not shown). With ethanol as an electron donor in 10 mM nitrate MM (Exp. 3), GN32#2 did not grow below pH 5 (Table 2), further validating that electron donor choice affects pH tolerance. For this isolate, pH range was also narrower at 100 mM vs. 10 mM nitrate (Table 2, Exps. 3 and 4). Lastly, growth of *Rizobium* isolates in nitrate MM typically

yielded more biomass (higher Max. OD_{600nm}) than *Castellaniella* or *Pseudomonas* isolates (Fig. 2).

Pseudomonas str. GN33#1 appeared to have the fastest generation time of all isolates at neutral pH in nitrate MM (Fig 2), having the narrowest pH range, growing from pH 6.0-8.0 in nitrate MM (Exp. 1), and the highest optimum pH (8.0) (Table 2). Aerobically, GN33#1 was capable of growth down to pH 5.5 (Table 2, Exp. 5), suggesting that nitrate, or accumulation of denitrification intermediates, may contribute to toxicity at slightly acidic pH values. Unlike *Rhizobium* isolates , growth at pHs > 6 was not affected by high initial [NO_3^-] (Table 2, Exps. 3 and 4).

All isolates were considered mesophilic in regards to their temperature ranges and optima and were capable of growth at low temperatures (4 and 16°C).

(ii) pH-dependent nitrate reduction and nitrite accumulation. In pH experiments 1-4 (Table 2), subsamples were removed from serum tubes periodically throughout growth curves to determine [NO_2^-] and [NO_3^-]. Nitrate reduction rates seemed to match closely with growth for all isolates (Fig. 2 -- nitrate data for Exps 1 and 2 are shown in Fig. 2; nitrate data for Exps. 3 and 4 showed similar trends and are thus not presented here). *Pseudomonas* str. GN33#1 reduced nitrate more quickly during growth than *Castellaniella* and *Rhizobium* isolates (Fig. 2), and did so optimally at slightly alkaline pH values. In 10 mM nitrate MM with ethanol, *Pseudomonas* GN33#1 accumulated more NO_2^- at pH 6 (max [NO_2^-] = 10.9 mM) than at pHs 7 or 8 (Table 3); furthermore, NO_2^- was not further reduced at pH 6 (data not shown). In 100 mM nitrate MM, *Psudomonas* GN33#1 accumulated higher [NO_2^-] at pHs 7 and 8 (max [NO_2^-] = 71.2 and 80.3 mM, respectively) than at pH 6 (max [NO_2^-] = 19.5 mM); however, as noted

in other experiments, NO_2^- was not reduced at pH 6, and after the observed NO_2^- accumulation, further nitrate reduction and growth were inhibited (data not shown).

Rhizobium and *Castellaniella* isolates, on the other hand reduced nitrate more slowly than *Pseudomonas* str. GN33#1 during growth, and optimally at pH 6.5 (Fig 2). For *Castellaniella* str. 4.5A2, nitrate reduction varied little between pHs 5.5-7.5, whereas acidic pH values had a greater effect on the lag time and rate of nitrate reduction and growth for *Rhizobium* str. GN32#2 (Fig. 2). Overall, much less NO_2^- accumulation was observed in *Castellaniella* and *Rhizobium* isolates than for *Pseudomonas* GN33#1 at all pH values.

(iii) Utilization of different electron donors and electron acceptors. All isolates were tested for growth on 28 different organic substrates; Maximum optical densities (Abs_{600nm}) for GN32#2 (typical of all *Rhizobium* isolates, unless otherwise indicated), 4.5A2 (typical of both *Castellaniella* isolates) and GN33#1 are shown in Figure 3. *Rhizobium* isolates had the highest maxmum ODs on nearly all substrates tested and were the only isolates to utilize sugars other than glucose as a growth substrate (Fig. 3). *Rhizobium* isolates grew best on sugars, and sugar alcohols (sorbitol and mannitol) but also grew well on most amino acids and organic acids tested, with the exceptions of glycine, succinate, and citrate (Fig 3). *Rhizobium* GN33#3, which differs phylogenetically from the other two *Rhizobium* isolates (Fig 1), was the only isolate of the three capable of growth on succinate (data not shown).

Pseudomonas str. GN33#1 and *Castellaniella* isolates 4.5A2 and 7.5A2 had similar substrate utilization profiles, growing best on amino acids and organic acids

(Fig 3). As well, *Pseudomonas* str. GN33#1 and *Castellaniella* isolates were capable of using benzoate as a sole carbon source.

Castellaniella str. 4.5A2 showed visible clearing of U(IV), a black precipitate, when UO_2 was added to nitrate MM (with N_2:CO_2 headspace) as an electron donor whereas *Rhizobium* str. GN32#2 and *Pseudomonas* str. GN33#1 did not. Though U(VI) was not measured and cultures have yet to be transferred, this serves as a preliminary indication that *Castellaniella* str. 4.5A2 may be capable of chemolithoautotropic growth with U(IV) as an insoluble electron donor and nitrate as an electron acceptor.

All isolates were capable of utilizing O_2, NO_3^-, NO_2^-, and N_2O as electron acceptors to support growth, whereas none of the isolates were capable of growth on any of the other electron acceptors tested. *Rhizobium* isolates showed a partial dissolution of iron gel and MnO_2 minerals; however reduced Fe(II) or Mn(II) could not be detected in any of these culture tubes.

(iv) Comparison of growth rates on nitrate and denitrification intermediates, nitrite and nitrous oxide. Isolates 4.5A2, GN32#2, and GN33#3 were all tested for growth on nitrate, NO_2^-, and N_2O at both pHs 5 and 7. All were capable of growth on each electron acceptor at pH 7 (Table 4). *Pseudomonas* str. GN33#1 had the fastest generation time on nitrate (2.6±0.15 hours), whereas *Castellaniella* str. 4.5A2 grew the most slowly on nitrate (Gen time = 8.6±1.3 hrs). Each isolate grew more slowly in 100 mM nitrate MM than in 10 mM nitrate MM, suggesting that nitrate or accumulating denitrification intermediates could be toxic during growth. Likewise, an increase in [NO_2^-] impaired growth rates of each isolate, though more so for

Pseudomonas GN33#1 and *Rhizobium* GN32#2 than for *Castellaniella* 4.5A2, which had the fastest generation time on NO_2^- (4.0±1.3 hours) (Table 4). *Rhizobium* str. GN32#2 was the most impaired with 50 mM NO_2^- – the lag time was long (several weeks, data not shown) and the average generation time was 95 hours. This isolate grew faster on N_2O than on nitrate or NO_2^-, and its growth on N_2O was faster than the other isolates (Gen time=3.4 ±0.12 hours).

At pH 5, only *Castellaniella* 4.5A2 and *Rhizobium* GN32#2 grew on nitrate; however, GN32#2 grew too slowly at pH 5 (with ethanol) for a generation time to be reported (Table 4). At pH 5, 4.5A2 was the only isolate of the three to grow on NO_2^- (and growth only occurred at 5 mM NO_2^-, after several weeks of a lag period) and N_2O (Table 4).

(v) Colony morphologies on different types of solid media. All isolates formed smooth, round colonies on solid media. On LB plates, GN33#1 formed pale yellow colonies within 24 hours (approx. 1 mm in diameter). *Castellaniella* isolates 4.5A2 and 7.5A2 formed small pin-point colonies on LB within 48 hours (barely visible); within 72 hours, colonies were pale yellow and 0.5-1 mm in diameter. *Rhizobium* isolates did not form colonies on LB plates; further tests revealed that these isolates also did not grow on other complex rich solid media, such as tryptic soy agar or nutrient agar. All isolates, however, formed colonies on MM plates with yeast extract (1 g/L) as a carbon source. As on LB, *Pseudomonas* and *Castellaniella* isolates formed colonies at 24 and 48-72 hours, and respectively, with distinguishing diameters, as noted above. *Rhizobium* isolates formed smooth, white colonies approx 1 mm in diameter after 48-72 hours on yeast extract MM plates.

Viable counts were performed from pure liquid cultures of 4.5A2, GN32#2, and GN33#1 and from a mixed culture of the three (mixed with known volumes of each pure culture) using LB plates and MM plates + sorbitol. As expected, in pure culture, *Pseudomonas* GN33#1 and *Castellaniella* 4.5A2 formed colonies on LB plates at 24 and 48-72 hours, respectively, and *Rhizobium* GN32#2 was the only isolate to form colonies only on MM plates + sorbitol (round, white, with a mucoid texture). From mixed cultures of all three isolates, GN33#1 and 4.5A2 could be distinguished on LB plates due to their respective colony morphologies and sizes, and GN32#2 could be counted from a mixed culture on MM+sorbitol plates. This method was used from a mixed culture with known cell densities of each isolate to accurately quantify CFUs/ml of each isolate from within the mixture (data not shown).

Growth and nitrate reduction in contaminated groundwater by pure cultures and mixed assemblages at pHs 5 and 7.

(i) Growth, nitrate reduction and nitrite accumulation in pH 5 FW021 GW.

Castellaniella sp. 4.5A2 was the only isolate of the three capable of growth in pH 5 FW021 GW in pure culture (Fig. 4); 4.5A2 also maintained its population within the mixed culture better than it did in pure culture, where viable counts began to decrease after 25 days (Fig. 4). *Rhizobium* sp. GN32#2, though incapable of growth in pure culture (dying off, below detectable levels within 46 days) grew in the mixed assemblage in pH 5 GW both with ethanol, as well as in the electron donor control (no ethanol added) after initially dying off (Fig 4). *Rhizobium* GN32#2, despite growing in the mixed culture + ethanol, remained < 1% of the total cells throughout the majority of the experiment. *Pseudomonas* str. GN33#1 had died off, below detectable

levels, by 25 and 64 days in pure culture and within the mixed assemblage + ethanol, respectively (Fig 4).

Cells in both 4.5A2 pure cultures and mixed assemblages in pH 5 GW appeared similar by phase contrast microscopy after 5 days of incubation; most cells were single rods whereas some formed large aggregates. The number of cell aggregates per field of view peaked between Days 17-46. By day 85, most cells appeared as longer, single rods in the 4.5A2 pure culture; however, in the mixed assemblage, many more cells were observed in odd arrangements, attached to each other or to precipitates.

Nitrate reduction was faster with less NO_2^- accumulation in the mixed assemblage + ethanol compared to *Castellaniella* str. 4.5A2 alone (Fig. 5, Table 5). Within the 85 days of the experiment, *Castellaniella* str. 4.5A2 had reduced 56% of the total nitrate in pure culture whereas 88% of the nitrate had been reduced in the mixed assemblage + ethanol. The zero-order nitrate reduction rate by the mixed assemblage + ethanol at pH 5 was 1.3 mM/day. Also, maximum and final NO_2^- concentrations were higher in bottles containing 4.5A2 alone vs. the mixed assemblage + ethanol (Table 5). Nitrate was not reduced in either the *Pseudomonas* or *Rhizobium* pure cultures, nor in the mixed assemblage – ethanol control at pH 5; however approximately 0.5-1 mM NO_2^- accumulated in each of these (Fig. 5). The final pH values of GW containing *Castellaniella* 4.5A2 pure culture, the mixed assemblage + ethanol, and the mixed assemblage – ethanol were 6.6, 6.8, and 5.6, respectively, whereas the pH of GN32#2 and GN33#1 pure cultures had remained unchanged throughout the experiment (final pHs = 5.05 and 5.1, respectively).

(ii) Growth, nitrate reduction and nitrite accumulation in pH 7 FW021 GW. All three isolates were capable of growth in pure culture and concomitant growth within mixed assemblages + ethanol in pH 7 FW021 GW (Fig. 4). In pure culture, and in mixed assemblages, both *Castellaniella* str. 4.5A2 and *Pseudomonas* str. GN33#1 grew for 6 days after which viable counts began to decrease (Fig. 4). *Rhizobium* str. GN32#2, on the other hand, was the only isolate that continued to grow within the mixed assemblage + ethanol after 6 days, and by the end of the experiment, this isolate dominated the mixed assemblage (64% of total cells). In pure culture, however, viable counts of GN32#2 rapidly decreased after only 4 days (Fig. 4).

Under the microscope, GN32#2 in pure culture in pH7 GW appeared to form mostly small aggregates of 2-5 cells, typically attached in a variety of shapes whereas 4.5A2 and GN33#1 mainly appeared as single rods or attached to large mineral chunks (probably carbonate minerals). A mixture of all these cell arrangements were seen in the mixed assemblage + ethanol.

In pH 7 GW, nitrate reduction proceeded more quickly in mixed assemblages + ethanol vs. any of the pure cultures alone (Fig 5). The zero-order nitrate reduction rate by the mixed assemblage + ethanol was 2.6 mM/day, twice faster than at pH 5 (Table 5). In pure culture, *Pseudomonas* str. GN33#1 has the fastest initial nitrate reduction, but this slowed after Day 6, at which point [NO_2^-] had increased to >20 mM (Fig. 5). By the end of the experiment, this isolate only reduced 56% of the total nitrate, and the final [NO_2^-] was 18 mM (Table 5). *Rhizobium* str. GN32#2 was the second fastest of the three isolates in regards to initial nitrate reduction rate; however, this activity stopped after Day 15, at which point [NO_2^-] had increased to > 20 mM

(Fig. 5). By the end of the experiment, GN32#2 reduced 71% of the total nitrate, and the final [NO_2^-] was 29 mM (Table 5). *Castellaniella* str. 4.5A2 was the slowest among the three isolates in regards to nitrate reduction (Fig. 5); however, this activity did not slow down or cease, as observed with the other two isolates, and by end of the experiment, 4.5A2 reduced 84% of the total nitrate, and the final [NO_2^-] was 7.7 mM (Table 5). The final [NO_2^-] in the mixed assemblage + ethanol was less than in any of the pure cultures alone (1.5 mM).

Nitrite MICs

MICs of NO_2^- were higher along with increasing pH for all isolates (Table 6), suggesting that NO_2^- is generally more toxic at more acidic pH values. Nitrite MICs for *Castellaniella* str. 4.5A2, for example, were 5, 50, and 250 mM at pHs 5, 6, and 7, respectively. 4.5A2 was the much more NO_2^- tolerant than other isolates, growing at [NO_2^-] ten-fold higher than all other isolates at each pH. Nitrite was more toxic to *Pseudomonas* str. GN33#1 and *C. metallidurans* str. CH34 under anaerobic conditions, in which nitrite tends to accumulate > 5 mM, according to *a priori* data obtained (Table 3, data not shown *C. metallidurans*). This was not observed, however, for isolates 4.5A2 and GN32#2, which tend to accumulate little [NO_2^-] at lower pH values (Table 3).

Kinetic parameters of denitrification

Results from controls showed that absorbance loss due to either cell extracts alone or any of the electron acceptors alone occurred within the first 15 seconds of each assay, after which absorbance was stable. Thus, absorbance measurements taken before 15 seconds (i.e. T_0) were excluded from rate calculations. Nitrate-reduction rates were 9.2

times greater using cell extracts of GN33#1 than whole cells. Likewise, nitrate reduction rates based on BV assays using cell extracts of 4.5A2 were 9.3 times greater than those calculated from whole cell assays using ethanol. It was therefore concluded that assays using reduced BV as an electron donor for cell-free extracts prepared using B-PER were sufficient to determine and compare the kinetics of nitrate, NO_2^-, and N_2O reduction of the three isolates.

Isolates *Castellaniella* str. 4.5A2, *Rhizobium* str. GN32#2, and *Pseudomonas* str. GN33#1 all had fairly high K_m values for nitrate, compared to NO_2^- and N_2O (Table 7), GN33#1 had the fastest V_{max} on nitrate, compared to NO_2^- and N_2O, and the fastest nitrate-reducing activity of any of the other isolates. Although it was hypothesized that 4.5A2 would have the fastest rates of nitrite reduction, it was actually GN32#2 that had the highest V_{max} on nitrite (Table 7), but the lowest affinity, while GN33#1 had the highest affinity for NO_2^-, but slowest V_{max}. Both GN32#2 and 4.5A2 each had much faster rates of NO_2^- reduction compared to nitrate reduction (Table 7), thus potentially explaining the low levels of NO_2^- that accumulate in growth medium for these two cultures, compared to GN33#3 (Table 3), which, on the other hand, had nearly 100-fold greater activity on nitrate over NO_2^- (Table 7). Both GN33#1 and 4.5A2 had comparably high rates of N_2O reduction, with high affinities for the gas (Table 7). Michaelis Menten constants for GN32#2 unfortunately could not be obtained with cell extracts on N_2O; though cell extracts exhibited some activity (max observed = 0.34 μmol e^-•min^{-1}•mg $protein^{-1}$), rates were not proportional to protein concentration, nor were Michaelis Menten kinetics observed (i.e. rate was not proportional to substrate concentration).

Discussion

Nitrate reduction rates and accumulation of denitrification intermediates are both important in regards to bioremediation of U-contaminated groundwater in that both can directly (or indirectly) affect both U(VI) reduction and U(IV) reoxidation, respectively, during biostimulation efforts. In this study, we have shown through simple pure and mixed culture experiments using denitrifying isolates cultivated from the OR-IFRC, that denitrification in acidic and neutral high-nitrate groundwater is optimal with mixed assemblages of denitrifying bacteria, with more complete and higher rates of nitrate reduction and less nitrite accumulation than with any pure culture alone (Fig 5, Table 5). Nitrite accumulation, while relevant to U(VI) oxidation, may also be critical in regards to the growth and survival of subsurface microorganisms, as concentrations > 5 mM are considered toxic to sulfate-reducing, syntrophic, and methanogenic bacteria (26, 46), as well as denitrifying bacteria in pure culture (2) and in activated sludge (23). In the latter studies, it was indicated that nitrite toxicity is dependent on nitrous acid (HNO_2) concentration, and is therefore pH-dependent. As well, our data indicate that nitrite inhibits growth of denitrifying bacteria at concentrations > 10 mM, and that nitrite toxicity is pH-dependent, being more toxic at acidic pH values (Table 6). Thus, the ability of mixed assemblages to limit nitrite accumulation compared to pure cultures (Fig 5) is likely important to growth and survival of not only denitrifying bacteria, but other subsurface microorganisms as well.

Mixed assemblage experiments using the three isolates *Castellaniella* str. 4.5A2, *Rhizobium* str. GN32#2, and *Pseudomonas* str. GN33#1 in OR-IFRC

groundwater showed comparable rates of nitrate reduction and amounts of nitrite accumulation to those seen in field and lab-scale experiments using natural sediment communities. Nitrite accumulation during biostimulation of Area 1 OR-IFC sediments often and typically far exceeds [NO_2^-] that are considered toxic, up to 130 mM, though typically between 10-20 mM (31, 43, 58). Similarly, the mixed assemblage accumulated [NO_2^-] of 4.8 and 18.8 mM at pHs 5 and 7, respectively (Table 5). Zero-order nitrate reduction rates during flow-through biostimulation experiments in Area 1 sediments typically range from 6-10 mM/day (31, 43). Our mixed assemblage results showed zero-order rates of 1.3 and 2.6 mM NO_3^-/day at pH 5 and 7, respectively, which is similar to that observed in Area 1 batch microcosms (1.125 mM/day) (43). Thus, the tri-culture used in our experiments may serve as a useful model to study natural denitrifying communities, and how environmental factors, such as pH, affect rates of nitrate reduction, nitrite accumulation, and growth and survival of microorganisms involved in these processes.

While co-culture experiments have been performed to study denitrification and have generated similar results (i.e. more complete denitrification and less denitrification intermediates) (42, 61, 63), it has been suggested that even in the breakdown of a single compound (i.e. nitrate), multiple species are likely involved and that it is difficult to identify which microorganisms are involved (63). In utilizing growth, kinetic, and nitrite MIC experiments, we have found that each isolate prefers different denitrification electron acceptors for growth (Table 4), with different rates of reduction for each (Table 7) and varies in regard to nitrite tolerance; these differences

lead us to believe that in the tri-culture, each genus may play a unique role in bioremediation.

Pseudomonas str. GN33#1 likely contributes mostly to nitrate reduction in neutral contaminated groundwater, until nitrite accumulation exceeds toxic levels. For example, during the OR-IFC groundwater experiment, nitrate reduction and nitrite accumulation of GN33#1 were nearly identical for the first 6 days, until nitrite concentration reached > 20 mM (Fig 5). In pure culture, viable counts and nitrate reduction rates decreased after this (Figs 4 and 5), suggesting it no longer had a competitive advantage in a mixed culture. Despite having the lowest affinity for nitrate, nitrate reduction rates are the fastest for this organism over the other two isolates in kinetic experiments (Table 7), and it had the fastest generation time on nitrate (Table 4), further validating its likely role as the key nitrate-reducer in mixed assemblages. However, it accumulates more nitrite at acidic pH values (Fig 2, Table 3), is less nitrite-resistant (Table 6), and has a narrower pH range (and higher optimal pH) than the other isolates (Table 2), *Pseudomonas* str. GN33#1 is likely only active in nitrate reduction in neutral and basic groundwater.

Castellaniella str. 4.5 A2 is likely a key player in nitrite reduction in acidic high-nitrate groundwater as well as under neutral conditions when nitrite accumulates to high levels. Though this isolate did not have the fastest V_{max} in regards to nitrite reduction at pH 7 (Table 7), it was shown to be the most nitrite-resistant (Table 6), and was even capable of growth on 5 mM nitrate at pH 5 (Table 4). This isolate typically accumulates low levels of nitrite at low pHs (Table 3); this, along with its enhanced nitrite tolerance compared to other isolates, may explain how its growth rate is

relatively unaffected at lower pH values (Fig 2). Even though this isolate is the slowest nitrate reducer under neutral conditions, it seems to be likely involved in nitrate reduction in pH 5 OR-IFRC groundwater, since the other two isolates alone were incapable of nitrate reduction under the same conditions. In all, it seems that nitrite and pH tolerance are likely two large contributing factors to the growth and survival of this isolate in OR-IFC groundwater under acidic and neutral conditions, thus potentially explaining the dominance of *Castellaniella* in clone libraries generated from OR-IFRC Area 1 sediments undergoing biostimulation (50, 58). This isolate may prove to be a useful model organism to study pH and nitrite tolerance, and further research is warranted in examining whether aggregate formation and motility are important for growth and survival of *Castellaniella* species and whether these isolates are indeed capable of chemoautolithotrophic growth with insoluble U(IV) and nitrate as an electron donor and acceptor, respectively.

Rhizobium str. GN32#2 is likely involved in later stages of denitrification in OR-IFRC high nitrate GW. First, this isolate was the only one among the three capable of growth in both pH 5 and 7 OR-IFRC groundwater after Days 17 and 6, in mixed assemblage experiments (Fig 4). Second, it has the fastest rate of nitrite reduction (Table 7), but since it is comparably lacking in nitrite tolerance, this isolate is might only contribute to nitrite reduction when nitrite concentrations remain low. Third, it has the fastest growth rate on N_2O (Table 4). Factors that may contribute to growth and survival of this isolate in groundwater may include, as with *Castellaniella*, its ability to accumulate low levels of nitrite under low-pH conditions (Table 3) and its wide pH range (Table 2). Also, this isolate has the widest substrate range, and seems

to be the most growth efficient (Figure 3), and preliminary data suggests it has the ability to solubilize insoluble Fe(III) and Mn(IV) minerals; the ability to solubilize minerals could be an advantage in low nutrient conditions.

Lastly, the work presented here provides some general implications for field scale bioremediation efforts. First, electron donor choice is important in acidic groundwater, as we have shown that donor type for both *Castellaniella* str. 4.5A2 and *Rhizobium* str. GN32#2 affects these isolates pH range of growth (Table 2). Similarly, electron donor choice has been shown to be critical to functional diversity of microbial communities and U(VI) reduction, with ethanol being a good electron donor choice (1). Second, nitrite accumulation is key, and must be alleviated to improve bioremediation of high nitrate- and U-contaminated groundwater, as it not only results in U(IV) oxidation as other studies have shown, but also has toxic effects on the microbial populations responsible for nitrate removal. Because of its nitrite reduction rates and nitrite tolerance, growth and survival of *Castellaniella* str. 4.5A2, or species closely related to this isolate, is likely important in keeping nitrite concentrations low in acidic high-nitrate groundwater undergoing biostimulation.

References

1. **Akob, D. M., H. J. Mills, T. M. Gihring, L. Kerkhof, J. W. Stucki, A. S. Anastacio, K. J. Chin, K. Kusel, A. V. Palumbo, D. B. Watson, and J. E. Kostka.** 2008. Functional diversity and electron donor dependence of microbial populations capable of U(VI) reduction in radionuclide-contaminated subsurface sediments. Appl Environ Microbiol **74:**3159-70.

2. **Almeida, J. S., S. M. Julio, M. A. M. Reis, and M. J. T. Carrondo.** 1995. Nitrite inhibition of denitrification by *Pseudomonas fluorescens*. Biotechnol Bioeng **46:**194-201.

3. **Anderson, R. T., H. A. Vrionis, I. Ortiz-Bernad, C. T. Resch, P. E. Long, R. Dayvault, K. Karp, S. Marutzky, D. R. Metzler, A. Peacock, D. C. White, M. Lowe, and D. R. Lovley.** 2003. Stimulating the in situ activity of *Geobacter* species to remove uranium from the groundwater of a uranium-contaminated aquifer. Appl Environ Microbiol **69:**5884-91.

4. **Armstrong, P. B., W. B. Lyons, and H. E. Gaudette.** 1979. Application of Formaldoxime Colorimetric Method for the Determination of Manganese in the Pore Water of Anoxic Estuarine Sediments. Estuaries **2:**198-201

5. **Baek, S. H., K. H. Kim, C. R. Yin, C. O. Jeon, W. T. Im, K. K. Kim, and S. T. Lee.** 2003. Isolation and characterization of bacteria capable of degrading phenol and reducing nitrate under low-oxygen conditions. Curr Microbiol **47:**462-6.

6. **Baggi, G., P. Barbieri, E. Galli, and S. Tollari.** 1987. Isolation of a *Pseudomonas stutzeri* strain that degrades o-xylene. Appl Environ Microbiol **53:**2129-32.

7. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressureized atmosphere. Appl Environ Microbiol **32:**781-91.

8. **Baldwin, B. R., A. D. Peacock, M. Park, D. M. Ogles, J. D. Istok, J. P. McKinley, C. T. Resch, and D. C. White.** 2008. Multilevel samplers as microcosms to assess microbial response to biostimulation. Ground Water **46:**295-304.

9. **Baumann, B., J. R. van der Meer, M. Snozzi, and A. J. Zehnder.** 1997. Inhibition of denitrification activity but not of mRNA induction in *Paracoccus denitrificans* by nitrite at a suboptimal pH. Antonie Van Leeuwenhoek **72:**183-9.

10. **Cavigelli, M. A., and G. P. Robertson.** 2000. The functional significance of denitrifier community composition in a terrestrial ecosystem. Ecology **81:**1402-1414.

11. **Chang, Y. J., P. E. Long, R. Geyer, A. D. Peacock, C. T. Resch, K. Sublette, S. Pfiffner, A. Smithgall, R. T. Anderson, H. A. Vrionis, J. R. Stephen, R. Dayvault, I. Ortiz-Bernad, D. R. Lovley, and D. C. White.** 2005. Microbial incorporation of 13C-labeled acetate at the field scale: detection of microbes responsible for reduction of U(VI). Environ Sci Technol **39:**9039-48.

12. **Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje.** 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res **33:**D294-6.

13. **DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen.** 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol **72:**5069-72.

14. **DeSantis, T. Z., Jr., P. Hugenholtz, K. Keller, E. L. Brodie, N. Larsen, Y. M. Piceno, R. Phan, and G. L. Andersen.** 2006. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Res **34:**W394-9.

15. **Edwards, L., K. Kusel, H. L. Drake, and J. E. Kostka.** 2007. Electron flow in acidic subsurface sediments co-contaminated with nitrate and uranium. Geochimica et Cosmochimica Acta **71:**643-654.

16. **Fernandez-Nava, Y., E. Maranon, J. Soons, and L. Castrillon.** 2008. Denitrification of wastewater containing high nitrate and calcium concentrations. Bioresour Technol **99:**7976-81.

17. **Fields, M. W., T. Yan, S. K. Rhee, S. L. Carroll, P. M. Jardine, D. B. Watson, C. S. Criddle, and J. Zhou.** 2005. Impacts on microbial communities and cultivable isolates from groundwater contaminated with high levels of nitric acid-uranium waste. FEMS Microbiol Ecol **53:**417-28.

18. **Finneran, K. T., M. E. Housewright, and D. R. Lovley.** 2002. Multiple influences of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediments. Environ Microbiol **4:**510-6.

19. **Foglar, L., F. Briski, L. Sipos, and M. Vukovic.** 2005. High nitrate removal from synthetic wastewater with the mixed bacterial culture. Bioresour Technol **96:**879-88.

20. **Gentile, M., T. Yan, S. M. Tiquia, M. W. Fields, J. Nyman, J. Zhou, and C. S. Criddle.** 2006. Stability in a denitrifying fluidized bed reactor. Microb Ecol **52:**311-21.

21. **Gentile, M. E., C. M. Jessup, J. L. Nyman, and C. S. Criddle.** 2007. Correlation of functional instability and community dynamics in denitrifying dispersed-growth reactors. Appl Environ Microbiol **73:**680-90.

22. **Glass, C., and J. Silverstein.** 1998. Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. Water Res **32:**831-839.

23. **Glass, C., J. Silverstein, and J. Oh.** 1997. Inhibition of denitrification in activated sludge by nitrite. Water Environment Research **69:**1086-1093.

24. **Gorby, Y. A., F. Caccavo Jr., and J. Bolton, H.** 1998. Microbial Reduction of Cobalt(III)EDTA- in the Presence and Absence of Manganese(IV) Oxide. Environ Sci Technol **32:**244-250.

25. **Goris, J., P. De Vos, T. Coenye, B. Hoste, D. Janssens, H. Brim, L. Diels, M. Mergeay, K. Kersters, and P. Vandamme.** 2001. Classification of metal-resistant bacteria from industrial biotopes as *Ralstonia campinensis* sp. nov., *Ralstonia metallidurans* sp. nov. and *Ralstonia basilensis* Steinle et al. 1998 emend. Int J Syst Evol Microbiol **51:**1773-82.

26. **Greene, E. A., C. Hubert, M. Nemati, G. E. Jenneman, and G. Voordouw.** 2003. Nitrite reductase activity of sulphate-reducing bacteria prevents their inhibition by nitrate-reducing, sulphide-oxidizing bacteria. Environ Microbiol **5:**607-17.

27. **Gu, B., H. Yan, P. Zhou, D. B. Watson, M. Park, and J. Istok.** 2005. Natural humics impact uranium bioreduction and oxidation. Environ Sci Technol **39:**5268-75.

28. **Holtan-Hartwig, L., P. Dorsch, and L. R. Bakken.** 2000. Comparison of denitrifying communities in organic soils: kinetics of NO−3 and N2O reduction. Soil Biology and Biochemistry **32:**833-843.

29. **Hwang, C., W. Wu, T. J. Gentry, J. Carley, G. A. Corbin, S. L. Carroll, D. B. Watson, P. M. Jardine, J. Zhou, C. S. Criddle, and M. W. Fields.** 2009. Bacterial community succession during in situ uranium bioremediation: spatial similarities along controlled flow paths. Isme J **3:**47-64.

30. **Hwang, C., W. M. Wu, T. J. Gentry, J. Carley, S. L. Carroll, C. Schadt, D. Watson, P. M. Jardine, J. Zhou, R. F. Hickey, C. S. Criddle, and M. W. Fields.** 2006. Changes in bacterial community structure correlate with initial

operating conditions of a field-scale denitrifying fluidized bed reactor. Appl Microbiol Biotechnol **71:**748-60.

31. **Istok, J. D., J. M. Senko, L. R. Krumholz, D. Watson, M. A. Bogle, A. Peacock, Y. J. Chang, and D. C. White.** 2004. In situ bioreduction of technetium and uranium in a nitrate-contaminated aquifer. Environ Sci Technol **38:**468-75.

32. **Kampfer, P., K. Denger, A. M. Cook, S. T. Lee, U. Jackel, E. B. Denner, and H. J. Busse.** 2006. *Castellaniella* gen. nov., to accommodate the phylogenetic lineage of *Alcaligenes defragrans*, and proposal of *Castellaniella defragrans* gen. nov., comb. nov. and *Castellaniella denitrificans* sp. nov. Int J Syst Evol Microbiol **56:**815-9.

33. **Kim, I. S., S. E. Oh, M. S. Bum, J. L. Lee, and S. T. Lee.** 2002. Monitoring the denitrification of wastewater containing high concentrations of nitrate with methanol in a sulfur-packed reactor. Appl Microbiol Biotechnol **59:**91-6.

34. **Korner, H., and W. G. Zumft.** 1989. Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuous culture of *Pseudomonas stutzeri*. Appl Environ Microbiol **55:**1670-6.

35. **Kristjansson, J. K., and T. C. Hollocher.** 1980. First practical assay for soluble nitrous oxide reductase of denitrifying bacteria and a partial kinetic characterization. J Biol Chem **255:**704-7.

36. **Li, X., and L. R. Krumholz.** 2008. Influence of Nitrate on Microbial Reduction of Pertechnetate. Environ Sci Technol **42:**1910-1915.

37. **Liu, Q. M., L. N. Ten, W. T. Im, and S. T. Lee.** 2008. *Castellaniella caeni* sp. nov., a denitrifying bacterium isolated from sludge of a leachate treatment plant. Int J Syst Evol Microbiol **58:**2141-6.

38. **Lovley, D. R., and E. J. Phillips.** 1986. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl Environ Microbiol **51:**683-689.

39. **Lovley, D. R., and E. J. Phillips.** 1987. Rapid assay for microbially reducible ferric iron in aquatic sediments. Appl Environ Microbiol **53:**1536-1540.

40. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res **32:**1363-71.

41. **Madden, A. S., A. C. Smith, D. L. Balkwill, L. A. Fagan, and T. J. Phelps.** 2007. Microbial uranium immobilization independent of nitrate reduction. Environ Microbiol **9:**2321-30.

42. **Marazioti, C., M. Kornaros, and G. Lyberatos.** 2003. Kinetic modeling of a mixed culture of *Pseudomonas denitrificans* and *Bacillus subtilis* under aerobic and anoxic operating conditions. Water Res **37:**1239-51.

43. **Michalsen, M. M., Aaron D. Peacock, Amanda N. Smithgal, David C. White, Anne M. Spain, Yamil Sanchez-Rosario, Lee R. Krumholz, Shelly D. Kelly, Kenneth M. Kemner, James McKinley, Steve M. Heald, Mary Anna Bogle, David B. Watson and Jonathan D. Istok.** 2009. Treatment of Nitric Acid-, U(VI)-, and Tc(VII)-Contaminated Groundwater in Intermediate-Scale Physical Models of an In Situ Biobarrier. Environ Sci Technol **Online Ahead of Print, February 6, 2009.**

44. **Moon, H. S., J. Komlos, and P. R. Jaffe.** 2007. Uranium reoxidation in previously bioreduced sediment by dissolved oxygen and nitrate. Environ Sci Technol **41:**4587-92.

45. **Murray, J. W.** 1974. Surface chemistry of hydrous manganese dioxide. J Colloid Interface Sci **46:**357-371.

46. **O'Reilly, C., and E. Colleran.** 2005. Toxicity of nitrite toward mesophilic and thermophilic sulphate-reducing, methanogenic and syntrophic populations in anaerobic sludge. J Ind Microbiol Biotechnol **32:**46-52.

47. **Oh, J., S. M. Yoon, and J. M. Park.** 2001. Denitrification in submerged biofilters of concentrated-nitrate wastewater. Water Sci Technol **43:**217-23.

48. **Rezania, B., N. Cicek, and J. A. Oleszkiewicz.** 2005. Kinetics of hydrogen-dependent denitrification under varying pH and temperature conditions. Biotechnol Bioeng **92:**900-6.

49. **Rich, J. J., R. S. Heichen, P. J. Bottomley, K. Cromack, Jr., and D. D. Myrold.** 2003. Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. Appl Environ Microbiol **69:**5974-82.

50. **Sanchez-Rosario, Y., A. M. Spain, and L. Krumholz.** In preparation. Microbial communities from multilevel sampler systems filled with synthetic matrix. University of Oklahoma, Norman, OK.

51. **Senko, J. M., T. A. Dewers, and L. R. Krumholz.** 2005. Effect of Oxidation Rate and Fe(II) State on Microbial Nitrate-Dependent Fe(III) Mineral Formation. Appl Environ Microbiol **71:**7172-7.

52. **Senko, J. M., J. D. Istok, J. M. Suflita, and L. R. Krumholz.** 2002. In-situ evidence for uranium immobilization and remobilization. Environ Sci Technol **36:**1491-6.

53. **Senko, J. M., Y. Mohamed, T. A. Dewers, and L. R. Krumholz.** 2005. Role for Fe(III) minerals in nitrate-dependent microbial U(IV) oxidation. Environ Sci Technol **39:**2529-36.

54. **Sepulveda-Torres, L. C., N. Rajendran, M. J. Dybas, and C. S. Criddle.** 1999. Generation and initial characterization of *Pseudomonas stutzeri* KC mutants with impaired ability to degrade carbon tetrachloride. Arch Microbiol **171:**424-9.

55. **Sikorski, J., M. Mohle, and W. Wackernagel.** 2002. Identification of complex composition, strong strain d iversity and directional selection in local Pseudomonas stutzeri populations from marine sediment and soils. Environ Microbiol **4:**465-76.

56. **Singleton, M. J., K. N. Woods, M. E. Conrad, D. J. Depaolo, and P. E. Dresel.** 2005. Tracking sources of unsaturated zone and groundwater nitrate contamination using nitrogen and oxygen stable isotopes at the Hanford site, Washington. Environ Sci Technol **39:**3563-70.

57. **Smibert, R. M., and N. R. Krieg.** 1994. Phenotypic Characterization, p. 607-654. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington, DC.

58. **Spain, A. M., A. D. Peacock, J. D. Istok, M. S. Elshahed, F. Z. Najar, B. A. Roe, D. C. White, and L. R. Krumholz.** 2007. Identification and isolation of a Castellaniella species important during biostimulation of an acidic nitrate- and uranium-contaminated aquifer. Appl Environ Microbiol **73:**4892-904.

59. **Stringfellow, W. T., and M. D. Aitken.** 1994. Comparative physiology of phenanthrene degradation by two dissimilar pseudomonads isolated from a creosote-contaminated soil. Can J Microbiol **40:**432-8.

60. **Sun, W., R. Sierra-Alvarez, N. Fernandez, J. L. Sanz, R. Amils, A. Legatzki, R. M. Maier, and J. A. Field.** 2009. Molecular characterization and in situ quantification of anoxic arsenite-oxidizing denitrifying enrichment cultures. FEMS Microbiol Ecol **68:**72-85.

61. **Takaki, K., S. Fushinobu, S. W. Kim, M. Miyahara, T. Wakagi, and H. Shoun.** 2008. Streptomyces griseus enhances denitrification by Ralstonia pickettii K50, which is possibly mediated by histidine produced during co-culture. Biosci Biotechnol Biochem **72:**163-70.

62. **Tanner, R. S.** 1997. Cultivation of Bacteria and Fungi, p. 52-60. *In* C. J. E. i. C. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach, and M. V. Walter (ed.), Manual of Environmental Microbiology. ASM Press, Washington, D.C.

63. **Van de Pas-Schoonen, K. T., S. Schalk-Otte, S. Haaijer, M. Schmid, H. Op den Camp, M. Strous, J. Gijs Kuenen, and M. S. M. Jetten.** 2005. Complete conversion of nitrate into dinitrogen gas in co-cultures of denitrifying bacteria 10th Nitrogen Cycling Meeting 2004. Bichemical Society.

64. **Vollack, K. U., E. Hartig, H. Korner, and W. G. Zumft.** 1999. Multiple transcription factors of the FNR family in denitrifying Pseudomonas stutzeri: characterization of four fnr-like genes, regulatory responses and cognate metabolic processes. Mol Microbiol **31:**1681-94.

65. **Vrionis, H. A., R. T. Anderson, I. Ortiz-Bernad, K. R. O'Neill, C. T. Resch, A. D. Peacock, R. Dayvault, D. C. White, P. E. Long, and D. R. Lovley.** 2005. Microbiological and geochemical heterogeneity in an in situ uranium bioremediation field site. Appl Environ Microbiol **71:**6308-18.

66. **Waring, S. A., and J. W. Gilliam.** 1983. The effect of acidity on nitrate reduction and denitrification in lower Coastal Plain soils. Soil Sci Soc Am J **47:**246-251.

67. **Watanabe, T., H. Motoyama, and M. Kuroda.** 2001. Denitrification and neutralization treatment by direct feeding of an acidic wastewater containing copper ion and high-strength nitrate to a bio-electrochemical reactor process. Water Res **35:**4102-10.

68. **Wilhelm, E., R. Battino, and R. J. q. Wilcock.** 1977. Low-pressure solubility of gases in liquid water. Chem Rev **77:**219-262.

69. **Wu, W. M., J. Carley, J. Luo, M. A. Ginder-Vogel, E. Cardenas, M. B. Leigh, C. Hwang, S. D. Kelly, C. Ruan, L. Wu, J. Van Nostrand, T. Gentry, K. Lowe, T. Mehlhorn, S. Carroll, W. Luo, M. W. Fields, B. Gu, D. Watson, K. M. Kemner, T. Marsh, J. Tiedje, J. Zhou, S. Fendorf, P. K. Kitanidis, P. M. Jardine, and C. S. Criddle.** 2007. In situ bioreduction of uranium (VI) to submicromolar levels and reoxidation by dissolved oxygen. Environ Sci Technol **41:**5716-23.

70. **Zumft, W. G., K. Dohler, H. Korner, S. Lochelt, A. Viebrock, and K. Frunzke.** 1988. Defects in cytochrome cd1-dependent nitrite respiration of transposon Tn5-induced mutants from *Pseudomonas stutzeri*. Arch Microbiol **149:**492-8.

71. **Zumft, W. G., and H. Korner.** 1997. Enzyme diversity and mosaic gene organization in denitrification. Antonie Van Leeuwenhoek **71:**43-58.

Table 1. Summary of cultivation approach for each OR-IFRC denitrifying isolate: geochemical data of isolation sources, push-pull experiment descriptions, and cultivation techniques.

Isolate ID	Isolation source and historical geochemical data[1]			Push-pull experiments and sampling description				Cultivation approach
	GW Well ID	pH	$[NO_3^-]$ (mM)	Push pull test dates	Injection solution	Ref	Samples used for cultivation	
GN32#1 GN32#2	FW032	5.22	23.3	Summer (2002)	Glucose-amended high $[NO_3^-]$ GW	(31)	Glucose-stimulated GW	Direct plating from GW onto plates containing glucose and NO_3^-
GN33#1 GN33#3	FW033	5.85	14.3	Summer (2002)	Glucose-amended high $[NO_3^-]$ GW	(31)	Glucose-stimulated GW	Direct plating from GW onto plates containing glucose and NO_3^-
4.5A2 7.5A2	FW028	4.40	167	Fall (2003)	Ethanol-amended high $[NO_3^-]$ GW	(58)	Ethanol-stimulated sediment	Plating from high $[NO_3^-]$ ethanol-amended denitrifying enrichment cultures at pHs 4.5 and 7.5

[1]All historical data is publicly available from the OR-IFRC website: http://www.esd.ornl.gov/orifrc/.

Table 2. pH and temperature ranges of OR-IFRC isolates grown aerobically or on NO_3^- with electron donors glucose, ethanol, or acetate.

Experimental test conditions			Growth range (optimum[5]) for OR-IFRC isolates		
Exp. #	E^- Acceptor/E^- Donor Pair	Range tested	*Castellaniella* str. 4.5A2	*Rhizobium* str. GN32#2	*Pseudomonas* str. GN33#1
pH experiments					
1	NO_3^- (10 mM)/Glucose (5 mM) [1, 2]	3.5-8.0	---	4.5-8.0 (6.5)	6.0-8.0 (8.0)
2	NO_3^- (20 mM)/Ethanol (50 mM) [3, 4]	4.5-8.0	4.5-8.0 (6.5)	---	---
3	NO_3^- (10 mM)/ Ethanol (25 mM)	4.0-8.5	4.0-8.5 (7.0)	5.0-8.5 (6.5)	6.0-8.5 (8.0)
4	NO_3^- (100 mM)/ Ethanol (100 mM)	4.0-8.5	4.5-8.5 (7.5)	5.5-8.0 (6.0)	6.0-8.5 (8.0)
5	O_2/Glucose (5 mM) [2]	4.5-8.0	---	5.0-8.0 (7.0)	5.5-8.0 (8.0)
6	O_2/Acetate (16.9 mM) [4]	4.5-8.0	5.5-8.0 (7.5)	---	---
Temp experiments					
7	O_2/Glucose (5 mM) [2]	4-42°C	---	16-42 (42)	4-42 (30)
8	O_2/Acetate (16.9 mM) [4]	4-42°C	16-37 (30)	---	---
9	NO_3^- (10 mM)/Glucose (5 mM) [2]	4-42°C	---	4-42 (37)	4-42 (30)
10	NO_3^- (10 mM)/Acetate (16.9 mM) [4]	4-42°C	4-37 (30)	---	---

[1]Test conditions represent electron acceptor and donors (and respective concentrations) that the following strains were isolated on and maintained: GN32#1, GN32#2, GN33#1, and GN33#3.

[2]Data for all *Rhizobium* isolates (GN32#1, GN32#2, and GN33#3) is represented by isolate GN32#2.

[3]Test conditions represent electron acceptor and donors (and respective concentrations) that the strains 4.5A2 and 7.5A2 were isolated on and maintained.

[4]Data for both *Castellaniella* isolates (4.5A2 and 7.5A2) is represented by isolate 4.5A2.

[5]Optimal growth was considered to be the pH or temp with the fastest generation time; in many instances, this differed from optimal growth as determined by maximum $O.D._{600nm}$, as many slow-growing cultures formed more biomass than fast-growing cultures of the same isolate.

Table 3. pH-dependent NO_2^- accumulation of OR-IFRC isolates during growth on glucose or ethanol and different concentrations of NO_3^-. Even though growth was tested at 0.5 pH unit intervals for all experiments, samples for anion analysis were pulled from growth curve tubes at 1.0 pH unit intervals (i.e. pHs 4.5, 5.5, 6.5, and 7.5 for the first three growth curve experiments and pHs 4, 5, 6, 7, and 8 for the remaining growth curve experiments). Blacked-out rows represent samples for which samples were not pulled for anion analysis.

pH	Max NO_2^- accumulation (mM) of each isolate at different pHs in liquid media during growth on NO_3^-								
	NO_3^- (20 mM) Ethanol (50 mM)[1]	NO_3^- (10 mM) Glucose (5 mM)[2]		NO_3^- (10 mM) Ethanol (25 mM)			NO_3^- (100 mM) Ethanol (100 mM)		
	4.5A2	GN32#2	GN33#1	4.5A2	GN32#2	GN33#1	4.5A2	GN32#2	GN33#1
4.0				0.85	NG[3]	NG[3]	NG[3]	NG[3]	NG[3]
4.5	1.1	0	NG[3]						
5.0				1.1	ND[4]	NG[3]	9.1	NG[3]	NG[3]
5.5	1.3	0	NG[3]						
6.0				0.18	1.4	10.9	9.7	15.7	19.5
6.5	0.85	1.7	2.9						
7.0				0	0	5.8	17.7	14.3	71.2
7.5	5.8	1.3	3.0						
8.0				2.9	0.06	5.5	5.6	ND[4]	80.3

[1]Original growth curves for isolate 4.5A2 were done on electron acceptor and donors (and respective concentrations) that this strain was isolated on and maintained.

[2]Original growth curves for isolates GN32#2 and GN33#1 were done on electron acceptor and donors (and respective concentrations) that these strains were isolated on and maintained

[3]NG=No growth; samples not taken for anion analysis.

[4]ND=Not determined. Growth occurred, but samples were not taken for anion analysis due to long lag phases and slow growth (i.e. in instances when growth did not occur until experiments were completed for other isolates at the same pH).

Table 4. Generation times for OR-IFRC isolates on different concentrations of NO_3^- and NO_2^-, and on 25 mM N_2O at pH 7 (all isolates) and pH 5 (*Castellelaniella* str. 4.5A2). Each experiment used ethanol as the electron donor.

Isolate	Generation time (hr)					
	NO_3^-		NO_2^-			N_2O
	10 mM	100 mM	5 mM	10 mM	50 mM	25 mM
pH 7						
Castellaniella str. 4.5A2	8.6±1.3	11±0.25	**4.0**±1.3	6.2±0.7	7.1±0.5	6.2±0.56
Rhizobium str. GN32#2	5.8±0.27	6.2±0.67	6.7±0.79	9.6±2.6	95±48	**3.4**±0.12
Pseudomonas str. GN33#1	**2.6**±0.15	7.1±0.5	4.7±1.2	6.3±0.9	19±0.72	4.3±0.34
pH 5						
Castellaniella str. 4.5A2	18±6.0	32±5.3	49±23	--	--	24±3.7

Table 5. Summary of GW experiment, comparing growth and nitrate reduction in contaminated FW021 groundwater between a pure culture of Castellaniella str. 4.5A2 and mixed culture of three OR-IFRC isolates (4.5A2, GN32#2, and GN33#1) at pHs 5 and 7.

	pH 5		pH 7	
	4.5A2	Mixed assemblage	4.5A2	Mixed assemblage
Zero-order NO_3^- reduction rate (mM/day)	0.76	1.3	2.2	2.6
Max $[NO_2^-]$ (mM)	5.4	4.8	12	18
Final $[NO_2^-]$ (mM)	1.8	0.18	7.7	1.1
Final pH	6.6	6.8	7.2	7.4
Max cell no. (CFUs/ml)	$4x10^8$	$4x10^8$	$5x10^8$	$5x10^8$
Final cell no. (CFUs/ml)	$3x10^6$	$3x10^7$	$4x10^7$	$4x10^7$

Table 6. pH-dependent minimum inhibitory concentrations (MICs) of NO_2^- for OR-IFRC isolates and *Cupravidus metallidurans* str. CH34 (a heavy metal-tolerant organism) grown under aerobic and anaerobic conditions.

Organism	MIC[1] (mM NO_2^-)					
	Aerobic			Anaerobic (10 mM NO_3^-)[2]		
	pH 5	pH 6	pH 7	pH 5	pH 6	pH 7
Castellaniella str. 4.5A2	1	50	250	5	50	250
Rhizobium str. GN32#2	--	5	50	--	5	50
[2]*Pseudomonas* str. GN33#1	--	5	50	--	1	50
[2]*C. metallidurans* str. CH34	--	5	50	--	1	5

[1]MICs for these experiments was determined as the lowest concentration of NO_2^- at which no growth occurred over a two-week incubation. Concentrations of NO_2^- tested included: 0, 1, 5, 10, 50, 100, and 250 mM.
[2]These organisms accumulate >5mM NO_2^- under denitrifying conditions, thus, [NO_2^-] would likely increase from initial [NO_2^-], contributing further to NO_2^- toxicity. Under aerobic conditions, this would not be a factor.

Table 7. Michaelis Menten kinetic parameters, Vmax and Km, of *Pseudomonas* str. GN33#1, *Rhizobium* str. GN32#2, and *Castellaniella* str. 4.5A2 on NO_3^-, NO_2^-, and N_2O.

Organism	V_{max}[a, b, c]			K_m (μM)[a, c]		
	NO_3^-	NO_2^-	N_2O	NO_3^-	NO_2^-	N_2O
Castellaniella str. 4.5A2	1.51±0.28	6.93±0.99	12.8±2.2	139±70	43±22	22±14
Rhizobium str. GN32#2	0.288±0.061	10.6±1.9	---[d]	88±70	171±92	---[d]
Pseudomonas str. GN33#1	15.8±3.3	0.264±0.018	10.8±1.2	590±270	15.8±4.9	23.6±7.3

[a]V_{max} and K_m were calculated with non-linear regression modelling of the Michaelis Menten equation using SigmaPlot.
[b]Vmax units = μmol e^- transferred (from benzyl viologen) •min^{-1}•mg protein $^{-1}$ to each electron acceptor.
[c]Uncertainties are 95 % confidence intervals.
[d]Data not available; assays revealed very low activity. However, measurable activity was not relative to protein or substrate concentrations; thus Michaelis Menten kinetics were not observed. BV may not be able to act as an electron donor for this organism's N_2O reductase, a co-factor may be required, or the enzyme complex may not have been extracted by B-PER.

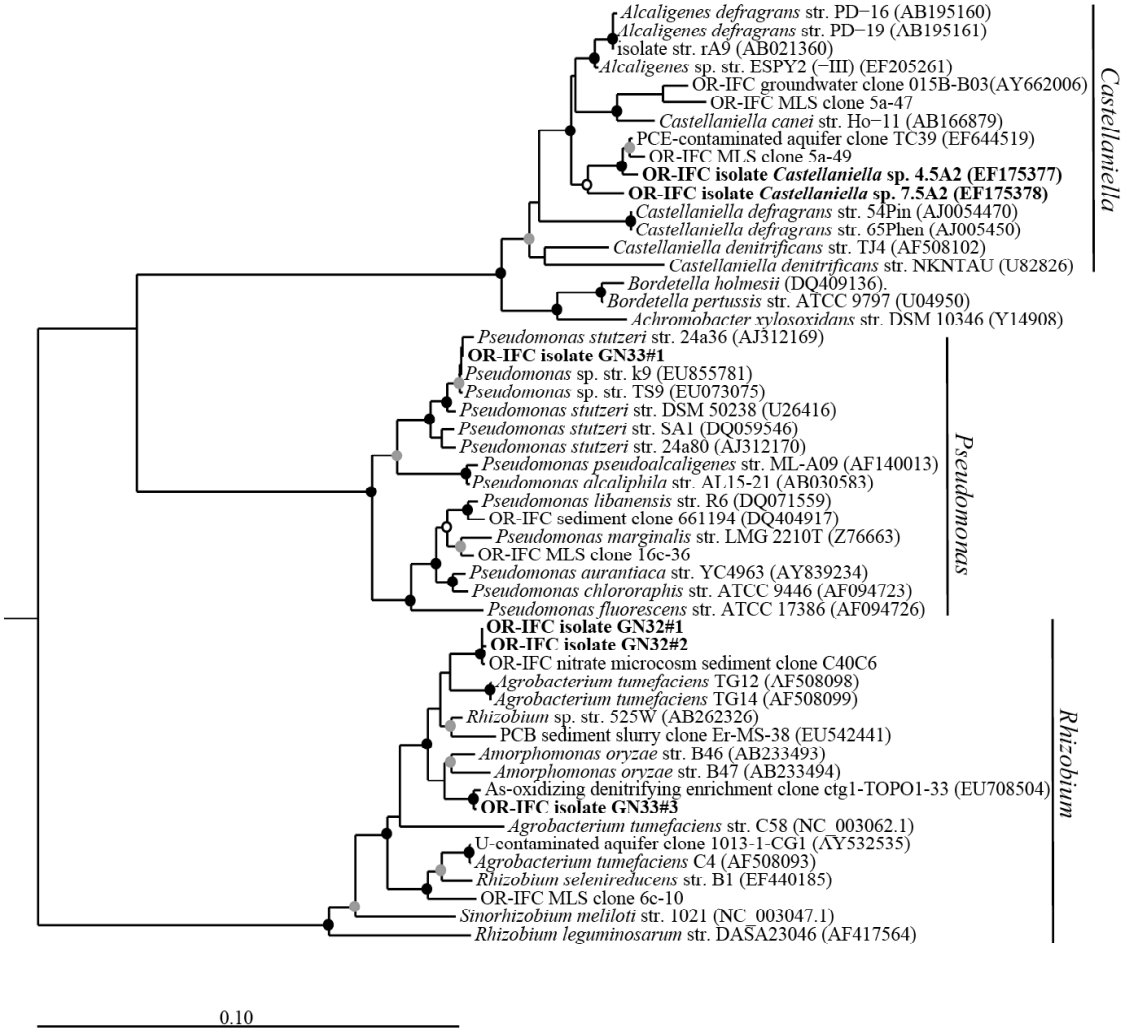

Figure 1. Distance phylogram 16S rRNA gene sequences from OR-IFRC *Proteobacteria* denitrifying isolates (shown in bold), belonging to the genera *Castellaniella* (*Betaproteobacteria*), *Pseudomonas* (*Gammaproteobacteria*), and *Rhizobium* (*Alphaproteobacteria*) and related sequences. The tree was rooted with the 16S rRNA gene sequence from *Geobacter metallireducens* (GenBack accession no. NC_007517.1). Bootstrap values (based on 1000 replicates) are shown at each node for branches with >90% (●), 70-89% ( ), and 50-69% (○) bootstrap support. Genbank accession numbers for each reference sequence are shown in parentheses, and clone sequences from the same site are denoted as "OR-IFRC" clones; similarly, clone sequences identified in a community study from OR-IFRC Area 1 multi-level samplers during biostimulation with ethanol are denoted as "OR-IFRC MLS clones" (sequences not published).

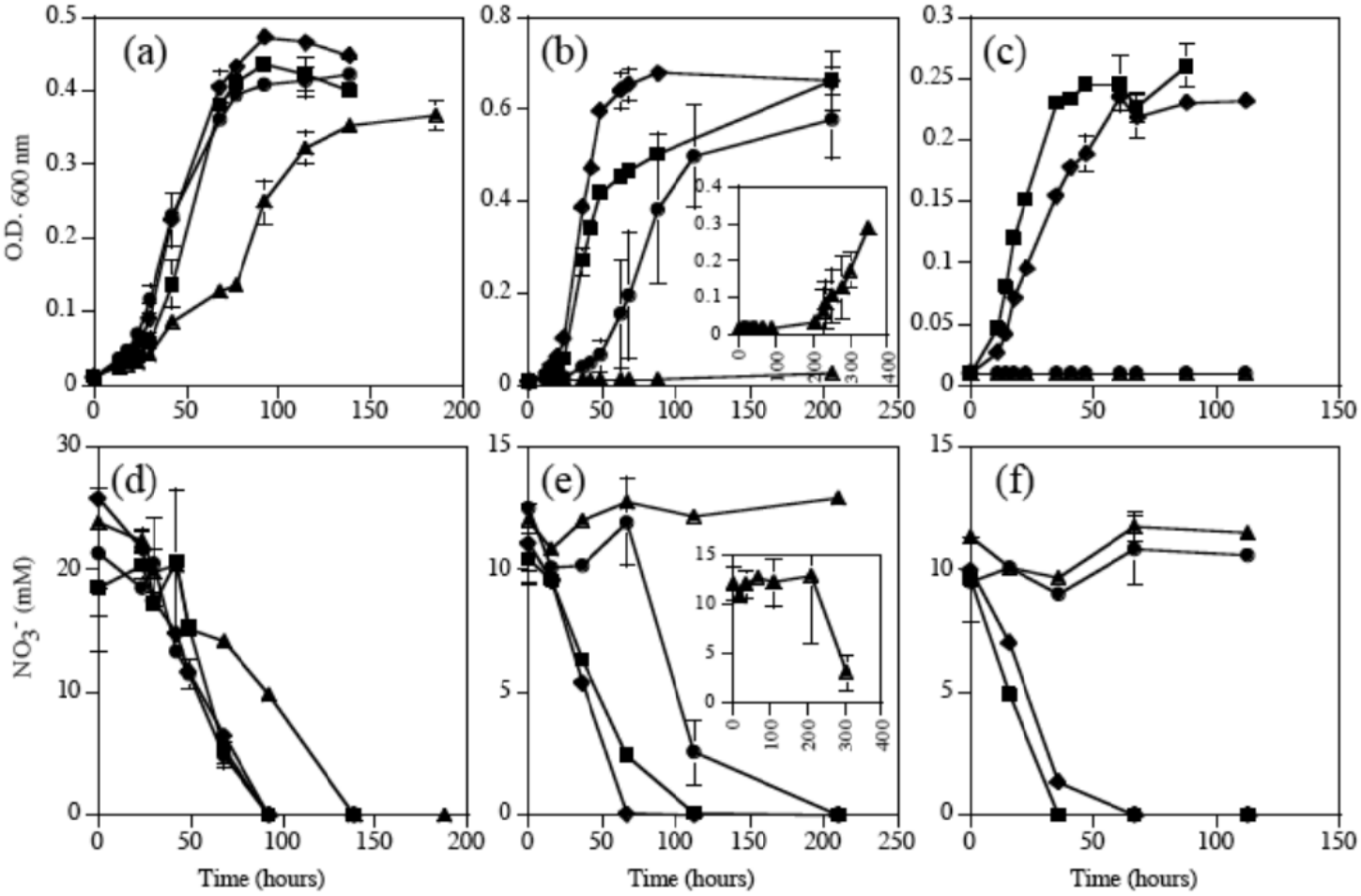

Figure 2. Examples of growth curves [(a), (b), and (c)] and NO_3^- reduction [(d), (e), and (f)] of isolates *Castellaniella* str. 4.5A2 [(a) and (d)], *Rhizobium* str. GN32#2 [(b) and (e)], , and *Pseudomonas* str. GN33#1 [(c) and (f)] at pHs 4.5 (▲), 5.5 (●), 6.5 (◆), and 7.5 (■)

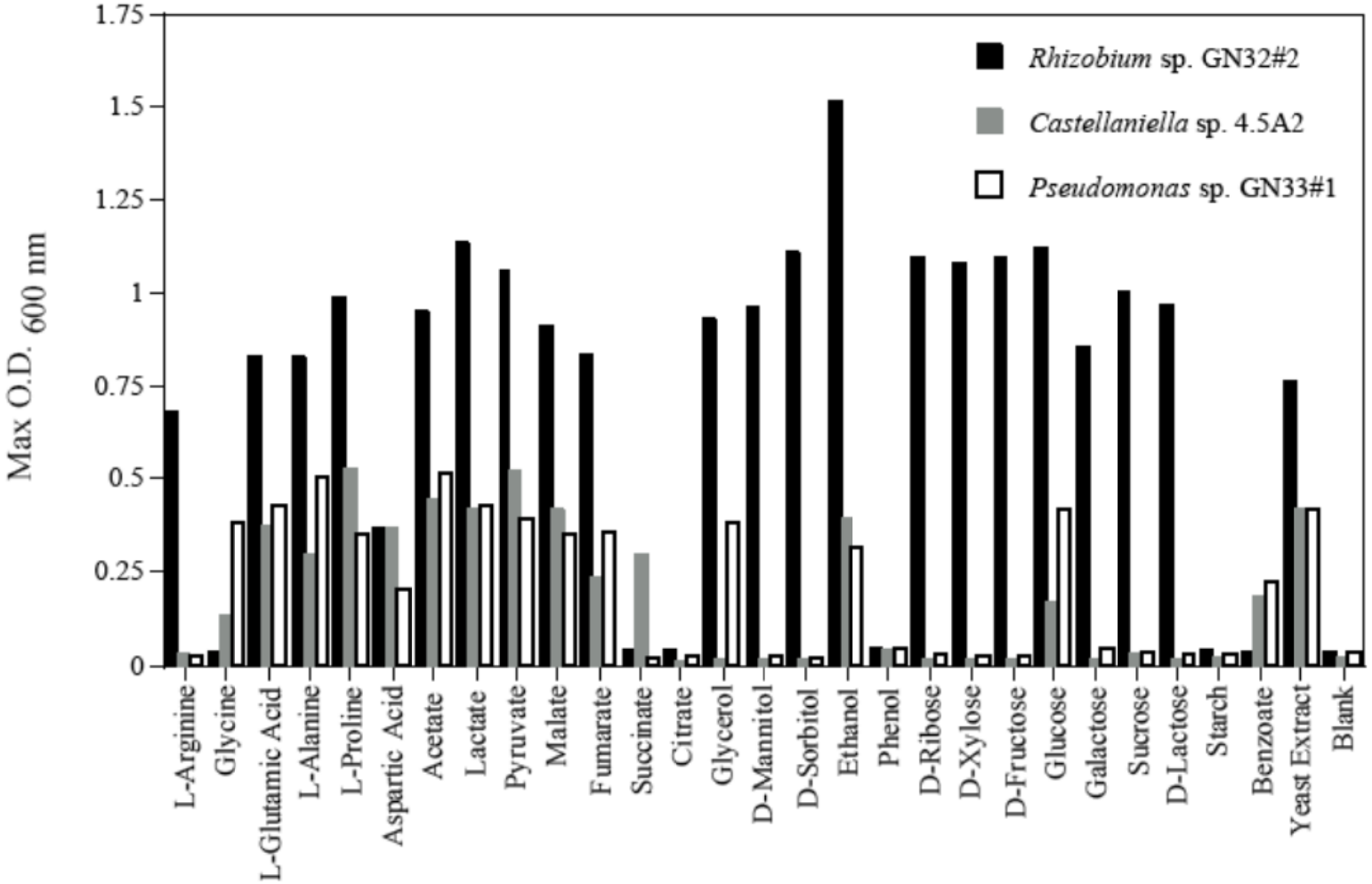

Figure 3. Growth of isolates 4.5 A2, GN32#2, and GN33#1 on different organic substrates, measured by optical density, OD, at 600 nm.

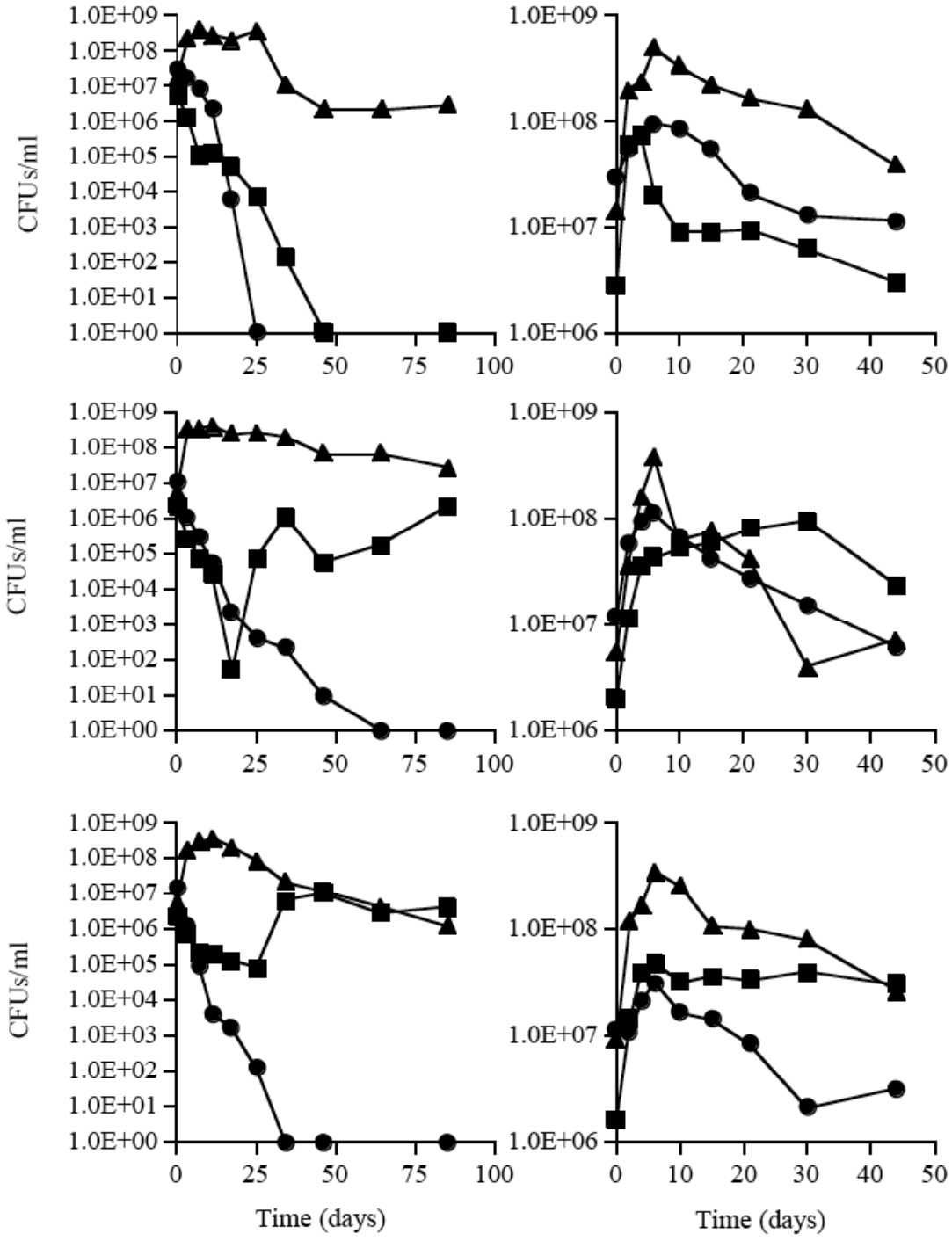

Figure 4. Growth of OR-IFC isolates, *Castellaniealla* str. 4.5A2 (▲), *Rhizobium* str. GN32#2 (■), and *Pseudomonas* str. GN33#1 (●) at pH 5 (left panel) and pH 7 (right panel) in ethanol-amended OR-IFC FW021 contaminated groundwater in pure culture (top panel) and mixed cultures (middle panel), and in un-amended OR-IFC groundwater in mixed culture (bottom panel).

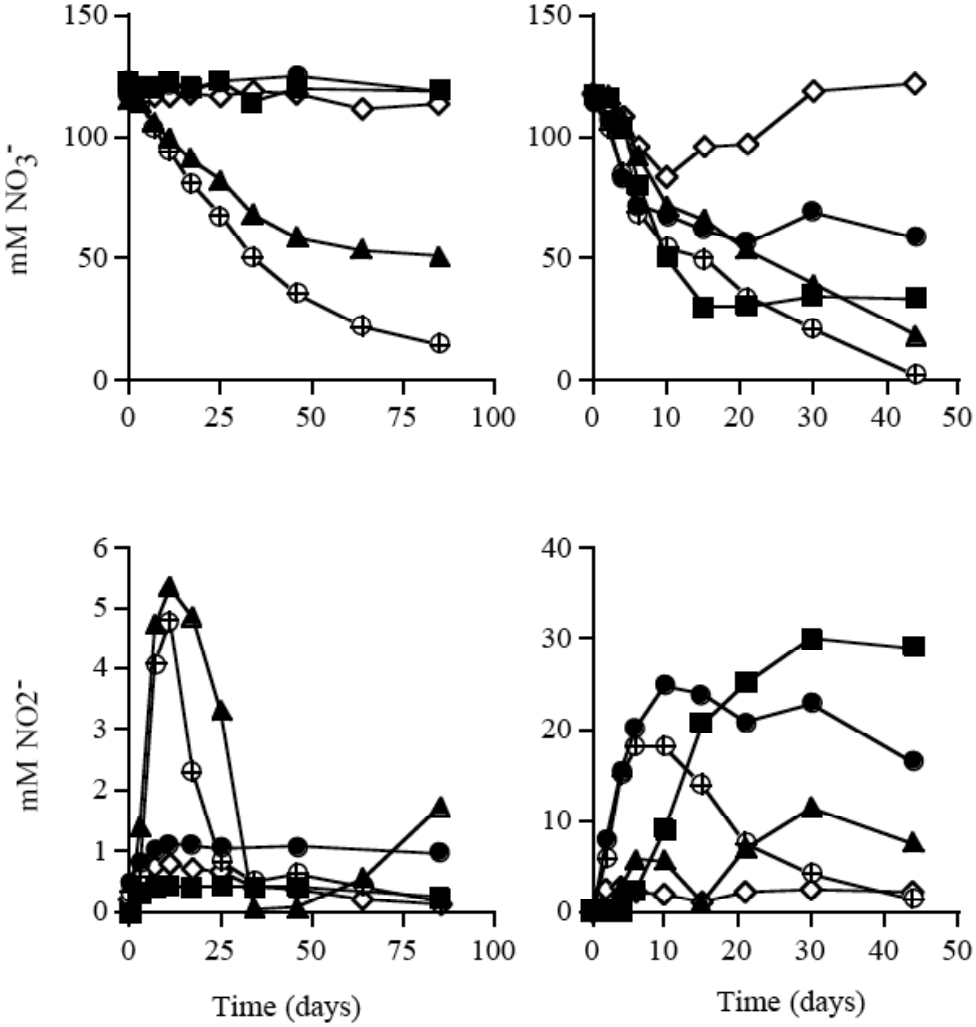

Figure 5. Nitrate reduction by OR-IFC isolates, *Castellaniealla* str. 4.5A2 (▲), *Rhizobium* str. GN32#2 (■), *Pseudomonas* str. GN33#1 (●), and mixed assemblages of the three isolates (⊕) at pH 5 (left panel) and pH 7 (right panel) in ethanol-amended OR-IFC FW021 contaminated groundwater. Nitrite accumulation for each group is shown in the bottom panel. Controls of mixed assemblages in un-amended FW021 groundwater are shown in each graph (◇).

APPENDIX I

Abundance, Composition, Diversity, and Novelty of Soil *Proteobacteria*

Abstract

Small subunit (16S) rRNA gene surveys generating large (e.g. >1000), near full-length 16S rRNA clones offer a unique opportunity for in-depth phylogenetic analysis to highlight the breadth of diversity within various major bacterial phyla encountered in soil. This study offers a detailed phylogenetic analysis of the *Proteobacteria*-affiliated clones identified from 13,001 nearly full-length 16S rRNA gene clones derived from Oklahoma tall grass prairie soil. *Proteobacteria* was the most abundant phylum in the community, and comprised 25% of total clones. The most abundant and diverse class within the *Proteobacteria* was *Alphaproteobacteria*, followed by the *Delta-*, *Beta-*, and *Gammaproteobacteria*. Members of the *Epsilon-* and *Zetaproteobacteria* were not detected in the dataset. Our analysis identified 15 novel order-level and 48 novel family-level *Proteobacteria* lineages. Additionally, we show that the majority of *Proteobacteria* clones in the dataset belong to orders and families containing no described cultivated representatives (50 and 65%, respectively). An examination of the ecological distribution of the six most abundant *Proteobacteria* lineages in this dataset with no characterized pure culture representatives provided important information regarding their global distribution and environmental preferences. This level of novel phylogenetic diversity indicates that our

understanding of the functions of soil microorganisms, even those belonging to phyla with numerous and diverse well-characterized cultured representatives such as the *Proteobacteria*, remains far from adequate.

Introduction

Small subunit (16S rRNA) gene-based surveys have clearly demonstrated that the scope of phylogentic diversity in soil is much broader than that implied using culture-based approaches (5, 16, 18, 24). Although having a remarkably stable phylum level diversity, soil is an extremely diverse ecosystem at the order, family, genus, and species levels (8), with multiple yet-uncultured lineages within virtually each of the major bacterial phyla in soil (e.g. *Proteobacteria*, *Acidobacteria*, *Actinobacteria*) (11). Detailed phylogenetic analysis and taxonomic placements of 16S rRNA gene sequences has traditionally been the main focus of soil diversity studies. However, with the availability of newer sequencing technology and curated databases and the subsequent creation of large (>1000) datasets, the focus of the data analysis process has recently shifted more towards computing more accurate estimates of species richness and evenness (20, 21, 23, 30), identification of novel bacteria phyla (6)**,** accessing members of the rare soil biosphere (6), and computational comparisons of communities between different soils (8, 21). Detailed phylogenetic analysis of these datasets has often been overlooked, either due to the short amplicon size created, or to the sheer number of clone sequences analyzed. This is unfortunate, since such datasets, especially those with near full-length 16S rRNA gene sequence, offer a unique opportunity for an in-depth evaluation of the phylogenetic diversities within each of the major bacterial phyla in soil.

In a recent study, a near full-length 16S rRNA gene clone library was constructed from Oklahoma tall grass prairie soil and 13,001 clones were sequenced (6). The most abundant phylum was shown to be the *Proteobacteria* as is typically

observed in soil libraries [for a review, see (11)]. The *Proteobacteria* encompass an enormous level of morphological, physiological and metabolic diversity, and are of great importance to global carbon, nitrogen, and sulfur cycling (13). Despite this phylum containing more validly described isolates than any other phylum (13), the vast majority of soil *Proteobacteria* are yet to be cultivated. In this study, we describe the composition of *Proteobacteria* clones from OK tall grass prairie soil, in which the majority of clones belong to family- and order- level lineages containing no characterized cultivated isolates, and compare the ecological distribution of some of the dominant uncharacterized orders whose functions in soil remain unknown.

Materials and Methods

Phylogenetic analysis of Kessler Farm soil (KFS) *Proteobacteria* 16S rRNA gene sequences.

The dataset used in this study initially consisted of 13,001 16S rRNA clone sequences from soil, described in a previous study (6). Briefly, a clone library (n = 13,001 clones) was constructed from 16S rRNA genes (PCR-amplified using primers 27F and 1391R) from community DNA extracted Kessler Farm Soil (KFS), which was collected from an undisturbed tall grass prairie preserve in Central Oklahoma. Sequences were binned into OTUs using a 97% similarity cutoff using DOTUR (22). Soil characteristics, and details of sampling, DNA extraction, PCR amplification, 16S rRNA clone library construction and sequencing, and initial phylogenetic classification of 16S rRNA sequences can be found in the original manuscript (6).

Sequences representative of each OTU identified as *Proteobacteria* in the original manuscript were aligned using Greengenes' NAST alignment tool (3, 4). Aligned KFS and closely related 16S rRNA sequences were imported into Greengenes May 2007 ARB database (3) using ARB software package (17). We used the on-line program Pintail (1) to screen individual sequences within the *Proteobacteria* dataset using suspicious sequences (those identified by Bellerophon (10) or those with unclear phylogenetic affiliation or that formed unusually long branches in neighbor-joining dendrograms) as the query sequence, and the closest cultured relative or a reliable closely-related abundant KFS OTU sequence (n>50) as the reference sequence. After removal of chimera, 2,675 *Proteobacteria* clones belonging to 479 OTUs were classified to the family taxonomic level using phylogenetic tree-building methods.

Initial placement of OTUs in already-named families according to the Hugenholtz taxonomic framework (3) was determined by parsimony placement of KFS clone sequences into the ARB universal dendrogram. Distance trees of each class within *Proteobacteria* were constructed using the neighbor-joining algorithm and Jukes-Cantor corrections using ARB software package (17) with filters available for each class of *Proteobacteria*. Branching of distance trees was also verified by constructing trees via the same methods using PAUP 4.0b10 software (Sinauer Associates, Sunderland, MA) and generating bootstrap values based on 1000 replicates. Final classifications of KFS OTUs into families, according to the Hugenholtz taxonomic outline (3), were determined by placement of each OTU into a bootstrap-supported (>50) already-named or novel family in constructed trees. In general, novel families were defined as a bootstrap-supported group of clone sequences (n>2) sharing approx. > 92-93% sequence similarity with each other but < 92-93% sequence similarity to sequences from an already-named family. Novel orders were defined similarly, using 90% as a general cutoff, though these values varied between each class of *Proteobacteria* (i.e. *Deltaproteobacteria* is more divergent than *Alpha* and *Betaproteobacteria*).

Ecological distribution of abundant KFS uncharacterized lineages.

We chose the six most abundant uncharacterized *Proteobacteria* order-level lineages (Deltaproteobacteria-KFS-6, EB1021, Ellin314, MND1, A21b, and Ellin339), and recorded the isolation source of all available environmental clone sequences belonging to each order. To determine what environmental clone sequences belonged in an order, we created distance trees in ARB using all sequences belonging to the order

based on the universal parsimony tree, using the May 2007 Greengenes database. Second, we used the BLAST algorithm on the NCBI website (in November, 2008) to search for more recently deposited sequences belonging to each order, using the "type sequence" (the environmental clone sequence after which the order was named, e.g. MND1) as the query and 90% similarity as a general cutoff.

Results and Discussion

Abundance and composition of *Proteobacteria* in KFS and other soils.

The *Proteobacteria*-affiliated clones in KFS represented 25% of the total 16S rRNA clone sequences (6) compared to an average of 40% abundance in all published soil studies analyzing >1000 16S rRNA sequences, including eight individual soil samples in addition to a composite collection of soil libraries compiled by Janssen (11) (Table 1). In these studies, *Proteobacteria* comprised 25-40% abundance (relative to total sequences) in clone libraries comprised of near full-length (or >300 bp) 16SrRNA sequences, and 42-50% abundance from shorter (~100 bp) fragments generated by pyrosequencing (Table 1). While such larger proportion of *Proteobacteria* in pyrosequencing-based studies might be a true reflection of the communities analyzed, it might also indicate the existence of a cloning bias or that classification based on small 16S rRNA gene fragments could lead to different taxonomic assignments than classification based on near to full-length sequences, as previously suggested (6). Nevertheless, *Proteobacteria* remains the most abundant soil phylum, regardless of the utilized approach, which aside from PCR-based clone libraries and pyrosequencing has included metagenomics (15, 26), fluorescent *in situ* hybridization (FISH) (31), microarray analysis (28).

The most abundant class (39% of total *Proteobacteria* clones) in KFS was *Alphaproteobacteria*, followed by *Delta*- (37%), *Beta*- (16%) and *Gammaproteobacteria* (7.6%). Among all large datasets (>1000 sequences) of PCR-amplified 16S rRNA genes from soil (Table 1), *Alphaproteobacteria* is typically the most abundant class, relative to total clone sequences, comprising 36 ± 15% of

Proteobacteria clones while *Gammaproteobacteria* is typically the least abundant (12 ± 6%). *Deltaproteobacteria* was overrepresented in KFS compared to other large soil datasets, whereas *Betaproteobacteria* was underrepresented (Table 1). *Epsilonproteobacteria*, which has not been detected in many of the large 16S rRNA soil libraries (Table 1) was not detected in KFS, suggesting that this class is either extremely rare in soil or is not ubiquitous as are the other classes within *Proteobacteria*. Likewise, the recently discovered class *Zetaproteobacteria*, which appears to have a limited ecological distribution and metabolic abilities (7), was undetected in KFS and other large soil clone libraries (Table 1).

Family and order-level diversities within KFS *Proteobacteria*.

The use of classifier programs, available from Greengenes and the Ribosomal Database Project (RDP) (2, 3), provide useful tools for initial classification of 16S rRNA gene sequences; however, inaccurate taxonomic assignments may be made without tree-building phylogenetic analyses, especially at the subphylum levels. In addition, uncertain placements of clones with low sequence similarity to their closest relative has been observed with both classification programs, resulting in outputs with multiple placement suggestions (Greengenes), or low confidence in order and family level affiliation outputs (RDP). Also, satisfactory identification and documentation of novel lineages requires detailed phylogenetic analysis and tree-building approaches. In this study, phylogenic associations at the class, order, and family levels were initially determined using both Greengenes and RDP classification programs, and were verified by parsimony analysis using the ARB software package and neighbor-joining analysis using PAUP 4.01b10. Using this combined approach, 120 family-

level lineages were identified belonging to 60 orders (Table 2). *Alphaproteobacteria* had the highest number of families and orders, consisting of 45 families within 29 orders, and was followed by *Deltaproteobacteria* (33 families within 15 orders) (Table 2, Figures 1 and 2). *Beta-* and *Gammaproteobacteria* were less diverse, containing 23 and 19 families within five and 11 orders, respectively (Table 2, Figures 3 and 4). This pattern of order and family level diversity rankings between various *Proteobacteria* classes is in agreement with the diversity ranking estimated from the same datasets based on rarefaction curve analysis and diversity ordering approaches of KFS $OTU_{0.03}$ (29).

Prevalence of uncharacterized and novel lineages within KFS *Proteobacteria*. The vast majority of KFS *Proteobacteria* clones belonged to uncharacterized lineages (families or orders containing no validly described species); in total, 50 and 65% of KFS *Proteobacteria* clones belonged to uncharacterized orders and families, respectively (Table 2). It is important to note, however, that among the *Alpha-*, *Beta-*, and *Gammaprotebacteria*, some microorganisms have been cultivated among these uncharacterized lineages, but have not been characterized nor validly described (Figures 1-3). Indeed, within all *Proteobacteria* classes in KFS with the exception of *Alphaproteobacteria*, the most abundant orders contained no cultivated or characterized pure cultures. The most abundant order in *Alphaproteobacteria* was *Bradyrhizobiales* (Figure 1), which consisted of 463 clones (39 OTUs) and contained the most abundant OTU in the KFS dataset (n=204). The most abundant orders in *Deltaproteobacteria* were EB1021 (310 clones, 20 OTUs) and novel order *Deltaproteobacteria*-KFS-6 (210 clones, 9 OTUs) (Figure 2), neither of which contain

any cultivated microorganisms. The dominant orders in *Beta*- and *Gammaproteobacteria* in KFS were MND1 and Ellin339, respectively (Figures 3and 4), which are also uncharacterized lineages. *Deltaproteobacteria* contained the highest number of clones belonging to undescribed lineages, with 637 clones (64%) belonging to uncharacterized orders and 848 clones (85%) belonging to uncharacterized families. These *Deltaproteobacteria* lineages were comprised solely of environmentental clone sequences, none containing any cultivated representatives, suggesting that soil *Deltaproteobacteria* may be extremely difficult to cultivate in pure culture in the laboratory using standard heterotrophic growth media.

In addition, KFS contained numerous novel lineages within the *Proteobacteria* dataset (Table 2). In total, 15 novel orders and 48 novel families among the four classes were named in this study (Figures 1-4; for detailed descriptions of *Proteobacteria* KFS OTU phylogenetic affiliations, including all novel lineages, see Supplementary Table 1). The large number of novel family and orders identified in a single clone library clearly suggests that global soil *Proteobacteria* diversity is far broader than our current database collection of sequences and that the potential of identifying novel lineages within the soil rare biosphere using large clone libraries is just starting to be realized. Likewise, despite *Proteobacteria* being the most abundant soil phylum, containing more validly described species than any other phylum, the functions of the majority of *Proteobacteria* in soil remain yet to be revealed.

Ecological distribution of abundant uncharacterized order-level lineages. Because the majority of KFS *Proteobacteria* clones belong to families and orders with no characterized representatives, the functions of these groups of microorganisms in

soils cannot be delineated by simply determining closest relatives of sequences. To gain insight into the rarity of and ecological distribution of uncharacterized lineages within *Proteobacteria*, we chose the six most abundant KFS uncharacterized orders, *Deltaproteobacteria*-KFS-6 (*Deltaproteobacteria*, n=210), EB1021 (*Deltaproteobacteria*, n=310), Ellin314 (*Alphaproteobacteria*, n=103), MND1 (*Betaproteobacteria*, n=198), A21b (*Betaproteobacteria*, n=99), and Ellin339 (*Gammaproteobacteria*, n=99) and mapped their distribution among different environmental categories using data available from 16S rRNA sequences deposited into GenBank. We found that these six lineages, collectively, have been identified in 174 different sampling sites that fall into 30 general environmental categories, the most abundant of which was soil, while many samples also came from aquatic and subsurface ecosystems (Table 3; for details and references for each study, see Supplementary Table 2). Originally, we had hypothesized that uncharacterized orders which were more abundant in KFS would be more widespread in the environment; however there was no linear correlation between abundance and the no. sampling sites among which uncharacterized orders were detected ($R = -0.088$, $p = 0.43$).

The two *Deltaproteobacteria* orders were the most abundant of the uncharacterized orders; however, novel order *Deltaproteobacteria*-KFS-6 was detected in only four sites, all from soil. EB1021 contained the most clones out of any of the KFS uncharacterized orders, and was detected in 52 total samples from 15 different ecosystem types. This order was detected in 25 out of the 61 different soil sample sites but was detected in 90% of the deep sea sediment sites (Table 3, Supplementary Table 2) and both of the marine sponge studies. Interestingly, among

aquatic environments, EB1021 was detected in all sediment ecosystems (freshwater, estuarine, and marine) but was not detected in any of the overlying water ecosystems, suggesting EB1021 could be preferentially distributed in anoxic ecosystems. Thus, members of EB1021 might be living in anoxic or hypoxic microenvironments within soil aggregates, and the use of anaerobic techniques could prove useful in trying to cultivate members of EB1021.

From the *Alphaproteobacteria*, uncharacterized order Ellin314 was detected in more ecosystem types than any of the other KFS uncharacterized orders (Table 3, Supplementary Table 2). Most notably, members of this order have been detected in 75% of samples detected from anaerobic enrichments or consortia degrading organic pollutants. Like EB101, Ellin314 was detected in 25 of the 61 soil sites, and was more frequently detected in aquatic sediments rather than overlying water, including 60% of the deep sea sediment sites. Unlike EB1021, however, organisms belonging to Ellin314 have been cultivated but not characterized (12).

From the *Betaproteobacteria*, MND1 (the dominant order in KFS *Betaproteobacteria*) was detected 84 different samples sites, more than any of the other KFS uncharacterized orders (Table 3, Supplementary Table 2), being detected more frequently in soil, aquatic, and subsurface ecosystems, which suggests that MND1 may be diverse in function and/or capable of a wide range of environmental conditions. MND1 was detected in 18 of the 25 total subsurface sites, which is triple the number of any other KFS uncharacterized order. Originally, MND1 was first detected in in ferromanganous-coated sediment (12, 25), but it shows no preferential distribution towards either aerobic vs. anaerobic environments. A21b

(*Betaproteobacteria*) has a similar distribution pattern to MND1, but is detected in fewer samples, and has been rarely documented among subsurface community studies, and has not been detected in any marine environments to date (Table 3, Supplementary Table 1). Like A21b, Ellin339 (the dominant order in KFS *Gammaproteobacteria*) was rare in subsurface sites and was not detected in any marine samples. However, unlike other KFS uncharacterized orders, Ellin339 was detected among more freshwater sites and was the only order detected in several acid mine drainage sites (Table 3, Supplementary Table 1). Also, Ellin339 was detected in an acid-impacted lake (19) and an extremely acidic river (9), suggesting this uncharacterized order likely contains acid-tolerant or acidophilic bacteria.

This study highlights the importance of detailed subphylum level phylogenetic analysis of large 16S rRNA datasets, a process that is increasingly overlooked in favor of automated phylum-level assignment. The discovery and documentation of 15 novel orders and 46 novel families within the *Proteobacteria* in a single dataset indicates that even in phyla with multiple cultured representatives, the breadth of the subphylum level diversity is not completely understood. Finally, our survey of the ecological distribution of six abundant, yet-uncultured *Proteobacteria* orders suggests that most of these uncharacterized lineages may be ecologically important in not only soil but many ecosystems globally, and that specific enrichment and isolation approaches that have rarely been tested (e.g. acidic, hypoxic, or anoxic conditions) might prove useful in obtaining these lineages in pure cultures.

Acknowledgements

This work has been supported by the National Science Foundation microbial observatories program (grant no. MCB_0240683 and EF0801858), DOE small laboratory science program, and OSU start-up funds to MSE.

References

1. **Ashelford, K. E., N. A. Chuzhanova, J. C. Fry, A. J. Jones, and A. J. Weightman.** 2005. At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. Appl Environ Microbiol **71:**7724-36.

2. **Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje.** 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res **33:**D294-6.

3. **DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen.** 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol **72:**5069-72.

4. **DeSantis, T. Z., Jr., P. Hugenholtz, K. Keller, E. L. Brodie, N. Larsen, Y. M. Piceno, R. Phan, and G. L. Andersen.** 2006. NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic Acids Res **34:**W394-9.

5. **Dunbar, J., S. Takala, S. M. Barns, J. A. Davis, and C. R. Kuske.** 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. Appl Environ Microbiol **65:**1662-9.

6. **Elshahed, M. S., N. H. Youssef, A. M. Spain, C. Sheik, F. Z. Najar, L. O. Sukharnikov, B. A. Roe, J. P. Davis, P. D. Schloss, V. L. Bailey, and L. R. Krumholz.** 2008. Novelty and uniqueness patterns of rare members of the soil biosphere. Appl Environ Microbiol **74:**5422-8.

7. **Emerson, D., J. A. Rentz, T. G. Lilburn, R. E. Davis, H. Aldrich, C. Chan, and C. L. Moyer.** 2007. A novel lineage of proteobacteria involved in formation of marine Fe-oxidizing microbial mat communities. PLoS ONE **2:**e667.

8. **Fulthorpe, R. R., L. F. Roesch, A. Riva, and E. W. Triplett.** 2008. Distantly sampled soils carry few species in common. Isme J **2:**901-10.

9. **Garcia-Moyano, A., E. Gonzalez-Toril, A. Aguilera, and R. Amils.** 2007. Prokaryotic community composition and ecology of floating macroscopic filaments from an extreme acidic environment, Rio Tinto (SW, Spain). Syst Appl Microbiol **30:**601-14.

10. **Huber, T., G. Faulkner, and P. Hugenholtz.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics **20:**2317-9.

11. **Janssen, P. H.** 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl Environ Microbiol **72:**1719-28.

12. **Joseph, S. J., P. Hugenholtz, P. Sangwan, C. A. Osborne, and P. H. Janssen.** 2003. Laboratory cultivation of widespread and previously uncultured soil bacteria. Appl Environ Microbiol **69:**7210-5.

13. **Kersters, K., P. De Vos, M. Gillis, J. Swings, P. Vandamme, and E. Stackebrandt.** 2006. Introduction to the Proteobacteria, p. 3-37, The Prokaryotes, vol. 5. Springer New York.

14. **Lesaulnier, C., D. Papamichail, S. McCorkle, B. Ollivier, S. Skiena, S. Taghavi, D. Zak, and D. van der Lelie.** 2008. Elevated atmospheric CO2 affects soil microbial diversity associated with trembling aspen. Environ Microbiol **10:**926-41.

15. **Liles, M. R., B. F. Manske, S. B. Bintrim, J. Handelsman, and R. M. Goodman.** 2003. A census of rRNA genes and linked genomic sequences within a soil metagenomic library. Appl Environ Microbiol **69:**2684-91.

16. **Lipson, D. A., and S. K. Schmidt.** 2004. Seasonal changes in an alpine soil bacterial community in the colorado rocky mountains. Appl Environ Microbiol **70:**2867-79.

17. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res **32:**1363-71.

18. **Ovreas, L., and V. V. Torsvik.** 1998. Microbial diversity and community structure in two different agricultural soil communities. Microb Ecol **36:**303-315.

19. **Percent, S. F., M. E. Frischer, P. A. Vescio, E. B. Duffy, V. Milano, M. McLellan, B. M. Stevens, C. W. Boylen, and S. A. Nierzwicki-Bauer.** 2008. Bacterial community structure of acid-impacted lakes: what controls diversity? Appl Environ Microbiol **74:**1856-68.

20. **Quince, C., T. P. Curtis, and W. T. Sloan.** 2008. The rational exploration of microbial diversity. Isme J **2:**997-1006.

21. **Roesch, L. F. W., R. R. Fulthorpe, A. Riva, G. Casella, A. K. M. Hadwin, A. D. Kent, S. H. Daroub, F. A. O. Camargo, W. G. Farmerie, and E. W. Triplett1.** 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. The ISME Journal **1:**283-290.

22. **Schloss, P. D., and J. Handelsman.** 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl Environ Microbiol **71:**1501-6.

23. **Schloss, P. D., and J. Handelsman.** 2006. Toward a census of bacteria in soil. PLoS Comput Biol **2:**e92.

24. **Smit, E., P. Leeflang, S. Gommans, J. van den Broek, S. van Mil, and K. Wernars.** 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Appl Environ Microbiol **67:**2284-91.

25. **Stein, L. Y., M. T. La Duc, T. J. Grundl, and K. H. Nealson.** 2001. Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. Environ Microbiol **3:**10-8.

26. **Tringe, S. G., C. von Mering, A. Kobayashi, A. A. Salamov, K. Chen, H. W. Chang, M. Podar, J. M. Short, E. J. Mathur, J. C. Detter, P. Bork, P. Hugenholtz, and E. M. Rubin.** 2005. Comparative metagenomics of microbial communities. Science **308:**554-7.

27. **von Mering, C., P. Hugenholtz, J. Raes, S. G. Tringe, T. Doerks, L. J. Jensen, N. Ward, and P. Bork.** 2007. Quantitative phylogenetic assessment of microbial communities in diverse environments. Science **315:**1126-30.

28. **Yergeau, E., S. A. Schoondermark-Stolk, E. L. Brodie, S. Dejean, T. Z. Desantis, O. Goncalves, Y. M. Piceno, G. L. Andersen, and G. A. Kowalchuk.** 2008. Environmental microarray analyses of Antarctic soil microbial communities. Isme J **In Press**.

29. **Youssef, N. H., and M. S. Elshahed.** 2008. Diversity rankings among bacterial lineages in soil. Isme J **In Press**.

30. **Youssef, N. H., and M. S. Elshahed.** 2008. Species richness in soil bacterial communities: a proposed approach to overcome sample size bias. J Microbiol Methods **75:**86-91.

31. **Zarda, B., D. Hahn, A. Chatzinotas, W. Schonhuber, A. Neef, R. I. Amann, and J. Zeyer.** 1997. Analysis of bacterial community structure in bulk soil by in situ hybridization Arch Microbiol **168:**185-192.

Table 1. Comparison of the composition and abundance of *Proteobacteria* in Kessler Farm soil to other soils among published studies analyzing >1000 PCR-amplified 16S rRNA sequences.

Soil Sample	Type of community analysis	No. sequences analyzed	Avg length (bp)	% abundance of *Proteobacteria*	% of each class within *Proteobacteria*					Ref
					α	*β*	*γ*	*δ*	*ε*	
Oklahoma tallgrass prairie (KFS) soil	Clone library	13,001	>1300	25	39	16	7.6	37	0	(6)
Wisconsin trembling aspen rhizosphere soil	Clone library									(14)
ambient [CO_2]		1155	>1300	37	55	27	9.0	8.8	0	
elevated [CO_2]		1132	>1300	40	58	25	11	6.9	0	
Minnesota farm soil	Clone library	1,700	>1300	32	35	23	17	25	0	(26, 27)
Review of 21 different soils from various locations	Clone library	2,290	>300	39	48	25	5.9	21	0.1	(11)
Soil samples from different regions	Pyro-sequencing									(21)
Brazil		26,140	103	50	24	30	10	36	0	
Florida		28, 328	102	48	17	42	25	16	0.21	
Illinois		31,818	104	42	24	40	17	19	0	
Canada		53,533	102	47	26	39	8.6	27	0.21	
			Average	**40**	**36**	**30**	**12**	**22**	**0.058**	
			St Dev	**8.0**	**15**	**8.9**	**6.1**	**11**	**0.092**	

Table 2. Composition and novel and uncharacterized lineages within the different classes of *Proteobacteria*.

Lineage	No. clones	No. OTUs	No. orders	No. novel orders	% clones in uncharacterized orders	No. families	No. novel families	% clones in uncharacterized families
***Proteobacteria* (total)**	**2675**	**479**	**60**	**15**	**50**	**120**	**48**	**65**
Alpha-	1043	168	29	8	28	45	14	37
Delta-	998	165	15	6	64	33	15	85
Beta-	432	78	5	1	69	23	14	85
Gamma-	202	68	11	0	58	19	5	66

Table 3. Global ecological distribution of six abundant uncharacterized order-level lineages from KFS.

Ecosystem Type	No. sampling sites detected from each ecosystem type						
	Total	*Deltaproteobacteria*-KFS-6	EB1021 (δ)	Ellin314 (α)	MND1 (β)	A21b (β)	Ellin339 (γ)
Soil Ecosystems:	**61**	**4**	**25**	**25**	**32**	**23**	**27**
Soil/Rhizosphere (uncontaminated)	34	3	16	11	15	16	18
Organic-contaminated soil	11	1	2	5	6	1	5
Soil from cold/Polar regions	7		1	3	5	3	1
Metal/radionuclide mill tailings	4		4	3	3		2
Wetland soils	3		2	3	2	2	1
Peat soils	2				1	1	
Aquatic ecosystems:	**54**	**0**	**18**	**16**	**25**	**12**	**14**
Freshwater	11			1	5	1	7
Freshwater sediments	9		4	4	5	4	3
Coastal/Estuarine sediments	10		4	1	5	2	3
Marine water	2				2		
Deep sea sediment	10		9	6			
Wastewater/Activated sludge	9		1	2	6	4	1
Drinking water	3			2	2	1	
Subsurface ecosystems:	**25**	**0**	**4**	**6**	**18**	**1**	**2**
Subsurface groundwater/sediment	10		3	2	7		1
Contaminated subsurface	15		1	4	11	1	1
Extreme ecosystems:	**14**	**0**	**2**	**5**	**6**	**1**	**6**
Caves	4			3	3	1	1
Acid Mine Drainage	5						5
Volcanic/terrestrial mats	3		1	2	2		
Hot springs	2		1		1		
Animal-associated ecosystems:	**10**	**0**	**2**	**3**	**3**	**3**	**2**
Animal GI tract	4			1	1	2	1
Marine sponges	2		2				
Oyster shell	1				1		
Deep sea octacoral	1			1	1	1	
Freshwater sponge	1			1			
Human skin	1						1
Other ecosystems:							
Anaerobic organic-degrading enrichments/consortia	4		1	3		1	
Air	3			2			1
Waste-gas biofilter	1					1	
Decayed velvetleaf seed	1						1
Biofilm on O_2-transfer membrane	1			1			

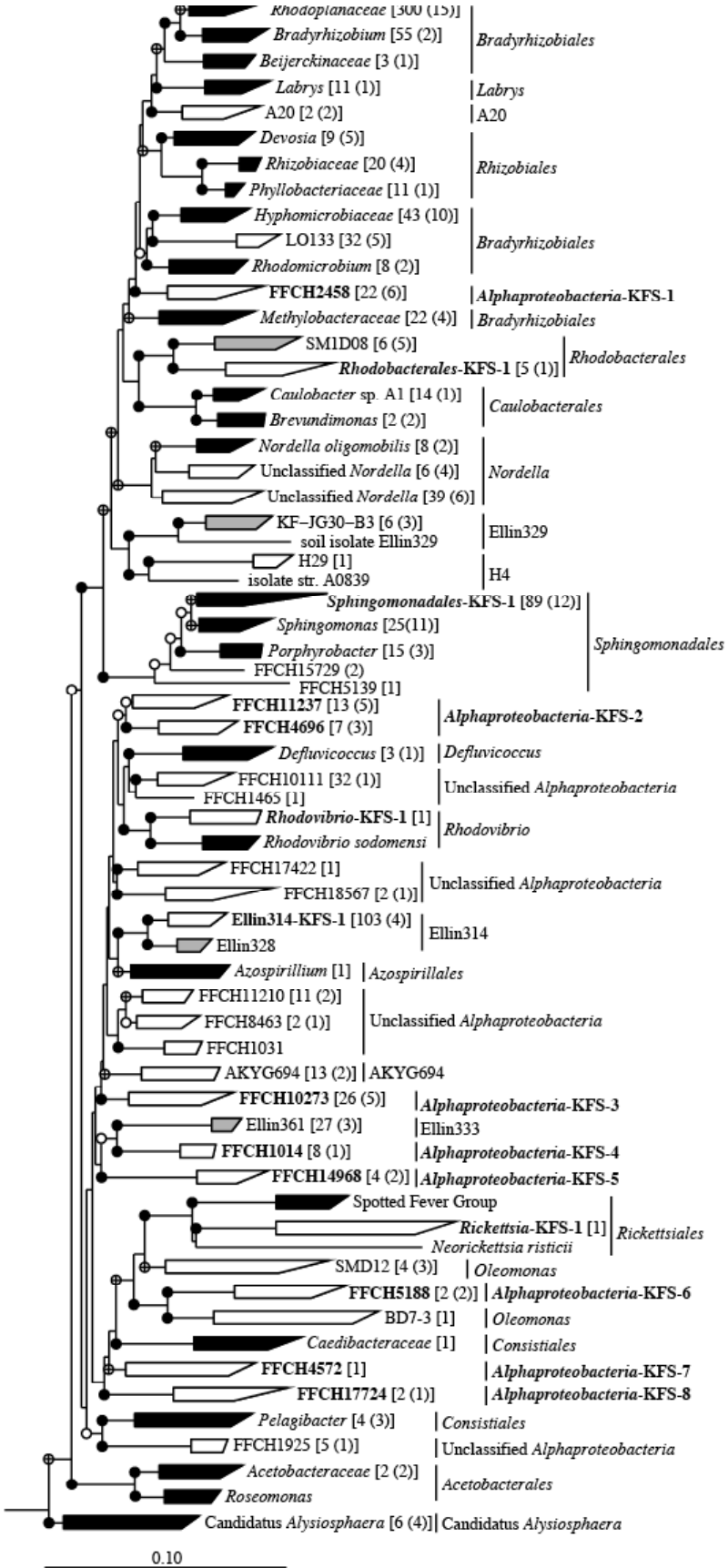
Rhodoplanaceae [300 (15)]
Bradyrhizobium [55 (2)]
Beijerckinaceae [3 (1)]
Bradyrhizobiales
Labrys [11 (1)]
Labrys
A20 [2 (2)]
A20
Devosia [9 (5)]
Rhizobiaceae [20 (4)]
Phyllobacteriaceae [11 (1)]
Rhizobiales
Hyphomicrobiaceae [43 (10)]
LO133 [32 (5)]
Rhodomicrobium [8 (2)]
Bradyrhizobiales
FFCH2458 [22 (6)]
Alphaproteobacteria-KFS-1
Methylobacteraceae [22 (4)]
Bradyrhizobiales
SM1D08 [6 (5)]
Rhodobacterales-KFS-1 [5 (1)]
Rhodobacterales
Caulobacter sp. A1 [14 (1)]
Brevundimonas [2 (2)]
Caulobacterales
Nordella oligomobilis [8 (2)]
Unclassified Nordella [6 (4)]
Unclassified Nordella [39 (6)]
Nordella
KF–JG30–B3 [6 (3)]
soil isolate Ellin329
Ellin329
H29 [1]
isolate str. A0839
H4
Sphingomonadales-KFS-1 [89 (12)]
Sphingomonas [25(11)]
Porphyrobacter [15 (3)]
FFCH15729 (2)
Sphingomonadales
FFCH5139 [1]
FFCH11237 [13 (5)]
FFCH4696 [7 (3)]
Alphaproteobacteria-KFS-2
Defluvicoccus [3 (1)]
Defluvicoccus
FFCH10111 [32 (1)]
FFCH1465 [1]
Unclassified Alphaproteobacteria
Rhodovibrio-KFS-1 [1]
Rhodovibrio sodomensi
Rhodovibrio
FFCH17422 [1]
FFCH18567 [2 (1)]
Unclassified Alphaproteobacteria
Ellin314-KFS-1 [103 (4)]
Ellin328
Ellin314
Azospirillium [1]
Azospirillales
FFCH11210 [11 (2)]
FFCH8463 [2 (1)]
FFCH1031
Unclassified Alphaproteobacteria
AKYG694 [13 (2)]
AKYG694
FFCH10273 [26 (5)]
Alphaproteobacteria-KFS-3
Ellin361 [27 (3)]
Ellin333
FFCH1014 [8 (1)]
Alphaproteobacteria-KFS-4
FFCH14968 [4 (2)]
Alphaproteobacteria-KFS-5
Spotted Fever Group
Rickettsia-KFS-1 [1]
Neorickettsia risticii
Rickettsiales
SMD12 [4 (3)]
Oleomonas
FFCH5188 [2 (2)]
Alphaproteobacteria-KFS-6
BD7-3 [1]
Oleomonas
Caedibacteraceae [1]
Consistiales
FFCH4572 [1]
Alphaproteobacteria-KFS-7
FFCH17724 [2 (1)]
Alphaproteobacteria-KFS-8
Pelagibacter [4 (3)]
Consistiales
FFCH1925 [5 (1)]
Unclassified Alphaproteobacteria
Acetobacteraceae [2 (2)]
Roseomonas
Acetobacterales
Candidatus Alysiosphaera [6 (4)]
Candidatus Alysiosphaera
0.10

Figure 1 (previous page). Distance phylogram of *Alphaproteobacteria* KFS OTU sequences based on aligned near full-length 16S rRNA gene sequences (approx. 1350 base pairs) from KFS clone library as well as representative sequences from each family-level lineage downloaded from GenBank, totaling 329 sequences, with each clade shown representing a family-level lineage (unless otherwise noted), consisting of at least two sequences. The tree was rooted with the 16S rRNA gene sequence from *Chloroflexus aurantiacus* (GenBack accession no. AJ308501). Bootstrap values are based on 1000 replicates and are shown to the left of each branch with bootstrap support >90% (●), 70-89% (⊗), and 50-69% (○). Black clades represent families with characterized, described cultivated representatives. Gray and unfilled clades represent uncharacterized families, consisting of clone sequences and sequences from unpublished or uncharacterized isolates (gray) or only environmental clone sequences (unfilled). Numbers aside each clade denote the number of clone sequences and OTUs detected from each family in the KFS clone library. Orders, according to Hugenholtz taxonomy and the Greengenes ARB May, 2007 database, are shown to the right of families. Novel lineages are shown in bold, with novel orders labeled as *Proteobacteria* class-KFS-# (e.g. *Alphaproteobacteria*-KFS-1). Novel families within novel orders are labeled according to clone names (e.g. FFCH2458), and novel families within characterized orders are labeled as order name-KFS-# (e.g. *Sphingomonadales*-KFS-1).

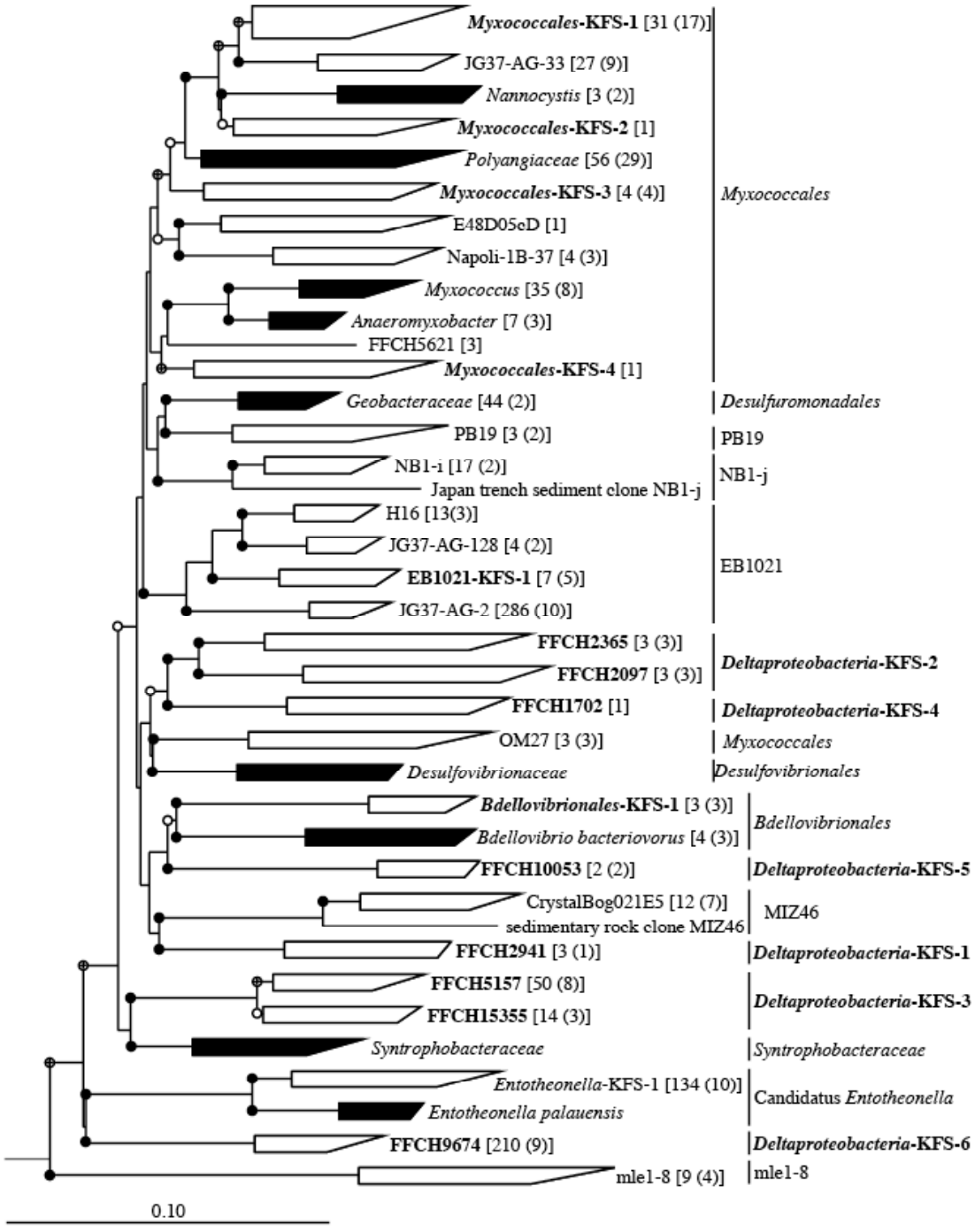

Figure 2. Distance phylogram of *Deltaproteobacteria* KFS OTU sequences based on aligned near full-length 16S rRNA gene sequences from KFS clone library as well as representative sequences from GenBank, totaling 241 sequences. Tree construction and notations are the same as described in Figure 1.

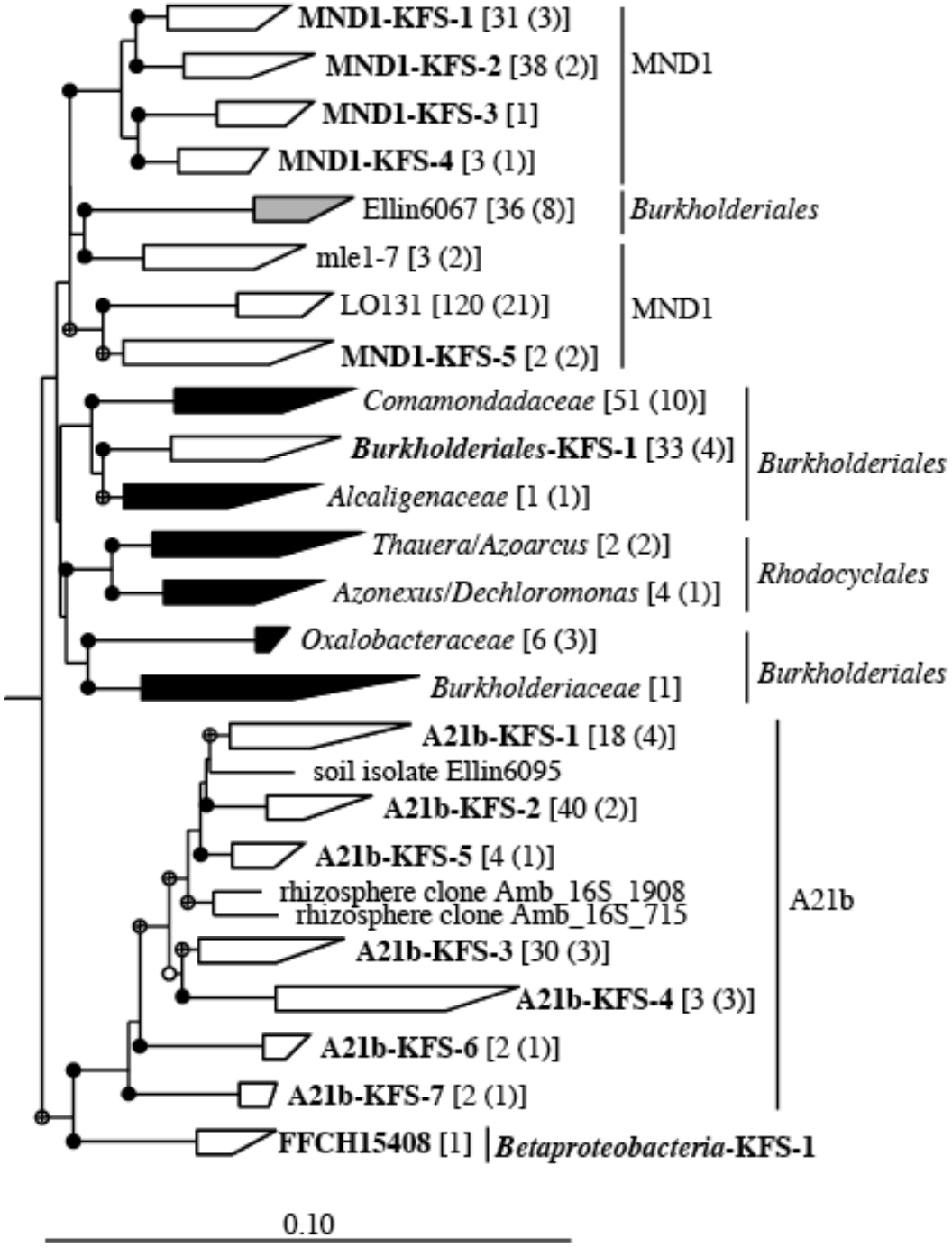

Figure 3. Distance phylogram of *Betaproteobacteria* KFS OTU sequences based on aligned near full-length 16S rRNA gene sequences from KFS clone library as well as representative sequences from GenBank, totaling 128 sequences. Tree construction and notations are the same as described in Figure 1.

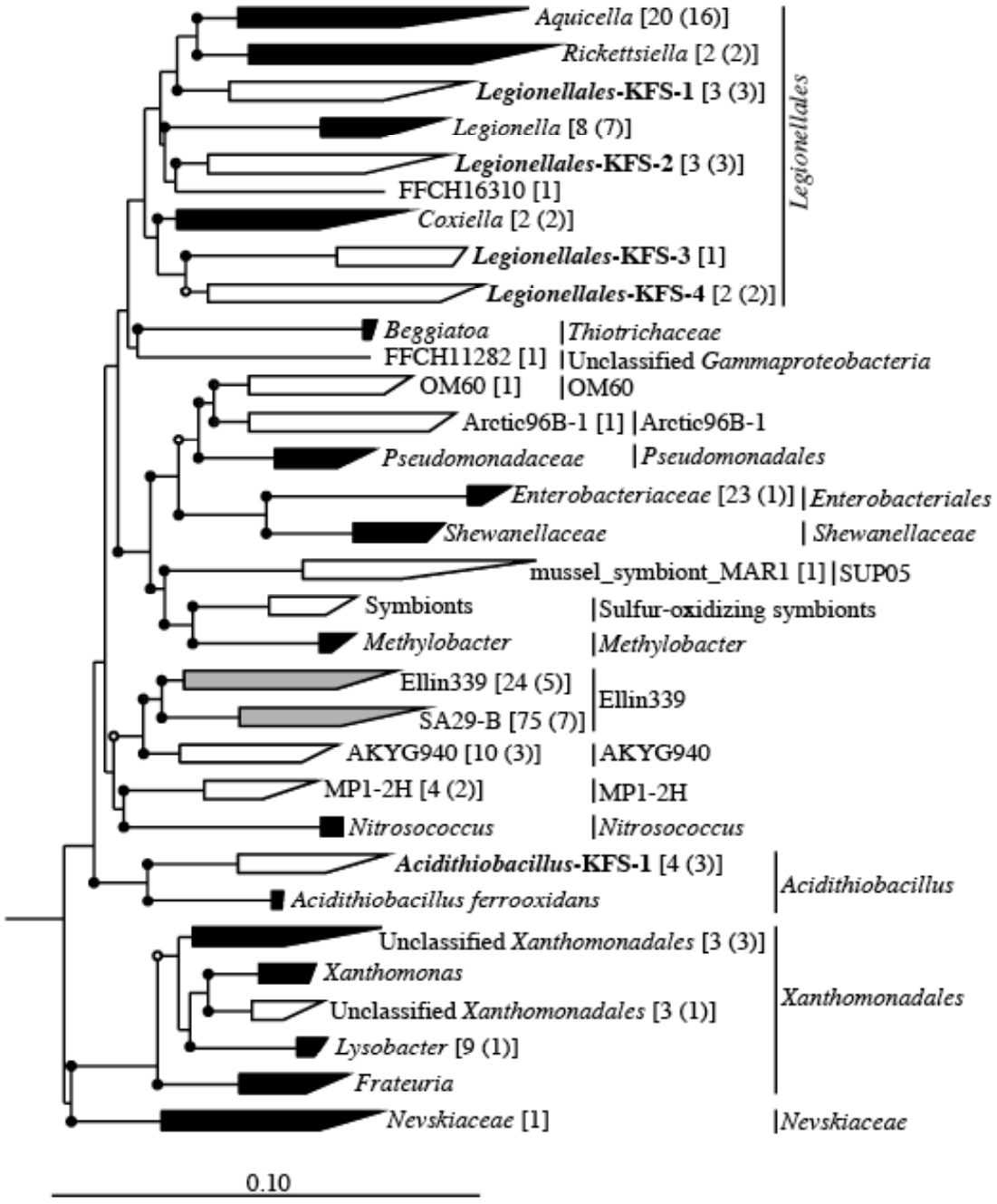

Figure 4. Distance phylogram of *Gammaproteobacteria* KFS OTU sequences based on aligned near full-length 16S rRNA gene sequences from KFS clone library as well as representative sequences from GenBank, totaling 183 sequences. Tree construction and notations are the same as described in Figure 1.

APPENDIX II

Phylogeny and Environmental Distribution of the Phylum *Fibrobacteres*

Phylogeny of the Phylum *Fibrobacteres*

The phylum *Fibrobacteres* currently consists of three classes circumscribed on the basis of phylogenetic analysis of 16S rRNA gene sequences and one cultivated class *Fibrobacteres* class nov. *Fibrobacteres* is the type class and contains a single order, family and genus.

The phylum *Fibrobacteres* is most closely related to the *Bacteroidetes* [*Cytophaga-Flavobacterium-Bacteroides* (CFB)] phylum based on signature sequences of proteins (2-4). Phylogenic analyses based on 16S rRNA gene sequences supports the relatedness of *Fibrobacteres* to this phylum, but indicate it is even more closely related to a candidate division TG3 (5, 6), and also shares a common ancestor with *Gemmatimonadetes* (Figure 1). The phylum contains three classes (Table 1, Figure 2), only one of which contains cultivated isolates and is formally named.

The class *Fibrobacteres* is circumscribed on the basis of 16S rRNA sequences. The class contains the single order *Fibrobacterales*. The order *Fibrobacterales* is circumscribed on the basis of phylogenetic sequences (Figure 2). The order contains the sole family, *Fibrobacteraceae*. The type genus of this family is *Fibrobacter*

The first pure culture of *Fibrobacter* was described as *Bacteroides succinogenes* by (8). (10) considered *Bacteroides succinogenes* to belong in the genus *Ruminobacter*, but the name *Ruminobacter succinogenes* was not validly published. More recently, phylogenetic analysis showed that strains of *Bacteroides succinogenes* were phylogenetically distinct from other species of *Bacteroides* necessitating the formation of a new genus (9). Sequences of small subunit rRNA of several strains were shown to have less than 72% similarity with *Bacteroides fragilis* providing evidence that these organisms constituted a distinct evolutionary line of descent at the phylum level.

There are currently no phenotypic characteristics that are useful for distinguishing the two species *F. intestinalis* and *F. succinogenes*; rather, small subunit rRNA analysis must be used (Figure 2). *F. succinogenes* subsp. *succinogenes* can be distinguished from *F. succinogenes* subsp. *elongatus* based on cell morphology, the former being ovoid and the latter more slender and rod shaped.

Within the genus, strains of *F. succinogenes* subsp. *succinogenes* and *F. succinogenes* subsp. *elongatus* have a 16S rRNA sequence similarity of 95.3 –98.1(1) and DNA hybridization of less than 20%. Between the two species, the 16S rRNA similarity is 91.8 to 92.9, with a DNA hybridization of less than 10%. The mol% G+C content of DNA of the genus is 45 – 51%. The type species is *Fibrobacter succinogenes* (9).

Environmental Distribution of the Phylum *Fibrobacteres*

Although all of the currently extant species were isolated from the rumen or other locations of the gastrointestinal tract, 16S rRNA genes related to the *Fibrobacteres* have been observed in a number of environments (Table 1, Figure 2). It is therefore likely that this phylum is much more broadly distributed in the environment than indicated based on the habitat of the pure culture representatives. Clones within the class *Fibrobacteres* (but not belonging to *Fibrobacterales*) were detected in an acid-impacted lake and a sulfidic cave stream biofilm (Figure 2), as well the termite species *Macrotermes gilvus* (7). The class-level lineage *Fibrobacteres*-2 was originally named a subphylum of *Fibrobacteres* and is solely composed of clone sequences derived from the gut of different species of termites (5). The third class-level lineage of *Fibrobacteres*, denoted here as "Environmental Clones," consists of clones detected in both soil and water downstream of manure (Figure 2). Though only three sequences are shown in Figure 2, unpublished sequences of <1000 bp belonging to this class-level lineage have been deposited in GenBank and also come from soil and water sources.

References

1. **Amann, R. I., C. Lin, R. Key, L. Montgomery, and D. A. Stahl.** 1992. Diversity among *Fibrobacter* isolates: Towards a phylogenetic classification. . System. Appl. Microbiol. **15:**23-31.

2. **Griffiths, E., and R. S. Gupta.** 2001. The use of signature sequences in different proteins to determine the relative branching order of bacterial divisions: evidence that Fibrobacter diverged at a similar time to *Chlamydia* and the *Cytophaga–Flavobacterium–Bacteroides* division. Microbiology **147:**2611-2622.

3. **Gupta, R. S.** 2004. The phylogeny and signature sequences characteristics of *Fibrobacteres, Chlorobi,* and *Bacteroidetes*. Crit Rev Microbiol **30:**123-43.

4. **Gupta, R. S., and E. Lorenzini.** 2007. Phylogeny and molecular signatures (conserved proteins and indels) that are specific for the *Bacteroidetes* and *Chlorobi* species. BMC Evol Biol **7:**71.

5. **Hongoh, Y., P. Deevong, S. Hattori, T. Inoue, S. Noda, N. Noparatnaraporn, T. Kudo, and M. Ohkuma.** 2006. Phylogenetic diversity, localization, and cell morphologies of members of the candidate phylum TG3 and a subphylum in the phylum *Fibrobacteres*, recently discovered bacterial groups dominant in termite guts. Appl Environ Microbiol **72:**6780-8.

6. **Hongoh, Y., P. Deevong, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, C. Vongkaluang, N. Noparatnaraporn, and T. Kudo.** 2005. Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. Appl Environ Microbiol **71:**6590-9.

7. **Hongoh, Y., L. Ekpornprasit, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, N. Noparatnaraporn, and T. Kudo.** 2006. Intracolony variation of bacterial gut microbiota among castes and ages in the fungus-growing termite *Macrotermes gilvus*. Mol Ecol **15:**505-16.

8. **Hungate, R. E.** 1950. The anaerobic mesophilic cellulolytic bacteria. Microbiol. Mol. Biol. Rev. **14:**1-49.

9. **Montgomery, L., B. Flesher, and D. Stahl.** 1988. Transfer of Bacteroides succinogenes (Hungate) to *Fibrobacter* gen. nov. as *Fibrobacter succinogenes* comb. nov. and description of *Fibrobacter intestinalis* sp. nov. International Journal of Systematic Bacteriology **38:**430-435.

10. **Prevot, A. R.** 1966. Manual for the classification and determination of the anaerobic bacteria. Lea and Febiger, Philadelphia, PA.

Table 1. Taxonomic divisions within the phylum *Fibrobacteres*

Lineage	Delineation [Max (Avg ± StdDev)][1]		Isolation Source	
	Within lineage	To *Fibrobacter succinogenes* str. S85[2]	Environmental clones	Cultivated isolates
Phylum: *Fibrobacteres*	0.29 (0.18 ± 0.06)	0.25 (0.15 ± 0.07)		
Class I: *Fibrobacteres*	0.18 (0.10 ± 0.06)	0.16 (0.08 ± 0.06)		
Order I: *Fibrobacterales*	0.11 (0.07 ± 0.03)	0.09 (0.05 ± 0.03)		
Family I: *Fibrobacteraceae*				
Genus I: *Fibrobacter*				
F. succinogenes	0.06 (0.05 ± 0.01)	0.06 (0.04 ± 0.02)	Equine cecum Bovine rumen	Ovine rumen Bovine rumen
F. intestinalis	0.04	0.09 (0.09 ± 0.00)	NA	Ovine rumen Rat cecum Porcine cecum
Unclassified *Fibrobacteres*	0.18 (0.14 ± 0.03)	0.16 (0.13 ± 0.02)	Termite gut Sulfidic cave stream biofilm Acid-impacted lake	NA
Class II: *Fibrobacteres*-2	0.14 (0.09 ± 0.02)	0.22 (0.20 ± 0.01)	Termite gut	NA
Class III: Environmental clones	0.19 (0.14 ± 0.04)	0.25 (0.22 ± 0.02)	Soil Water*	NA

[1]Delineation values are derived from a distance matrix of all sequences in Figure 1, based on Jukes Cantor-corrected distances. Maximum delineation values refer to the maximum pairwise distance between any two sequences within a given lineage; average delineations are the average distance between any two sequences from Figure 1 within a given lineage.

[2]Pairwise distances of sequences from all lineages were compared to F. *succinogenes* str. S85 as a reference.

*Water source=10m downstream from manure.

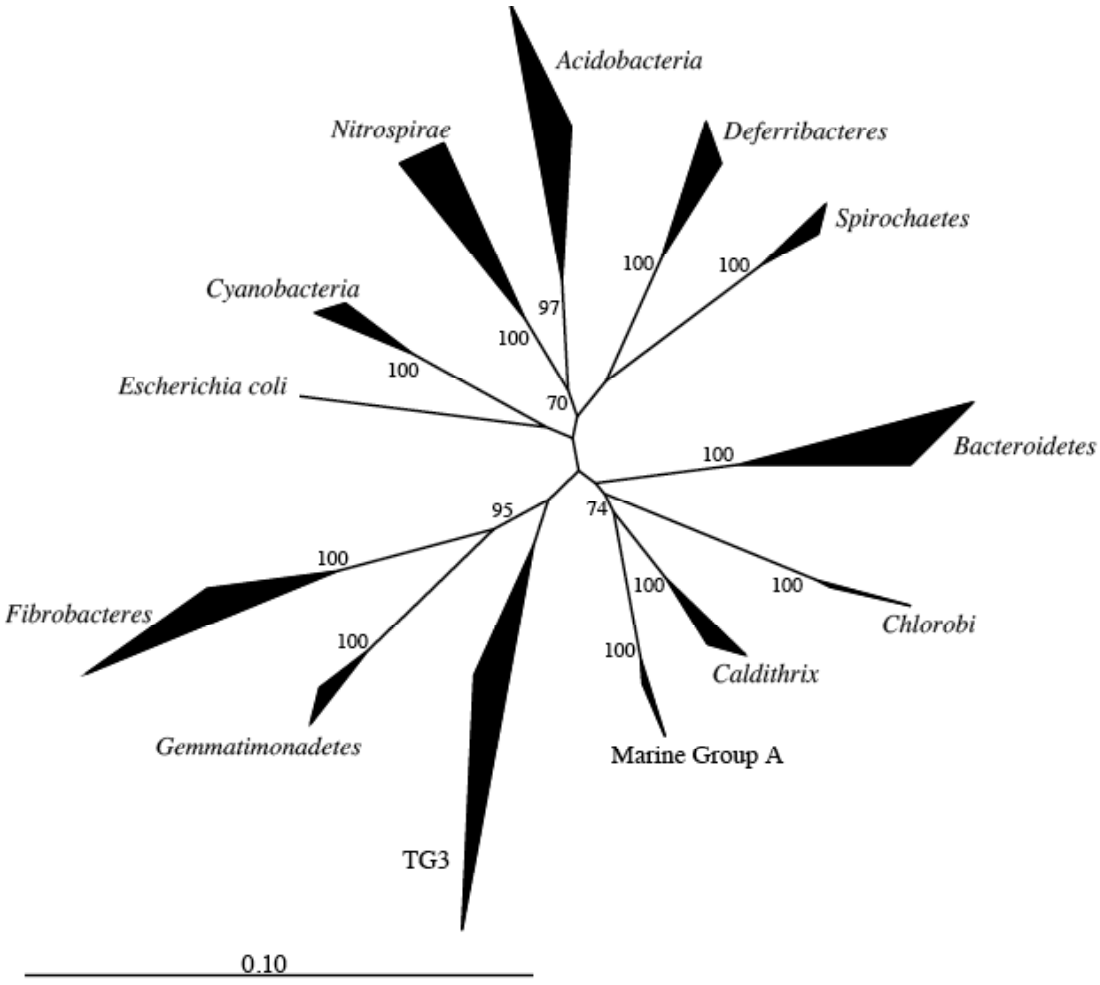

Figure 1. Maximum likelihood phylogentetic tree of the phylum *Fibrobacteres* in relation to closely related phyla. The tree was constructed from two or more aligned nearly full-length (>1300 bp) 16S rRNA gene sequences from each phylum-level lineage using the fastDNAml method. Bootstrap values >50 are shown to the left or above corresponding branches and are based on 1,000 replicates.

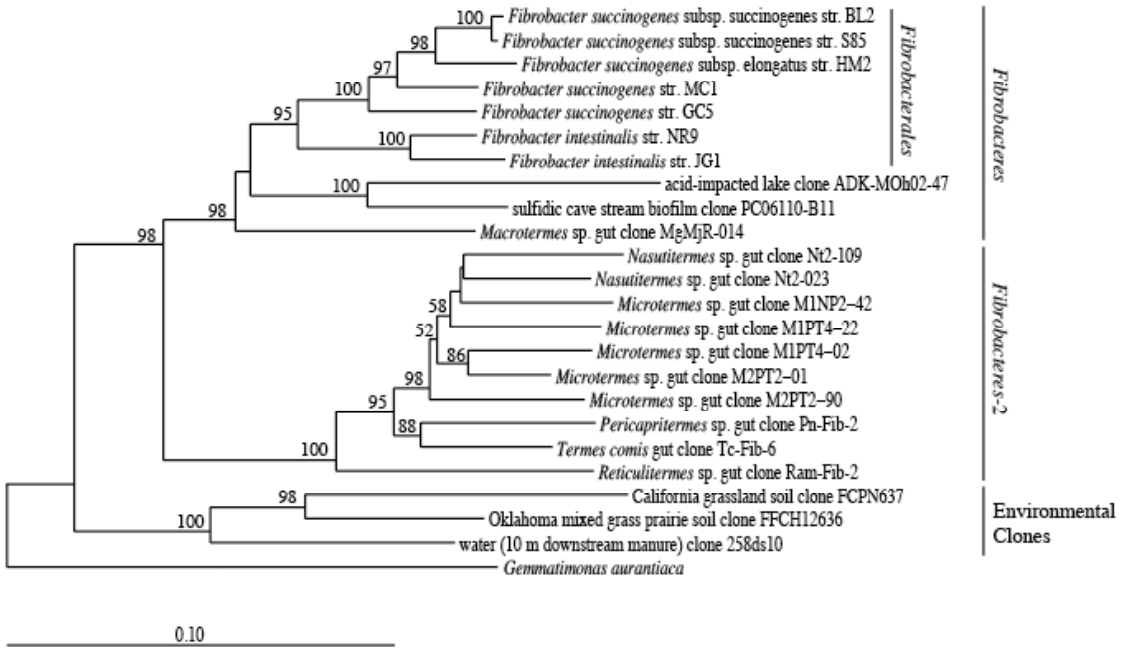

Figure 2. Distance phylogram of species within the three classes of the phylum *Fibrobacteres*. Environmental clone sequences are only included in lineages with no cultivated representatives. The tree was constructed from nearly full-length (>1300 bp) 16S rRNA gene sequences using the neighbor-joining algorithm with Jukes-Cantor corrected distances. Bootstrap values >50 are shown above corresponding branches and are based on 1,000 replicates.