MICROBIAL DIVERSITY AND GROUNDWATER CHEMISTRY IN A PRISTINE AQUIFER

BY

THEODORE MATTHEW FLYNN

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Geology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2011

Urbana, Illinois

Doctoral Committee:

Professor Craig M. Bethke, Chair
Research Associate Professor Robert A. Sanford, Co-Director of Research
Professor Bruce W. Fouke
Assistant Professor Rachel J. Whitaker

ABSTRACT

The Mahomet aquifer, a pristine water resource spanning much of central Illinois, is home to a diverse ecosystem of bacteria and archaea. We examined this microbial community at 18 wells across the aquifer by filtering cells suspended in groundwater and trapping sediment-bound populations on sterile samplers incubated in the wells themselves. After extracting DNA directly from these samples, we characterized both the suspended and attached communities with terminal restriction fragment length polymorphism (T-RFLP) and large-scale sequencing of the 16S rRNA gene. Using multivariate statistics, we quantified how the distribution of bacterial populations corresponds to the geochemical zonation of groundwater.

Groundwater in this area of the Mahomet aquifer is divided into areas of high and low sulfate, and the concentration of dissolved methane in these areas is inversely proportional to that of sulfate. Although groundwater in every well we sampled was considerably richer in ferrous iron than sulfide, we found that the presence of iron-reducing bacteria does not exclude sulfate reducers as previously assumed. Instead, where the concentration of sulfate is high, sulfate reducers comprise a proportion of the bacterial community (18%) nearly equivalent to that of iron reducers (23%). Iron reducers only dominate the bacterial community in wells with sulfate concentrations of 0.2 mM or less, where clones classified as *Geobacter*, *Geothrix*, or *Desulfuromonas* represent more than half of all sequences analyzed. We observed, in contrast, no statistically significant relationship between the structure of the bacterial community and the concentration of either ferrous iron or dissolved sulfide. This result calls into question the use of iron and sulfide as indicators of the nature of subsurface bacterial activity. Sulfate reducers and iron reducers do not appear to be segregated into discrete zones in the aquifer, as commonly believed to occur as a result of competitive exclusion. Instead, we found the two groups co-existing in the subsurface in a relationship

that we argue is not only competitive, but mutualistic.

Through further use of sediment traps to evaluate the attached microbial community, we found that overall structure of bacterial communities in the Mahomet is resistant to a shift in the availability of sulfate. After one year of incubation within a well where the concentration of sulfate was either high (1.5 mM) or low (0.04 mM), sediment traps were switched between the two wells for an additional year of incubation. Despite the more than 40-fold change in the concentration of sulfate, these switched traps remained more similar to the community from the well in which they were initially incubated. While the overall community composition did not change significantly, certain bacterial groups associated with sulfate reduction (*Desulfobacter* and *Desulfobulbus*) were found to increase or decrease along with the concentration of sulfate. These results show that while the abundance of many populations is at least partly controlled by the evolutionary history of that particular community, certain functional groups of critical biogeochemical importance are sensitive to local changes. While measures of overall community similarity are useful, the specific abundance of these taxa must be accounted for.

"Dimidium facti qui cœpit habet: Sapere aude, incipe."

– Quintus Horatius Flaccus

ACKNOWLEDGMENTS

I owe a sincere debt of gratitude to my graduate advisors, Craig Bethke and Rob Sanford. Their guidance, patience, and inspiration were essential to the completion of my dissertation. Similar thanks goes to the other two members of my dissertation committee, Bruce Fouke and Rachel Whitaker. Their insightful comments and words of encouragement helped me greatly in this endeavor. I would also like to thank other faculty members at the University of Illinois, particularly Thomas Johnson and Craig Lundstrom in the Department of Geology, Kevin Finneran in the Department of Civil and Environmental Engineering, and Joanne Chee-Sanford in the Department of Natural Resources and Environmental Science. The research staff at the Illinois State Water Survey, particularly George Roadcap and Walt Kelly, provided me with invaluable wisdom about the Mahomet aquifer and taught me the ins and outs of sampling groundwater in the field. Research scientists Jorge Santo Domingo and Nick Ashbolt at the U.S. Environmental Protection Agency lent me considerable support, both intellectual and financial, that contributed greatly to the completion of this project.

Additional thanks goes to Scott Clark, Emily Berna, Derik Strattan, Matt Kyrias, Peter Berger, Brian Farrell, Michael Kandianis, Cathy Kandianis, Elizabeth Luber, Jenna Shelton, Paige Jany, Chris Salvatori, Shabbir Kheraluwala, Zoheb Mohammed, Jenny Brulc, Dion Antonopoulos, and James Davis, for help in the field, in the lab, and in life. The outstanding academic support teams of Shelley Campbell, Lori Baker, Barb Elmore, Marilyn Whalen, and Julie Dyar in the Geology Department and Janessa Gentry and Debbie Piper at the Institue for Genomic Biology. And finally, thanks to my parents, Tom and Rita, my brother Andrew, and my wife-to-be Trish. Thanks for your encouragement, your understanding, and, most of all, your patience.

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CHAPTER 1

INTRODUCTION

The waters that fill pore spaces between rocks and sediments in the Earth's subsurface are one of humankind's most important natural resources. Groundwater from municipal and private wells supplies approximately 40% of the drinking water used in the United States [Drever, 1997]. The chemical composition of groundwater has long been of interest to citizens as well as scientists. Much early work on the fundamentals of chemistry was undertaken in order to understand and exploit the supposed curative properties of mineral water from hot springs [Back et al., 1995]. And the stability of "India Pale Ale" brewed at Burton-on-Trent was found to be caused by the high concentration of sulfate in the groundwater used for brewing. This allowed this particular ale to survive for months at sea while other British beers, brewed using carbonate-rich groundwater from the Chalk aquifer in southern England, spoiled during their trip around Cape Horn [Lloyd, 1986]. More recent interest in groundwater chemistry comes largely from an interest in remediating aquifers that have become contaminated by anthropogenic pollutants [Borch et al., 2010]. Yet while chemists, physicians, brewers, and geologists have sought for centuries to elucidate the inorganic chemical reactions that occur in aquifers, only recently have they come to appreciate the critical role that microorganisms play in determining the chemistry of groundwater.

The presence of microbes in groundwater has been known since invention of the microscope, when Antoine van Leeuwenhoek observed the first "animalcules" in water drawn from his well [Chapelle, 2001]. Obtaining unequivocally pristine samples from the subsurface proved difficult, however, and the function of subterranean microbial life remained enigmatic until late in the 20th century. Studies such as those of Olson et al. [1981] found viable bacteria in waters from a well 2.2 km below the Earth's surface. The temperature of groundwater there exceeded 50°C, and the bacteria recovered did not grow at surface temperature and

were therefore unlikely to have been transported there during the drilling. Today, microbial life is known to exist throughout the Earth's subsurface, from the subzero sediments underneath glaciers [Bhatia et al., 2006; Mikucki et al., 2009; Skidmore et al., 2005] to the boiling waters around hydrothermal vents [Amend and Shock, 2001; Huber et al., 2007; Reysenbach et al., 2000]. Indeed, subsurface microorganisms are even thought to be the most likely candidates for extraterrestrial life on Mars [Fisk and Giovannoni, 1999; Hoehler et al., 2007].

Microbes' ubiquity in the subsurface arises from their ability to derive useable energy from a diverse array of oxidation/reduction reactions. Upon infiltrating the subsurface, groundwater quickly becomes depleted in dissolved oxygen, allowing microbes who respire using other electron acceptors to flourish (e.g. Chapelle [2001]). Rather than "breathing" oxygen, anaerobic microorganisms exploit the disequilibrium between other chemical components in groundwater, transferring electrons from reduced carbon compounds or H_2 to an electron acceptor such as ferric iron or sulfate. This generates energy for the cell in the form of ATP and produces reduced forms of the electron-accepting species, such as ferrous iron or sulfide. Whether by mineralizing toxic organic compounds such as benzene into harmless bicarbonate ions or by drastically lowering the solubility of radionuclides such as uranium through enzymatic reduction, microorganisms (bacteria and archaea) exert strong control over the chemistry of groundwater [Bethke, 2008].

Despite longstanding interest in the biogeochemistry of aquifers and recognition of the importance of microbial metabolism in this system, many important questions remain regarding the relationship between groundwater systems and the microbes that dwell there. One primary reason for this gap in knowledge is that the tools for assessing the massive diversity of microbial ecosystems have only recently become available. Since Carl Woese formulated a universal tree of life based on the 16S rRNA gene [Olsen et al., 1986; Pace, 1997; Woese, 1987], the use of nucleic acid sequences to identify and categorize microorganisms based on phylogeny rather than physiology has transformed scientists' understanding of the microbial world [Madsen, 1998, 2005]. No longer limited by the number of strains they could isolate and grow from samples of water and sediment, researchers can now fingerprint an entire microbial community directly using the DNA extracted from those same samples. Cloning and sequencing 16S rRNA genes taken from aquifer sediment and groundwater has

shown microbial communities in the subsurface are comprised of hundreds or thousands of different phylotypes, many of which have never been cultured directly [Falkowski et al., 2008; Sogin et al., 2006]. Molecular assays have yet to become routine in studies of aquifer biogeochemistry, and knowledge of how this diversity corresponds to active microbial processes remains limited.

The heterogeneity of aquifer sediments is an additional source of complexity for microbial populations in the subsurface. When not suspended in groundwater, bacteria in an aquifer colonize sediment particles and create differentiated communities known as biofilms. These dynamic multicellular entities house microscale heterogeneities in pH and ion activity that can differ markedly from bulk phase measurements [Marshall, 2006]. Most studies of microbial ecosystems in aquifers, however, focus solely on cells filtered from pumped groundwater, which at least partially ignores the populations that attach to sediment particles [Kieft et al., 2007; Lehman, 2007]. While it is known that attached populations constitute the majority of cells in the subsurface [Alfreider et al., 1997; Griebler et al., 2002; Harvey et al., 1984; Hazen et al., 1991; Marxsen, 1982] and some physiological differences have been observed between attached and suspended microbial communities in a contaminated aquifer [Reardon et al., 2004], few studies have examined phylogenetic differences between these two fractions in a quantitative sense. Chapter 2 describes how I used molecular phylogenetic techniques and multivariate statistics to compare the bacterial communities that colonize aquifer sediments with those found suspended in groundwater.

Besides containing distinct physical phases of groundwater and sediment, aquifers are commonly segmented into geochemical zones where groundwater displays a characteristic chemical composition [Barnes and Hem, 1973; Berner, 1981]. These zones are thought to reflect the activity of a single, predominant group of microbes such as iron reducers or sulfate reducers [Chapelle and Lovley, 1992; Chapelle, 2001; Cozzarelli and Weiss, 2007; Lovley et al., 1994]. Many researchers have attributed this segregation to competitive exclusion, a mechanism by which the functional group that respires the electron acceptor most energetically favorable among those available dominates microbial activity, by outcompeting other organisms [Chapelle et al., 1995]. Recent publications have even suggested threshold concentrations of ions such as sulfate or ferrous iron can be used to infer which functional group

is active in a particular area of an aquifer [Chapelle et al., 2009; McMahon and Chapelle, 2008]. Molecular analyses, however, commonly find a variety of functional groups comprising a significant portions of the diversity in a single sample from an aquifer, regardless of how the groundwater is classified by geochemical criteria [Fry et al., 1997; Kovacik et al., 2006]. DNA-based molecular methods amplify 16S rRNA genes from both active and dormant cells, however, and have so far provided little concrete information on how microbial activity corresponds to geochemical zones in aquifers. In Chapter 3, I address this issue directly by focusing the molecular analyses on the active, sediment bound bacteria in a pristine, geochemically-zoned aquifer.

Because the chemical composition of groundwater and aquifer sediment plays such a critical role in determining the extent of microbial activity, there is considerable interest in not only characterizing the structure of microbial communities in aquifers, but in understanding how microbial populations respond to changes in their environment. Some types of communities appear to be resilient over time despite large influxes of inorganic nutrients such as nitrate over several decades [Allison and Martiny, 2008; Bowen et al., 2011], while other communities in soil retain the imprint of agricultural development even after decades of laying fallow [Buckley and Schmidt, 2001]. Little yet is known about how microbial communities in aquifers react when faced with changes in the geochemistry of groundwater, and the impact of those changes on the community's function remain poorly understood [Botton et al., 2006]. Chapter 4 describes a field experiment wherein sterile sediment traps were placed for one year within two wells, one containing groundwater with a high concentration of sulfate (1.5 mM) and the other a low concentration of sulfate (0.04 mM). After 12 months, one trap from each well was transferred to the other and allowed to incubate in the new well for an equal amount of time. I then used molecular techniques combined with statistical analyses in order to determine how changes in community composition among the switched traps compares to traps which remained in their original well for the duration of the experiment.

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CHAPTER 2

ATTACHED AND SUSPENDED MICROBIAL COMMUNITIES IN A PRISTINE CONFINED AQUIFER[†]

2.1 Introduction

Microbes have been observed in the terrestrial subsurface since the invention of the microscope, but only in the last thirty years has their importance as mediators of many of the chemical reactions controlling groundwater chemistry begun to be fully appreciated [Bitton and Gerba, 1984; Chapelle, 2000]. Ubiquitous in near-surface sedimentary environments, microorganisms break down anthropogenic pollutants [Lovley, 1997; Mohn and Tiedje, 1992], control the solubility and bioavailability of toxic metals [Guha, 2004; Kelly et al., 2005; Kirk et al., 2004; Senko et al., 2002], and govern iron concentration and other aspects of water quality [Chapelle and Lovley, 1992; Park et al., 2006; Roden, 2006]. As the immense scope of microbial diversity has been gradually revealed [DeLong and Pace, 2001; Hugenholtz et al., 1998; Tiedje et al., 1999; Woese, 1987], we have come to view subsurface bacterial and archaeal species not as isolated populations, but as members of a complex interdependent community [Stahl et al., 2006; von Mering et al., 2007]. To accurately characterize the relationship between microbial activity and aquifer geochemistry, then, we must endeavor to sample microbial communities completely.

An aquifer generally contains many individual microbes, which can be grouped into various populations of microorganisms belonging to the same species. Together, the populations make up a metabolically and phylogenetically diverse microbial community. Microorganisms in the subsurface community are found attached to the solid grains in the aquifer, as well as suspended in the groundwater. Whereas the suspended biomass can be sampled readily and inexpensively by filtering microbes from water as it is pumped from a well, obtaining a

[†]The contents of this chapter were published previously in *Water Resources Research*, Vol. 44(7), W07425.

sample representative of the attached community presents special challenges. The attached microbes can be sampled by aseptic drilling [Kieft et al., 2007], but the process is expensive and time consuming, and even in well-controlled situations the samples may be subject to contamination [Chapelle, 2001; Cozzarelli and Weiss, 2007].

Given its simplicity and low cost, groundwater filtration has been used as the sole sampling method in many, if not most studies in aquifer microbiology. As is commonly recognized, however, there is little reason to believe a priori that suspended microbes can be expected to represent the subsurface community as a whole. Attached bacteria, for example, develop into biofilms within which redox conditions, and hence the microbial community, might differ significantly from that found in the bulk groundwater [Marshall, 2006]. Microbes attached to aquifer sediment may have better access to nutrients than suspended cells [Hazen et al., 1991], leading to differing community compositions. Iron-reducing bacteria depend on access to ferric iron in the sediment, and therefore might be expected to be poorly represented in the suspended community [Caccavo Jr. and Das, 2002]. And some microbes may simply attach themselves to solid surfaces more securely than others.

Recently, down-well incubation on a sterile substrate has emerged as an alternative method for sampling attached microorganisms [Alfreider et al., 1997; Griebler et al., 2002; Marxsen, 1982; Peacock et al., 2004; Reardon et al., 2004]. In this technique, an in situ sampler, which is simply a packet of sterilized sediment, is lowered into a well to the level of the open screen. The packet is left to be colonized by ambient microorganisms and, after several months, retrieved so the microbes attached to the sediment can be analyzed. The community recovered by the sampler is commonly taken as representative, at least in a general sense, of the community attached to the aquifer sediments themselves. Reardon et al. [2004], for example, found two wells where the in situ sampler recovered at least one population detected by aseptic drilling, but not in the sample filtered from the groundwater. Numerous populations undetected in groundwater in their study were found using the in situ samplers. In this study, we refer to samples collected using in situ samplers as attached communities, with the caveat that the extent to which the samples reflect the actual attached community in the aquifer remains to be fully demonstrated in the general case.

Several previous studies have compared attached and suspended communities in the field

using culture-based enumeration and direct cell counts [Bekins et al., 1999; Hazen et al., 1991; Hirsch and Rades-Rohkohl, 1990; Hirsch et al., 1992; Kölbel-Boelke et al., 1988; Lehman et al., 2001] or by measuring uptake rates of a radiolabeled substrate [Alfreider et al., 1997; Harvey et al., 1984; Holm et al., 1992]. In each case, the studies concluded that microbes suspended in groundwater are less representative of the subsurface community than those attached to sediments. Because of the nature of the techniques used in these early studies, however, it is difficult to interpret the results in a general or quantitative sense [Madsen, 2000], and the question of the relationship of the two subsurface communities remains largely open [Lehman et al., 2004].

Various non-culture-based techniques [Marsh, 2005; Olsen et al., 1986; Tiedje et al., 1999] have also been applied to study attached and suspended communities in the subsurface. The studies, however, have been qualitative [Pedersen et al., 1996], based on microbial biomarkers present in very low concentrations [Lehman et al., 2001, 2004], or focused on comparing contaminated and pristine areas of an aquifer [Reardon et al., 2004]. In this study, we employ in situ microbial samplers and molecular techniques to explore in a quantitative sense the phylogenetic relationship between the attached and suspended microbial communities in the Mahomet aquifer, a pristine glacial aquifer that serves as a regional water supply to much of central Illinois [Burch, 2008; Panno et al., 1994].

2.2 Materials and Methods

2.2.1 Sample Collection

We examined 19 observation wells in the east-central Illinois area of the Mahomet aquifer, a regional aquifer holding water with isotopic age in the range $1 - 20$ ka [Hackley, 2002], that is largely free of anthropogenic pollution [Mehnert et al., 2004]. We used in situ samplers to recover attached microbes, and filtered suspended microbes from the groundwater. Since buried glass slides came into use for sampling soil bacteria (e.g. Rossi et al. [1936]), microbiologists have appreciated that the choice of substrate can influence the composition of the microbial communities collected [Parkinson et al., 1971]. Not surprisingly, then, the

mineralogy of the sediments used to construct in situ samplers can affect the structure of the communities recovered [Mauck and Roberts, 2007; Reardon et al., 2004]. To minimize selective effects, we used washed, autoclaved sand from the Mahomet aquifer itself. The sand was recovered during the drilling of a private well into the Banner formation of the Mahomet. Storage containers were filled completely, to minimize contact with the atmosphere, and placed in ice. Upon return to the laboratory, the sediment was immediately washed to remove drilling mud and to minimize the re-oxidation of any ferrous iron sorbed to mineral surfaces. The washed sediment was then autoclaved at 121℃. We constructed the samplers by placing 110 g of wet sediment in a $4'' \times 8''$ AquaClear 20 mesh bag (Doctors Foster and Smith™, CD-120490), which we secured with construction-grade nylon mason line (Lehigh®, #BNT1812YRL). Samplers were then stored at −45℃ until use.

Before sampling a well, we pumped the groundwater at a rate of 8 l min^{-1} using a Grundfos® Redi-Flo II electric submersible pump. Once pH, temperature and conductivity measurements stabilized, indicating we were pumping water from the aquifer itself [Kieft et al., 2007], we filtered 2 l of groundwater through a Supor-200® polyethersulfone filter (Pall Life Sciences) to collect suspended bacteria. After collecting duplicate filter samples, we lowered an in situ sampler until it was level with the well screen and left it for between 98 and 137 days, after which it was retrieved. This interval was likely sufficient for microbes in the aquifer to colonize the samplers and reach steady state populations, which Griebler et al. [2002] found to have occurred after 14 weeks (98 days) of incubation. Both the filtered and in situ samples were placed immediately in a sterile Whirl-Pak® bag and frozen on dry ice for return to the lab, where they were stored at −80℃ pending analysis.

2.2.2 Genetic Analysis

We extracted high molecular weight genomic DNA from both samplers and filters with a benchtop procedure similar to that of Tsai and Olson [1991]. Using appropriate procedural negative controls, cells were lysed by incubating either one filter or 4 g sampler sediment at 37℃ in a lysis solution (0.15 M NaCl, 0.1 M Na_2 ethylenediaminetetraacetic acid (EDTA), pH=8.0) with 15 mg lysozyme per mL, followed by a second incubation in

STS solution (0.1 M NaCl, 0.5 M Tris-Cl (pH=8.0), and 10% sodium dodecyl sulfate). After freeze-thaw cycling between a 55℃ water bath and liquid nitrogen to further disrupt cell membranes, proteins were removed through extraction with equal volumes of phenol (pH=7.8), phenol:chloroform:isoamyl alcohol (25:24:1, pH=7.8) and chloroform:isoamyl alcohol (24:1). DNA, which remained in the aqueous phase while proteins partitioned into the organic phase, was precipitated in isopropanol and 2.5 M ammonium acetate, then resuspended in Tris-EDTA buffer (pH = 8.0). The amount and quality of DNA recovered was confirmed visually by staining a 0.8% agarose gel with ethidium bromide. Although the total number of cells or the biomass was not directly quantified on either, the amount of DNA recovered from the samplers and filtering was approximately equivalent.

We used terminal-restriction fragment length polymorphism (T-RFLP) [Liu et al., 1997; Marsh, 2005] analysis to create a community profile of the extracted DNA. We paired a fluorescently-labeled (6-carboxyfluorescein), *Bacteria*-specific forward primer (8F, 5′ - AGAGTTTGATCCTGGCTCAG - 3′) with the universal reverse primer (1492R, 5′ - GGTTACCTTGTTACGACTT - 3′). PCR amplification was performed in 100 µl reaction volumes (20 µl TaqMaster™ PCR enhancer, 10 µl TaqBuffer™ (500 mM KCl, 100 mM Tris-Cl [pH=8.3], and 15 mM $Mg(OAc)_2$), 8 µl of dNTPs (dATP, dTTP, dCTP, and dGTP all at 10 mM), 10 µl each forward and reverse primer (4 µM), 0.50 µl *Taq* polymerase (Eppendorf MasterTaq®), 4 µl of template genomic DNA per tube, and 38 µl of HyPure™ Molecular Biology Grade water). Amplification began with a denaturing hot start of 4 minutes at 95℃ and was followed by 30 cycles of the following: 30 sec of denaturing at 94℃, 30 sec for primer annealing at 50℃, and 90 sec for extension at 72℃. The PCR was completed with a final extension step at 72℃ for 5 minutes. We purified our PCR product with QIAGEN's QIAquick® kit and digested the cleaned-up product with the restriction enzymes *Hha*I, *Msp*I, and *Rsa*I. We sent the digested and cleaned up DNA to the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign for fragment analysis by capillary electrophoresis.

From the fluorescent intensity of each terminal restriction fragment, we created a peak profile table of fragment size and intensity using GeneMapper® v3.7 (Applied Biosystems). For each sample, we eliminated the peaks with a size less than 50 or greater than 1000 base

pairs, the limits of the 1000 ROX™ (Applied Biosystems) size standards. This procedure prevents uncut DNA or large terminal fragments from contributing to the profile. We examined the profiles visually to ensure that these two types of fragments did not contribute significantly to the profile.

To ensure that only peaks representing actual populations were considered, we eliminated peaks with a height of less than 75 absorbance units. Furthermore, any samples with one or more peaks that exceeded the fluorescence threshold of $30,000$ absorbance units were reanalyzed at a lower DNA loading concentration. Finally, we normalized each individual peak height to the sum of all peak heights for the sample, in order to standardize the results for statistical comparison. We assume for the statistical analyses that each terminal fragment represents a single population [Rees et al., 2004].

2.2.3 Community Comparison

As with any technique that relies on PCR amplification of DNA, the results of T-RFLP analyses are subject to biases [Forney et al., 2004; Martin-Laurent et al., 2001; Sipos et al., 2007] and hence provide only a semi-quantitative characterization of the diversity of a microbial community [Bent et al., 2007; Blackwood et al., 2007; Fierer, 2007; Fierer and Jackson, 2006]. In this study, we are concerned not with determining diversity, but in comparing the composition of different communities. Since the analysis of each sample presumably entails the same amplification biases, T-RFLP analysis is believed to provide a robust method for drawing comparisons among microbial communities [Hartmann and Widmer, 2007; McGuinness et al., 2006; Moeseneder et al., 1999; Osborn et al., 2000]. Whereas the peaks in a T-RFLP profile may not reflect the complete species richness of a given sample, they nonetheless allow the importance of major populations to be compared among samples [Blackwood et al., 2007; Dunbar et al., 2000].

We began by calculating the average number of T-RFLP peaks found in the attached and suspended samples and then calculated the percentage of the total peak height shared between the attached and suspended profiles of each well. We then used a series of multivariate statistical analyses to quantify the similarities and differences between the attached and

suspended communities. Our analyses are based on the Bray-Curtis similarity coefficient [Bray and Curtis, 1957], which is calculated from the heights of peaks shared by two communities and useful for comparing T-RFLP profiles [Rees et al., 2004]. The approach, first used in macroecological studies [Clarke and Warwick, 2001], has been applied successfully in microbial ecology [Klaus et al., 2007; Wolsing and Priemé, 2004]. The coefficient is equal to zero for a sample with no peaks in common, and to one for identical samples.

After calculating the Bray-Curtis similarity coefficient for each pair of samples, we ranked the coefficients in decreasing order and used multidimensional scaling (MDS) to display differences in community composition among the different wells. MDS is a non-metric plotting method where the distance between any two points on the plot is inversely proportional to their similarity. The multidimensional plot is then compressed into two dimensions. After 50 replicate analyses, we chose the orientation that best represents the matrix of similarity coefficients [Kruskal, 1964]. Statistical analyses was performed using the Primer-6 software package (Primer-E Ltd., Plymouth, UK).

We used the analysis of similarity (ANOSIM) method to quantify the differences at each well between the composition of the attached and suspended microbial community. In this method, an R-value is computed by taking the difference in the mean Bray-Curtis coefficient of comparisons within a sample group and the mean value of comparisons between the two groups, and dividing it by the number of comparisons. The closer a calculated R-value for a pair of sample groups is to one, the more divergent the groups are. We tested the null hypothesis (H_0) that there is no difference between two groups by calculating R for 10^6 random permutations of the group assignments. The randomly computed R-values are commonly distributed normally around a mean of zero. Therefore, if the calculated R-value lies outside this distribution, we can confidently reject the null hypothesis [Clarke and Warwick, 2001].

We used the SIMPER (similarity percentage) function [Clarke and Warwick, 2001] to calculate the extent to which each individual population contributes to the dissimilarity coefficient, which is the inverse of the Bray-Curtis coefficient. The function identifies peaks detected primarily in one sample group, but not the other [Rees et al., 2004]. Primer-6 ranks the populations from most to least responsible for the dissimilarity of two sample

groups. The highest ranked populations, then, are those whose peaks are notable by their presence in one group and their absence in the other. We then performed in silico restriction enzyme digests on the 16S rRNA gene sequences from a clone library created from the attached community (see Chapter 2) using the computer program Cleaver [Jarman, 2006]. By calculating the terminal fragment size of each clone in the library, we were able to assign a taxonomy to some of the dominant terminal fragments identified in the SIMPER analysis.

2.3 Results

We were able to extract DNA from samples taken at each of the 19 wells studied. For 15 wells, we were able to extract amplifiable DNA from both the in situ samplers and the filters. For two of the wells we extracted DNA from only the in situ sampler; for another two, amplifiable DNA was obtained from only the groundwater. A small amount of DNA was also extracted from an unused sampler, but T-RFLP analysis indicated that it had no peaks in common with either the attached or suspended samples.

Direct comparison of the T-RFLP profiles for the attached and suspended communities at each well showed that a number of terminal restriction fragments (T-RFs) were present in both communities, but each contained unique T-RFs (Figure 2.1). This figure shows results for a single well, but in a qualitative sense is representative of all. Neither community appeared to have greater population richness than the other, as the median average number of terminal fragments was 51 for the attached samples, and 49 for the suspended. On average, 15% (standard deviation ±6%) of the populations at each well were detected in both communities. Shared T-RFs represent 36% (±20%) of the total peak height for the attached communities, and 38% (±17%) of this value for the suspended community. Whereas shared populations represent significant proportions of the attached and suspended communities, these results show that neither community is fully representative of the overall microbial community in the aquifer.

The average of the Bray-Curtis similarity coefficients for comparison of the attached to suspended communities at the wells studied was 0.201. This value is considerably less than the average similarity within the attached (0.302) or suspended (0.299) communities

from different wells. The MDS plot (Figure 2.2A) furthermore shows systematic differences between the attached and suspended microbial communities. The stress of ordination on these plots ranges from $0.13 - 0.20$, which, while not ideal, does not indicate that the plots are significantly distorted [Rees et al., 2004]. These results demonstrate that the profile of an attached community is more similar to the attached communities at other wells than it is to the profile of the suspended community at the same well, and vice-versa. The attached and suspended communities in the aquifer therefore differ consistently from each other.

In the ANOSIM, the calculated R-values for the *Rsa*I, *Hha*I, and *Msp*I restriction digests are 0.724, 0.822, and 0.877, respectively, showing a strong divergence of the attached and suspended sample groups, with some slight overlap. None of the 10^6 random permutations gave an R-value larger than the calculated value, indicating the results are statistically significant. SIMPER calculations identified two peaks in particular that are primarily responsible for differentiating the T-RFLP profiles of the attached communities from those of the suspended communities. In silico digests of cloned 16S rRNA gene sequences identified the peaks as the T-RFs of organisms with 100% genus-level similarity [Cole et al., 2007] to *Geobacter* and *Geothrix*. In the attached communities, peaks representing these organisms have an average abundance of 15% and 6%, respectively, of the total peak height, and a combined abundance of less than 1% in the suspended communities (Figures 2.2B and 2.2C). Organisms in these genera respire ferric iron [Lovley et al., 2004], a terminal electron acceptor that is highly insoluble at the pH of $7.5 - 7.7$ in this area of the Mahomet aquifer, and therefore likely to benefit from attachment and physical proximity to the sediment. The major populations of suspended bacteria identified by SIMPER were not identified in the clone library.

2.4 Discussion

The attached communities in our study were derived entirely by colonization of the in situ samplers by suspended microbes, yet attached and suspended samples from the same well share on average only one-third of their microbial community. If the communities found on the samplers were simply a result of random adhesion by ambient bacteria, the attached communities would not differ systematically from the suspended microbes. As we observe

in the MDS plots, however, there is a distinct, phylogeny-based grouping of the attached and suspended communities. This differentiation must result from selective colonization and growth by active microorganisms.

There are a number of potential reasons most of the bacteria in the groundwater were not detected on the in situ samplers. The suspended microbes could be inactive, they may prefer the groundwater environment, or they may be out-competed on sediment surfaces by other bacteria. Although 16S rRNA gene-based methods do not allow us to determine which individual populations are active in a particular sample, we can infer that the attached populations are metabolically active because selective colonization of a sterile substrate would not otherwise be possible. Further investigation with methods such as fluorescent in situ hybridization (FISH) or stable isotope probing (SIP) would be needed to further define the specific activity of populations within the suspended community. Regardless, the utility of the samplers clearly goes beyond simple observation of the attached community, representing a possible method to parse out actively metabolizing and reproducing populations from dormant ones.

The most consistent phylogenetic difference between attached and suspended communities in our study is seen in the absence from the profiles for many of the suspended samples of peaks representing *Geobacter* and *Geothrix*. We detected peaks representing *Geobacter* or *Geothrix*, or both, in all 17 of the in situ samplers from which we were able to extract DNA, but in fewer than half of the groundwater samples from the same wells. This result indicates that metal-reducing bacteria, while not completely absent from the groundwater, are associated primarily with sediment. This observation has important implications for groundwater sampling methods, since bacteria that respire insoluble electron acceptors such as ferric iron may be underrepresented in microbiological studies in which only groundwater is sampled.

For example, geochemical studies indicate that iron-reducing bacteria may release arsenic to groundwater in the Mahomet aquifer by consuming the ferric oxides to which the arsenic has sorbed [Kelly et al., 2005]. Microbiological study of the aquifer relying only on sampling the suspended community only, however, might not detect the presence of iron reducers, which in turn could lead investigators to incorrectly discount this mechanism of arsenic

release. The results of this study, then, underscore the advantages of sampling from aquifers both the suspended and attached communities whenever time and resources allow, because sampling only groundwater can provide results that are incomplete at best, and misleading at worst.

2.5 Figures

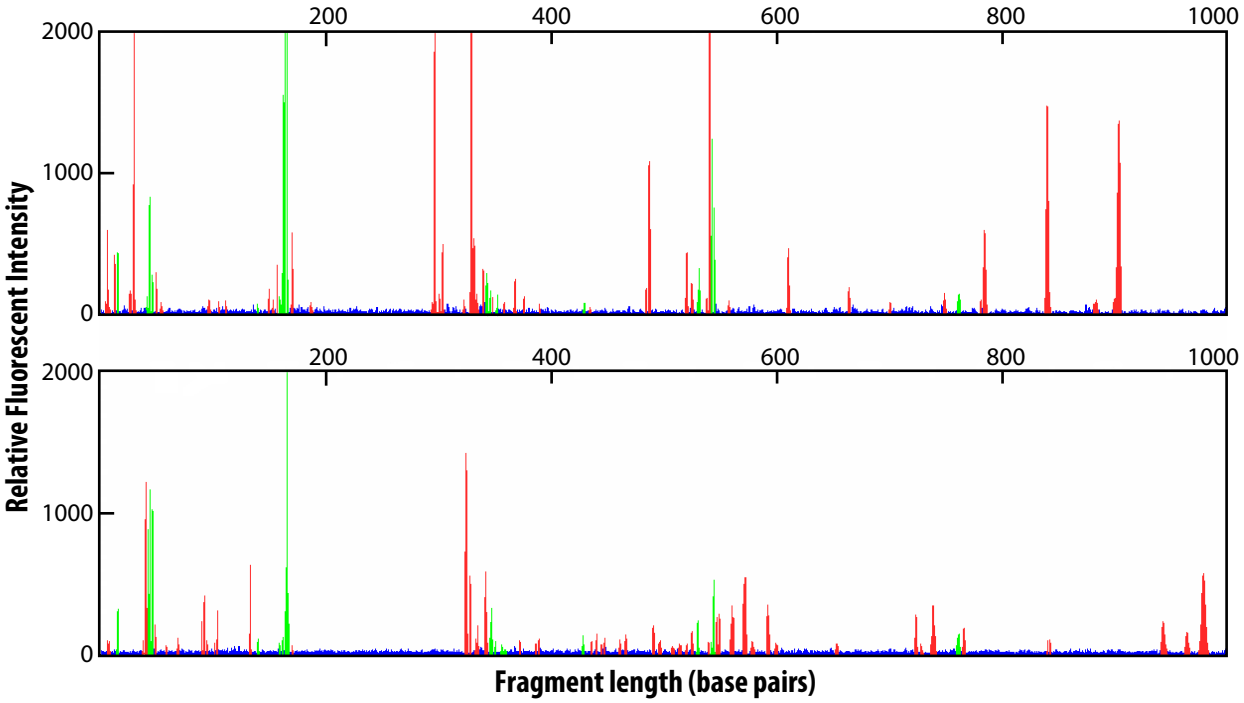

Figure 2.1: T-RFLP profiles of the (top) attached and (bottom) suspended communities from a single well (VER-94B). Green peaks represent T-RFs detected in both the attached and suspended communities, whereas red peaks show T-RFs detected in only one or the other.

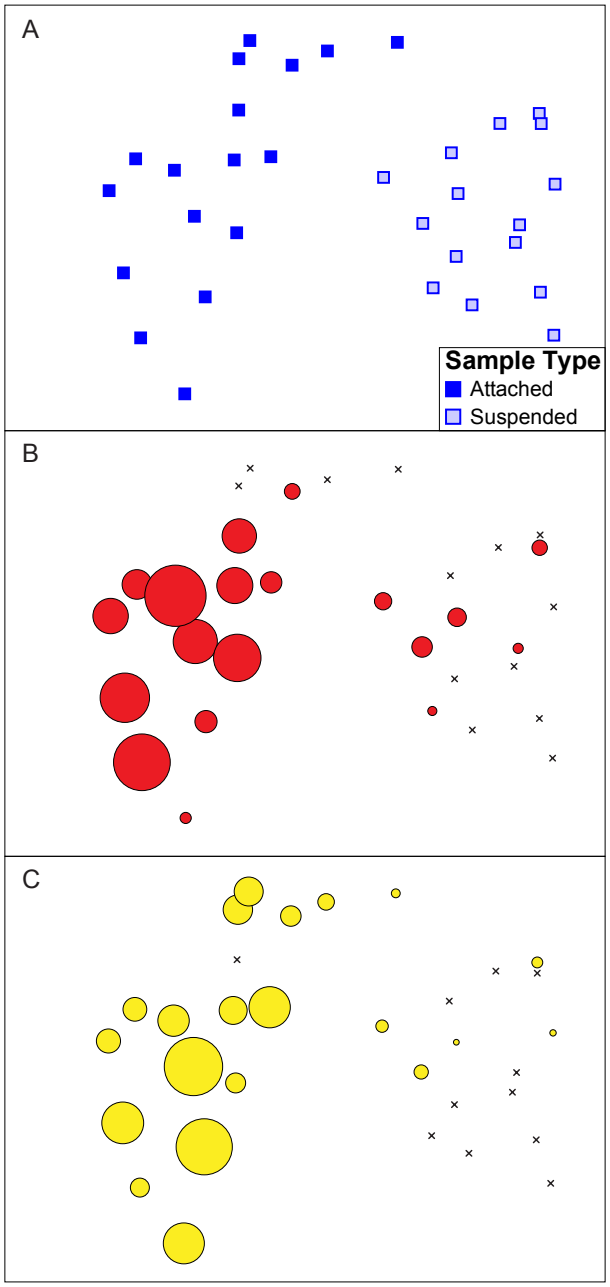

Figure 2.2: (A) Multidimensional scaling ordination of Bray-Curtis similarity coefficient rankings for attached (squares) and suspended (triangles) microbial communities. Stress, the amount of strain imposed by fitting a multidimensional ordination into two dimensions, is equal to 0.15. (B and C) Same ordination as Figure 2.2A but with each data point overlain with a circle representing the relative proportion of a specific T-RF in that particular sample. Circles in Figure 2.2B indicate the relative proportion of a T-RF of the same size as the in silico digest of a clone belonging to the genus *Geobacter*. Circles in Figure 2.2C are similarly representative of a clone belonging to the genus *Geothrix*. Communities in Figures 2.2B and 2.2C in which the *Geobacter* or *Geothrix* T-RF was not detected are indicated by a cross.

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CHAPTER 3

THE ACTIVE BACTERIAL COMMUNITY IN A PRISTINE, CONFINED AQUIFER

3.1 Introduction

Iron-reducing and sulfate-reducing bacteria exert strong control upon water chemistry in anoxic aquifers [Chapelle, 2000]. These functional groups control groundwater quality [Kirk et al., 2009], rates of mineral weathering [Park et al., 2009], and the fate and transport of toxic metals, metalloids, and organics [Kirk et al., 2004; Marshall et al., 2009]. Hydrologists are therefore keenly interested in better understanding the activity of these bacteria in the subsurface.

Like all respiring microorganisms, iron reducers and sulfate reducers capture energy by catalyzing the transfer of electrons from an initially reduced electron-donating species such as H2 or acetate to an initially oxidized electron acceptor. Iron-reducing bacteria make use of ferric minerals as electron acceptors, producing aqueous ferrous iron, whereas sulfate-reducing bacteria transfer electrons to sulfate ions, generating dissolved sulfide. These reactions have not been observed to proceed abiotically at low temperature [Lovley and Chapelle, 1995], so ferrous iron and sulfide in groundwater provide direct evidence for the activity of each group [Chapelle and Lovley, 1992; Jones et al., 1989].

Aquifers are commonly segmented into geochemical zones within which the activity of a single functional group, such as the sulfate reducers or iron reducers, is thought to predominate [Chapelle, 2001; Cozzarelli and Weiss, 2007]. Many researchers have attributed this segregation to competitive exclusion. By this mechanism, the functional group that can respire the electron acceptor most energetically favorable among those available dominates microbial activity, by outcompeting other organisms [Chapelle et al., 1995]. Iron reduction can provide more energy per electron transferred than sulfate reduction, for example, de-

pending on the ferric mineral being reduced, and the pH [Bethke et al., 2011]. Iron reducers might therefore exclude sulfate reducers by maintaining electron donor levels too low for the latter to respire [Lovley and Phillips, 1987]. Hydrologists therefore traditionally interpret groundwater rich in ferrous iron to indicate a zone in which iron reducers are active at the expense of sulfate reducers [Chapelle and Lovley, 1992; Chapelle et al., 2009]. Sulfidic groundwater, in contrast, is taken to result from the dominance of bacterial sulfate reduction, as might occur where iron reducers lack access to reducible ferric minerals.

In recent years, molecular methods have revealed the immense diversity of microbial communities in groundwater [Madsen, 2000]. Phylogenetic analyses commonly show that a wide variety of functional groups make up significant portions of the overall microbial community in an aquifer, regardless of how the groundwater is classified by geochemical criteria [Fry et al., 1997]. Near Cerro Negro, New Mexico, for example, groundwater chemistry points to sulfate reduction as the dominant form of respiration [Fredrickson et al., 1997; Krumholz et al., 1997, 2002], but nucleic acids recovered from the wells contain 16S rRNA and functional genes from not only sulfate reducers, but iron reducers and aerobes as well [Kovacik et al., 2006].

The apparent contradiction between the concept of exclusionary zoning and the molecular diversity observed in the subsurface might be explained if many of the microbes in an aquifer are dormant. At Cerro Negro, for example, sulfate reducers but not the iron reducers or aerobic bacteria might be active. Phylogenetic techniques shed little light on this question, because they amplify genes from both active and dormant cells [Madsen, 1998]. Most molecular studies in aquifer microbiology, furthermore, reveal only part of the subsurface community. Analyses are almost invariably made of cells filtered from groundwater, even though at any point in time only a small fraction of the community is suspended [Lehman, 2007]. Iron-reducing bacteria are physically associated with the insoluble ferric minerals they use as electron acceptors [Richter et al., 2008; Williams et al., 2010], and therefore may be underrepresented in groundwater [Flynn et al., 2008]. Phylogenetic studies, for these reasons, have provided little concrete information about how the activities of various functional groups of microorganisms are distributed throughout the subsurface.

We take a direct approach to these issues by studying the active, sediment bound bacteria

in a pristine confined aquifer. Using porous bags seeded with sterilized aquifer sediment, we trapped the active bacteria indigenous to 18 wells completed in the Mahomet aquifer, a regional water supply in east-central Illinois. Groundwater there is anoxic and has been categorized as iron reducing [Kelly et al., 2005]. We placed these in situ samplers - "bug traps" - at the depth of the well screens, where they were submerged in groundwater flowing along the aquifer.

Retrieving the traps after several months, we characterized the community of attached bacteria using terminal-restriction fragment length polymorphism (T-RFLP), and by analyzing bacterial sequences from 16S rRNA gene clone libraries. Our methodology did not target archaea, so the results provide information about bacteria in the aquifer, but not methanogens. Using multivariate statistics, we then quantified how differences in the bacterial community structure observed among wells are related to the concentrations of ferrous iron, sulfide, and sulfate in groundwater. In this way, we show how the distribution of microbial activity in the aquifer varies with these aspects of groundwater chemistry.

3.2 Materials and Methods

3.2.1 Sampling Area

We obtained water and microbial samples from 18 observation wells completed in the Mahomet aquifer. The Mahomet is composed of glacial sands and gravels interbedded with glacial till, which forms the confining layers [Kempton et al., 1991]. The bedrock underlying the north-central part of our sampling area is composed of pyritic coals and shales, whereas bedrock to the south and east is largely carbonate [Panno et al., 1994]. Locally, saline, sulfate-rich groundwater from the coal and shale passes upward and mixes into the dilute meteoric groundwater of the Mahomet [Hackley et al., 2010]. The mixing produces a range of sulfate concentrations across the study area, as shown in Figure 3.1.

3.2.2 Sample Collection

Before sampling, we purged each well by pumping the groundwater at a rate of 8 L min^{-1} with a Grundfos® Redi-Flo II electric submersible pump. The wells had been completed with screens 5 feet long, with 0.5 mm slots. We waited until pH, temperature and specific conductivity measurements of the pumped groundwater stabilized before taking water samples for geochemical analyses and deploying the microbial samplers. The bug traps were composed of washed, autoclaved aquifer sand previously collected from a Mahomet aquifer borehole. After lowering a trap into the until it reached the level left open to the aquifer, we left it submerged in flowing groundwater to be colonized by indigenous microbes. We left the traps in the well for between 98 and 137 days, a time period which Griebler et al. [2002] found to be sufficient for microbial populations colonizing sterile sediment in a similar aquifer to reach a steady state. Groundwater flow in this part of the aquifer is slow, < 1 m yr^{-1}, and the concentrations of key ions such as sulfate have been observed in nearby wells to be stable over many months [Kyrias, 2010]. Upon retrieval, the in-situ samplers were immediately frozen on dry ice in a sterile Whirl-Pak® bag for return to the lab, where they were stored at –80°C pending analysis.

In a previous study, Flynn et al. [2008] found that although the initially sterile bug traps are colonized solely by organisms suspended in groundwater, the bacterial communities attached to the traps are distinct from those that remain suspended. This selective colonization suggests the bacterial populations that colonize the traps are physiologically active and may be growing. By examining these attached communities separately from those found in the groundwater, we confine our analysis to what we assume to be the more active taxa in the aquifer. While the technical limitations inherent to DNA sequence analysis do not allow us to determine the specific activity of all populations in a particular well, bug traps still provide a direct method of sampling actively growing microbes in the subsurface.

3.2.3 Chemical Analysis of Groundwater

We filtered all groundwater samples in the field using a 0.2 µm Supor-200® polyethersulfone filter (Pall Life Sciences, Ann Arbor, MI). Filters were frozen immediately in the field on

dry-ice and stored prior to processing at –80℃. Samples for iron analysis were preserved by acidification to a pH < 2 using concentrated HCl. Water samples for sulfide analysis were collected directly from flowing groundwater using a degassed syringe, then injected into an N_2-filled bottle containing 20% zinc acetate, to prevent oxidation. Samples for dissolved organic carbon (DOC) analyses were stored in amber glass bottles and preserved using sulfuric acid (0.5% v/v). We stored the samples on ice for transport to the lab and kept them refrigerated until they could be analyzed.

We measured the concentrations of major anions (F^-, Cl^-, Br^-, NO_3^-, PO_4^{3-}, SO_4^{2-}) in groundwater using a Metrohm Advanced ion chromatograph (Metrohm USA Inc., Houston, TX). Our analysis protocol using this instrument has a detection limit of 10 µM. We measured ferrous and total iron concentrations using the ferrozine method [Viollier et al., 2000], and sulfide concentrations using the methylene blue method (Hach®). The detection limits were 1 µM for ferrous iron and 0.15 µM for sulfide. DOC analyses were performed at the Illinois Sustainable Technology Center using a Shimadzu TOC-VCPN carbon analyzer. The method has a detection limit of 0.4 mg kg^{-1}.

3.2.4 Molecular Microbial Analyses

We extracted total community DNA from the microbial cells attached to our in situ samplers and those filtered from the groundwater using a method described previously [Flynn et al., 2008]. Briefly, DNA was extracted from either 4 g of sediment or an entire filter through direct enzymatic lysis combined with freeze-thaw disruption, phenol/chloroform extraction and precipitation in isopropanol. We confirmed the amount and quality of DNA recovered using agarose gel electrophoresis. Although we did not directly quantify the number of cells attached to the samplers, the amount of DNA recovered from each was approximately equivalent.

3.2.5 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

We generated community profiles of the bacterial community using terminal restriction fragment length polymorphism (T-RFLP) analysis of the 16S rRNA gene using DNA extracted from the attached and suspended bacterial cells. This technique distinguishes microbial populations within a sample by tagging a PCR-amplified gene with a fluorescent marker, then cutting the gene into fragments using a series of restriction enzymes; the size of a tagged fragment is characteristic of the population from which the gene was obtained [Liu et al., 1997]. We amplified the 16S rRNA gene using PCR with a forward primer specific to bacteria (8F, 5′ - AGAGTTTGATCCTGGCTCAG - 3′) and a universally conserved sequence as a reverse primer (1492R, 5′ - GGTTACCTTGTTACGACTT - 3′). To create the fragment profile, we digested PCR product from DNA from each well in two separate reactions using two restriction enzymes (*Rsa*I and *Msp*I) that cut at different 4-base nucleotide sequences. The use of two separate digestions ensures that the tagged fragments from phylogenetically distinct microbial populations differ in length [Marsh, 2005]. A full description of the PCR reaction parameters and T-RFLP data analysis can be found in Flynn et al. [2008].

3.2.6 16S rRNA gene Clone Library

Primers 8F and 787R (5′ - GGACTACCAGGGTATCTAAT - 3′) [Buchholz-Cleven et al., 1997; Revetta et al., 2011] were used to amplify 16S rRNA genes for sequencing. Amplification reactions contained 5 U of Ex Taq® DNA polymerase (Takara Bio USA, Madison, WI), 5 µl of 10X concentrated Ex Taq™ Buffer, 4 µl of a 2.5 mM mixture of dNTPs, 3 µl each of forward and reverse primers (2.0 µM stock concentration), and 2 µl of template DNA (50 µl total volume). Amplification conditions for the bacterial assay included an initial denaturation step of 4 min at 94℃, followed by 35 cycles of 30 sec at 94℃, 30 sec at 56℃, and 1 min at 72℃, with a final extension step of 7 min at 72℃. Amplification products were screened using gel electrophoresis and GelStar® dye (BioWhittaker Molecular Applications, Rockland, ME).

Bacterial 16S rRNA gene PCR products were cloned into the pCR4.1 TOPO TA vector following the manufacturer's instructions (Invitrogen™, Carlsbad, CA). Transformed cells

were grown on Luria-Bertani agar plates containing 100 mg mL^{-1} ampicillin. Random colonies were screened for the presence of inserts of right size using M13 primers and gel electrophoresis. A total of 4,162 clones were sequenced from both the forward and reverse M13 primers using the BigDye® Terminator chemistry (Applied Biosystems, Foster City, CA) as described by [Lu et al., 2008].

Raw sequence chromatograms were checked for quality and assembled into contiguous sequences (contigs) using Sequencher® v4.10.1 (Gene Codes Corp, Ann Arbor, MI), then screened for chimeric assemblies using Bellerophon [Huber et al., 2004]. Any chimeric contigs were discarded and not considered in downstream analyses. Non-chimeric sequences were aligned using the alignment algorithm implemented in the program Mothur [Schloss et al., 2009] against a high-quality reference alignment of unique, chimera-checked reference sequences from the 12 October 2010 release of the Greengenes database [DeSantis et al., 2006]. Mahomet aquifer sequences from the Mahomet that did not align well or contained ambiguous base calls were discarded. The taxonomy of the final 4,029 16S rRNA gene sequences was classified to the genus level in Mothur using the Greengenes nomenclature [Schloss et al., 2009]. Restriction enzyme digests of the cloned sequences were created in silico using the program Cleaver [Jarman, 2006] in order to compare the distribution of cloned sequences with the T-RFLP data.

3.2.7 Statistical Analyses

We used multivariate statistics to quantify differences among the T-RFLP profiles from each well. We normalized the height of peaks in each profile to the total peak height [Rees et al., 2004]. For every possible pairing of wells, we computed the Bray-Curtis similarity coefficient [Bray and Curtis, 1957] representing similarity between the profiles using Primer 6. We computed a total of 435 Bray-Curtis coefficients, one for each pairing among the 30 T-RFLP profiles from the 18 wells studied (11 profiles were from duplicate samplers). We ranked the coefficients from most to least similar.

In this method, we computed a statistical measure (R_{ANOSIM}) that measures the similarity of bacterial communities from two groups of wells. The closer R_{ANOSIM} is to one, the more

divergent is the community structure of the two groups. Values of $R_{ANOSIM} > 0.75$ indicate two groups have largely distinct community structures, R_{ANOSIM} of $0.25 - 0.75$ indicates some overlap between the groups, and an $R_{ANOSIM} < 0.25$ shows communities within the two groups overlap significantly [Ramette, 2007]. We used Monte Carlo simulations to test the null hypothesis that there is no difference between two groups by calculating R_{ANOSIM} for 106 random group permutations. Randomly calculated values of R_{ANOSIM} values are distributed normally around a mean of zero; therefore, if the calculated R_{ANOSIM} lies far outside this distribution, we can confidently reject the null hypothesis [Clarke and Warwick, 2001]. The percentage of these randomly computed values is reported as p.

We plotted the distance matrix of similarity coefficients using multidimensional scaling (MDS). MDS plots portray the coefficients in a non-metric configuration, without specific axes. The rendering technique arranges the coefficients in three-dimensional space so that the distance between any pair of points is inversely proportional to their similarity. The technique then compresses the arrangement into two-dimensional space, seeking the orientation that best represents the matrix of similarity coefficients [Kruskal, 1964]. We ran MDS renderings 50 times from differing starting points, to help assure we had identified global rather than local optima. The calculations were made using the Primer–6 software package (Primer-E Ltd., Plymouth, UK).

Using the SIMPER (similarity percentage) function [Clarke and Warwick, 2001; Rees et al., 2004], we calculated the extent to which individual populations contribute to the dissimilarity between two sample groups. SIMPER identifies peaks important in one sample group but not another [Rees et al., 2004], then ranks them from most to least responsible. To identify the bacterial population associated with a peak identified by SIMPER, we performed in silico restriction enzyme digests on the 16S rRNA gene clone sequences from several wells. By predicting the size of the terminal fragment from each clone in the library, we could identify the bacterial strain that contributed a given fragment.

3.3 Results

The concentrations of sulfate, ferrous iron, sulfide and dissolved organic carbon (DOC) in groundwater sampled in this study vary broadly depending on the source well (Table 3.1). Dissolved oxygen was not detected in any well. Sulfate concentration ranges from below detection (0.01 mM) to about 10.7 mM; the average concentration is 1.7 mM. Panno et al. [1994] categorized groundwater in this region that contains $[SO_4^{2-}] > 0.2$ mM as high sulfate (denoted HS), and waters with less than this amount as low sulfate (LS). For this study we classified waters with < 0.03 mM sulfate, the nominal limiting concentration for bacterial sulfate reduction [Lovley and Klug, 1986], as containing negligible sulfate, denoted as NS (Figure 3.1).

We detected both ferrous iron and sulfide in groundwater from 14 of the 18 wells we sampled (Table 3.1). Ferrous iron concentration ranged from below detection (< 1.0 µM) to 28 µM, with an average of 16 µM. Sulfide levels varied from below detection (< 0.15 µM) to 2.5 µM, averaging 0.6 µM. The molar ratio of ferrous iron to sulfide was > 6 at each well in which both ions were detected, and ranged as high as 470. According to the criteria of Chapelle et al. [2009], a ratio greater than 6 indicates iron-reducing conditions are predominant.

Bacterial communities associated with attached and suspended bacteria were profiled using T-RFLP analysis. To compare community profiles we used ANOSIM to determine if the assemblage of bacterial populations present in the HS, LS, and NS wells are significantly different from one another, or if the groupings of community profiles are due to random chance. Based on the magnitude of the calculated values of R_{ANOSIM}, we found bacterial communities from NS wells were largely distinct from those in both HS and LS wells. The magnitude of R_{ANOSIM} for these comparisons ranged from $0.401-0.606$ (Table 3.2), indicating the groups being compared have distinctly different community structure with some overlap [Rees et al., 2004]. Monte Carlo simulations found values of p to be $< 5\%$ in comparisons for both restriction enzymes, indicating the differences between bacterial communities in NS wells and those in HS and LS wells are statistically significant. Comparison of HS and LS wells found a small ($R_{ANOSIM} = 0.170 - 0.245$), but still statistically significant

difference between communities in the two groups (Table 3.2). ANOSIM comparisons of the bacterial communities filtered from groundwater in HS, LS, and NS wells found no systematic differences in community structure (data not shown).

Differences in the composition of the attached communities among all 18 wells relative to sulfate concentration can be visualized by plotting a distance matrix of pairwise similarity coefficients using MDS (Figure 3.2A). Communities from HS areas of the aquifer fall into a discrete group in the MDS plot, separate from clusters associated with LS and NS wells. The clustering indicates similar distributions of bacterial populations among the wells within the three groups (Figure 3.2B). The MDS plots generated showing the concentration of ferrous iron and dissolved sulfide, in contrast, show no such clustering or statistical relationship (Figure 3.3). The bacterial community structure in the Mahomet thus appears unrelated to the measured concentration of either iron or sulfide in groundwater.

Sequencing of 16S rRNA genes yielded a total of 4,049 cloned sequences from the 18 wells analyzed. Members of the δ–Proteobacteria dominate the attached bacterial communities in the Mahomet (Figure 3.4). The differentiation between HS, LS, and NS wells is driven here by differences in the distribution of sequences most closely related to known iron-reducing and sulfate-reducing bacteria. Populations related to known iron reducers (the genera *Geobacter*, *Desulfuromonas*, and *Geothrix*) become more abundant with decreasing sulfate concentrations. Sequences from these groups comprise 23% of all bacterial sequences in HS wells, 35% in LS wells, and 53% in NS wells. Conversely, clones classified within the genera *Desulfobulbus* and *Desulfobacter*, clades of the δ–Proteobacteria comprised of sulfate reducers, become less abundant as sulfate concentrations decrease. In HS wells, they represent 18% of all bacterial sequences, 8% in LS wells, and 4% in NS wells.

Using SIMPER analysis of T-RFLP profiles, the populations most responsible for differentiating communities in HS wells from those in LS and NS wells belong to the genus *Geothrix*, on the basis of Greengenes phylogenetic classification [DeSantis et al., 2006] and in silico digests of cloned 16S rRNA gene sequences (Table 3.3). Using T-RF peak height as an indication of the relative abundance of a bacterial population, the T-RFs associated with *Geothrix* represent, on average, 12% and 13% of the total peak height in LS and NS wells but only 5% of the total peak height in HS wells. Other iron reducers with a 100%

probability of belonging to the genera *Desulfuromonas* or *Geobacter*, respectively, are also more predominant in the LS and NS wells (Table 3.3). Overall, T-RFs from groups classified as iron reducers (*Geobacter*, *Desulfuromonas*, and *Geothrix*) represent 32% and 34% of the total peak height on average in NS and LS wells, but only 18% of the total peak height in HS wells. Because of the multiplicity of T-RF lengths associated with *Desulfobulbus* and *Desulfobacter* spp., their relative contribution to the total peak height could not be accurately determined.

3.4 Discussion

Groundwater in the Mahomet aquifer falls squarely within the iron-reducing category of [Chapelle et al., 2009], because the water is considerably richer in ferrous iron than dissolved sulfide (Table 3.1). In areas of high sulfate (HS) groundwater, nonetheless, 16S rRNA sequences related to sulfate reducers are nearly as abundant as those related to iron reducers (Figure 3.4). Even in low sulfate (LS) and negligible sulfate (NS) wells, where the availability of sulfate approaches the nominal limit needed to derive energy from sulfate reduction [Lovley and Phillips, 1987], sulfate reducers still comprise a significant fraction of the bacterial community. This suggests that despite the apparent predominance of iron-reducing conditions in the Mahomet, sulfate reduction likely occurs alongside iron reduction.

Sulfate concentration is the primary discriminant of the active bacterial community in the Mahomet aquifer, according to the statistical analyses (Figure 3.2, Table 3.2). Bacterial communities from wells with little sulfate in the groundwater are statistically similar to each other, as are the communities from high sulfate wells. The low-sulfate communities, however, differ markedly from those living in high sulfate water.

Variation in the abundance of iron reducers and sulfate reducers accounts for most of the differentiation among bacterial communities in the Mahomet. Three genera of iron-reducing bacteria, *Geothrix*, *Geobacter*, and *Desulfuromonas*, together comprise more than half of the bacterial community in NS wells, but average only one-quarter to one-third of the total community in LS and HS wells, respectively (Figure 3.4, Table 3.3). Conversely, sulfate reducers classified as *Desulfobacter* and *Desulfobulbus* are more abundant in wells with a

high concentration of sulfate.

This inverse relationship between the relative proportion of iron reducers and sulfate reducers and the concentration of sulfate may arise from competition for electron donors in the aquifer. While the enzymatic reduction of poorly crystalline ferric minerals such as ferrihydrite can yield more energy per mole than sulfate reduction [Lovley et al., 2004], kinetic modeling of microbial respiration indicates the amount of energy available from the reduction of ferric minerals such as goethite is nearly equal to the energetic yield of sulfate reduction [Bethke et al., 2011]. Ferrihydrite is abundant in soils, but is not commonly found in older, deeper aquifers such as the Mahomet [Cutting et al., 2009]. The presence of sulfate alone is not known to inhibit iron reducers directly [Komlos et al., 2008]. Given sufficient substrate, sulfate reducers appear to be able to compete effectively with iron reducers.

In contrast to the results for sulfate, the statistical analyses revealed no clear relationship between the concentration of either ferrous iron or sulfide in groundwater and the structure of the bacterial community in the aquifer (Figure 3.3). This lack of correlation is notable because hydrologists commonly take iron in groundwater as a direct indicator of the importance of iron reduction, and dissolved sulfide to reflect the activity of sulfate reducers. The relative proportion of these ions has been proposed as a means to differentiate iron-reducing conditions from sulfate-reducing conditions on the basis of groundwater chemistry alone [Chapelle et al., 2009], but our results reveal no such relationship.

The simultaneous activity of iron reducers and sulfate reducers observed in the Mahomet may explain the failure of the concentrations of iron and sulfide in groundwater to predict bacterial activity. Ferrous iron and sulfide react together to precipitate as mackinawite (FeS) and greigite (Fe_3S_4) [Gramp et al., 2010; Rickard and Luther, 2007]. The reaction strips the ions from solution in a nearly one-to-one ratio, leaving in solution only the species present in excess of the other. But the respective metabolisms do not supply these chemical species equally. Utilizing the same amount of electron donor iron reducers generate eight times as many ferrous ions as sulfate reducers produce sulfide ions. Where both groups are active, then, groundwater rich in iron and depleted in sulfide may develop even in aquifers where sulfate reduction predominates [Bethke et al., 2008]. Thus gauging the relative importance of iron reduction and sulfate reduction in aquifers like the Mahomet may be possible by

molecular biology assay, but not necessarily through geochemical inference.

Aquifer microbiologists already appreciate the importance of competition in controlling the ecological balance between iron-reducing and sulfate-reducing bacteria. The results of this study suggest the possibility of a mutualistic relationship, the condition under which two organisms benefit from each other's activity [Little et al., 2008]. Ferrous iron reacts with sulfide to form insoluble precipitates, keeping these reaction products from building up in groundwater. The reaction in this way limits product inhibition and maintains the thermodynamic drives for each group's metabolism [Bethke et al., 2011; Jin and Bethke, 2007, 2009]. The precipitation reaction, perhaps more significantly, prevents ferrous ions in the aquifer from building up to the point at which they can inhibit iron reduction by lowering the redox potential of ferric minerals in the aquifer [Larese-Casanova and Scherer, 2007; Williams and Scherer, 2004].

Even though sulfate reducers compete for electron donors in the Mahomet, these bacterial populations may promote the activity of iron reducers. Despite the nominally iron-reducing conditions, sulfate reducers seem to be thriving in the aquifer. Results of this study portray the microbiological zoning of the subsurface in a manner more nuanced and complex than can be appreciated from the theory of exclusionary zoning alone.

3.5 Tables

Table 3.1: Concentrations of sulfate, chloride, sulfide, ferrous iron, and dissolved organic carbon (DOC) in Mahomet aquifer groundwater.

Well[*]	$[\mathbf{SO_4^{2-}}]$[†] **(mM)**	$[\mathbf{Cl^-}]$[‡] **(mM)**	**DOC**[‡] **(mg/L)**	$[\mathbf{HS^-}]$[§] **(µM)**	$[\mathbf{Fe^{2+}}]$[¶] **(µM)**	$[\mathbf{Fe^{2+}}]$:$[\mathbf{HS^-}]$
Chm94A	0.07	0.02	3.58	0.71	20.2	46
Chm94B	0.58	0.02	2.20	1.00	20.2	33
Chm95A	0.14	0.02	2.11	2.56	14.9	10
Chm95B	0.04	0.03	2.02	2.13	12.2	9
Chm95C	0.11	0.28	0.48	0.41	14.9	59
Chm95D	n/d	0.06	1.62	0.61	24.2	65
Chm96A	0.41	0.01	1.25	0.58	14.9	42
Chm98A	n/d	0.73	4.24	0.72	21.5	49
Frd94A	0.98	0.07	< 0.4	0.25	5.1	34
Frd94B	0.05	0.02	< 0.4	n/d	17.2	–
Iro95A	1.50	0.08	3.28	0.75	16.2	36
Iro96A	4.23	0.10	n/a	n/a	n/a	n/a
Iro98B	4.68	0.33	n/a	0.06	28.1	726
Iro98C	0.04	0.85	2.33	0.66	14.9	37
Iro98D	0.72	0.26	1.89	0.82	28.1	57
Ver94A	4.57	0.25	1.83	0.26	21.5	136
Ver94B	10.73	0.14	1.11	n/d	n/d	–
Ver94C	0.23	0.03	1.10	0.21	20.2	154
Ver94D	0.18	0.04	0.85	0.43	n/d	–

[*]Well identification tag given by Illinois State Water Survey.
[†]The detection limit for sulfate and chloride is 0.01 mM
[‡]The detection limit for dissolved organic carbon (DOC) is 0.4 mg L^{-1}
[§]The detection limit for sulfide is 0.15 µM.
[¶]The detection limit for ferrous iron is 1.0 µM.

Table 3.2: Results from analysis of similarity (ANOSIM) comparing bacterial communities grouped together by the concentration of sulfate in local groundwater (HS = high sulfate, LS = low sulfate and NS = negligible sulfate).

groups compared		***Rsa*I**	***Msp*I**
HS – LS	R_{ANOSIM}	0.245	0.170
	p	0.2%	4.3%
HS – NS	R_{ANOSIM}	0.606	0.401
	p	$< 0.1\%$	2.4%
LS – NS	R_{ANOSIM}	0.557	0.422
	p	0.2%	0.9%

Table 3.3: Average percentage of total T-RFLP peak height for specific bacterial taxa in the Mahomet aquifer.

HS	**LS**	**NS**	**Class (ID%)**[‖]	**Genus (ID%)**[**]
5%	11%	13%	Acidobacteria (100%)	*Geothrix* (100%)
2%	3%	8%	Clostridia (100%)	*Fusibacter* (100%)
5%	9%	7%	δ–Proteobacteria (100%)	*Desulfuromonas* (100%)
8%	12%	14%	δ–Proteobacteria (100%)	*Geobacter* (100%)

[‖] As calculated by SIMPER.

[**] Phylogeny and classification identity percentage calculated using the RDP-II Classifier [Cole et al., 2007].

3.6 Figures

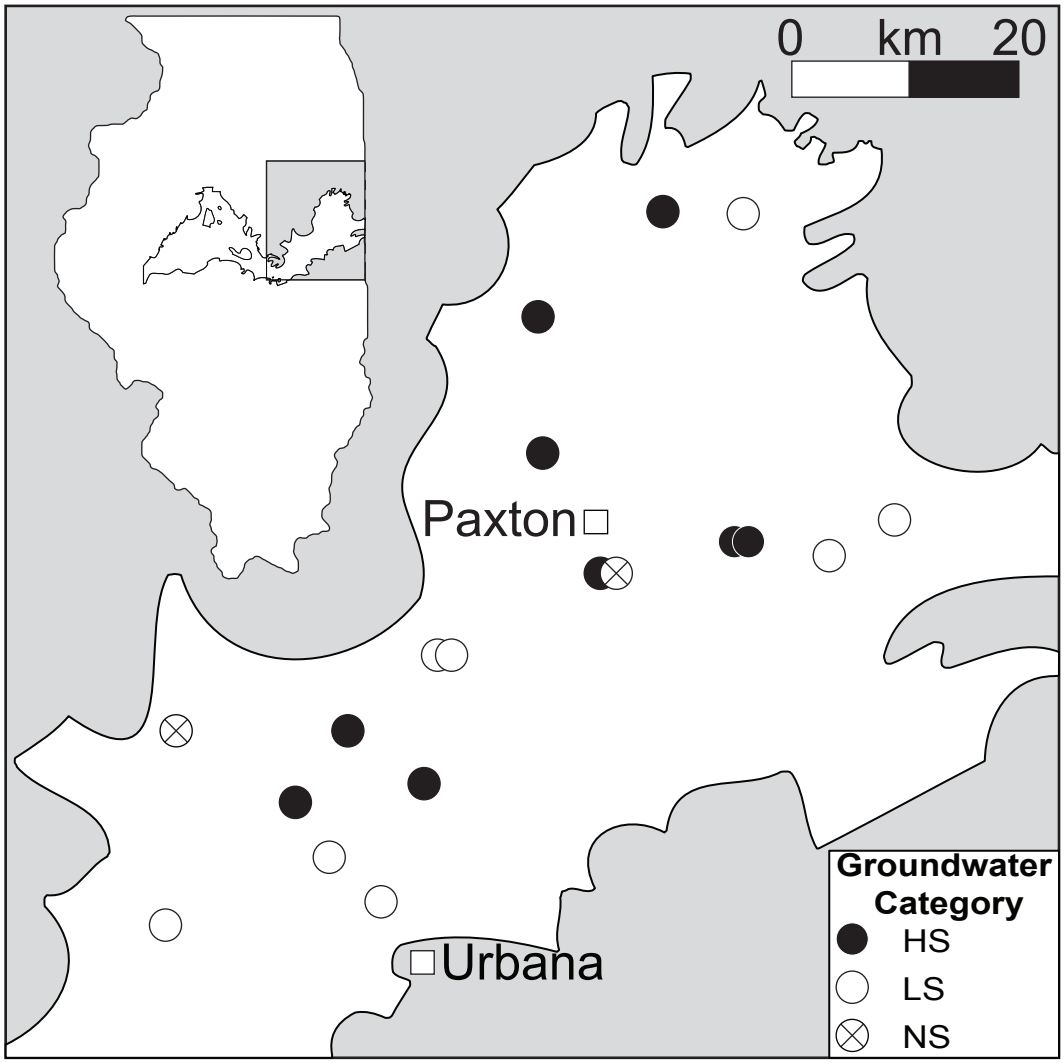

Figure 3.1: A map of the Mahomet aquifer indicating areas of high and low sulfate groundwater. Dots indicate the location of the wells sampled in for this study.

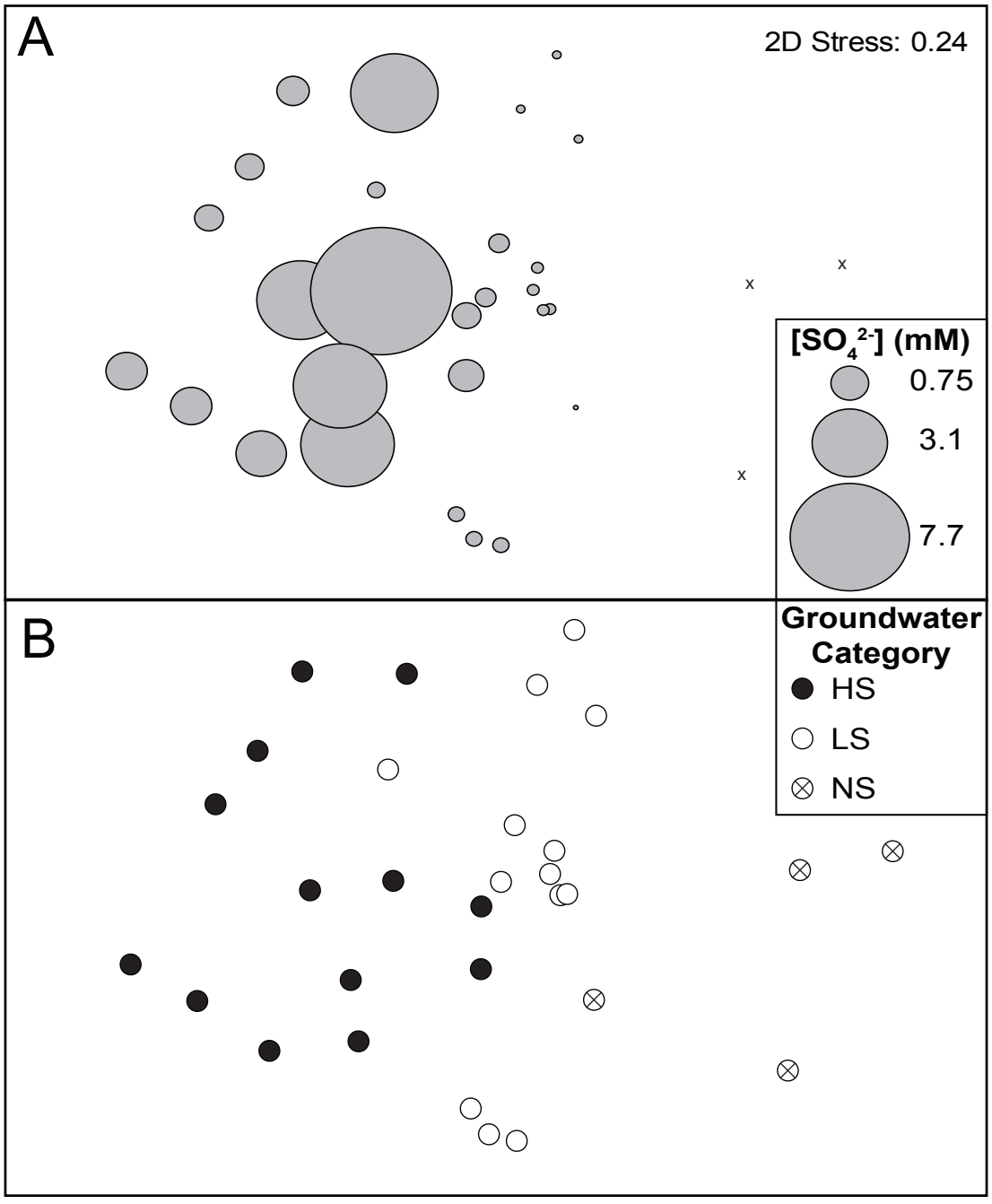

Figure 3.2: (A) Multidimensional scaling (MDS) ordination of the Bray-Curtis coefficients of similarity for active microbial communities in the Mahomet aquifer. The stress indicated in the upper right corner is the amount of strain imposed on the ordination when fitting it into two dimensions. The size of the circle representing a particular community is proportional to the concentration of sulfate in groundwater from the well where that sample was taken. (B) The same MDS ordination with each community labelled according to the concentration of sulfate in groundwater at the well from which the sample was taken. The amount of sulfate in HS wells is > 0.2 mM, is between $0.3 - 0.2$ mM in LS wells, and is less than 0.03 mM in NS wells.

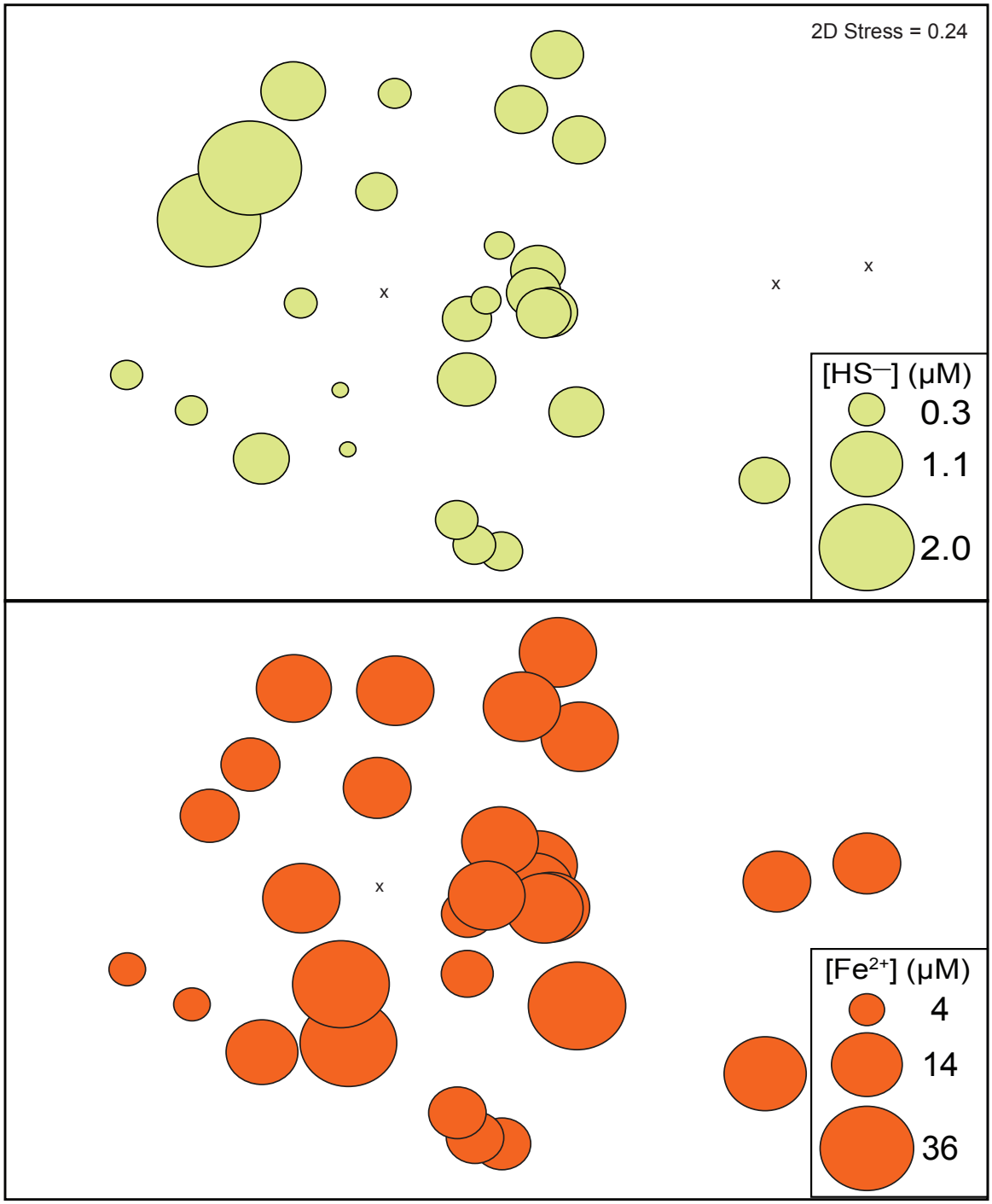

Figure 3.3: Non-metric multidimensional scaling (MDS) ordinations of the Bray-Curtis coefficients of similarity in the Mahomet aquifer. The size of a circle is proportional to the concentration of sulfide (top) or ferrous iron (bottom) in groundwater from the well where the community being represented was taken. No measurable sulfide or ferrous iron was detected in communities marked with by an "x." Stress indicated is the amount of strain required to fit the ordination into two dimensions

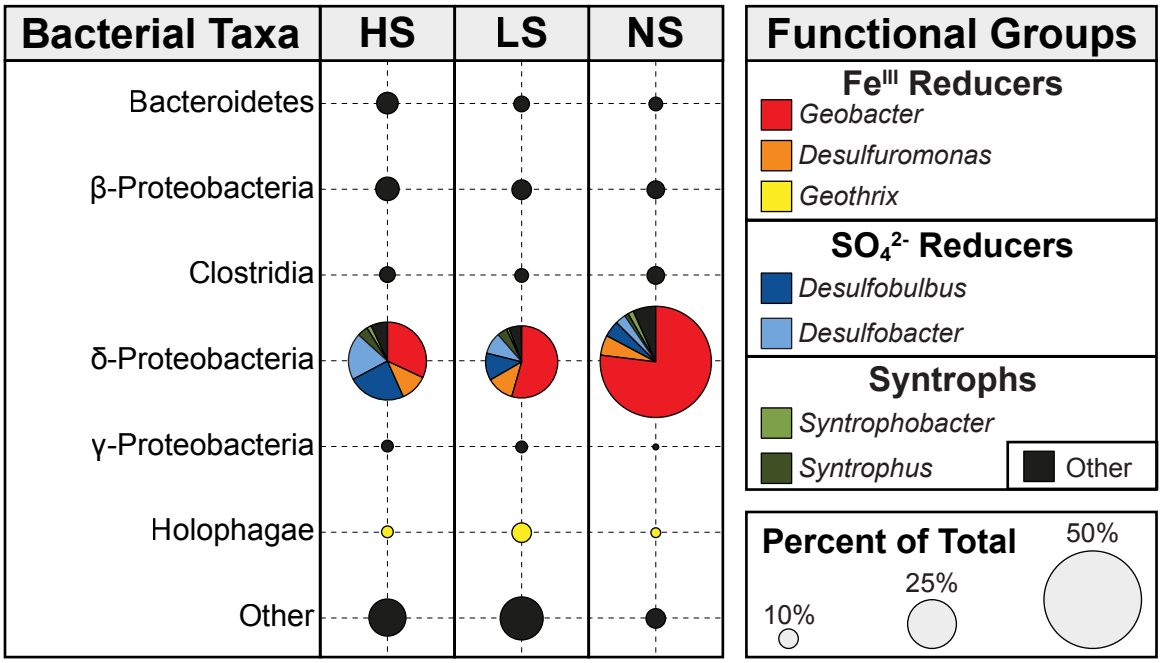

Figure 3.4: Taxonomy and relative distribution of bacterial populations attached to the sediment of in situ samplers. Sequences were classified to the genus level in Mothur [Schloss et al., 2009] according to the Greengenes taxonomic nomenclature [DeSantis et al., 2006]. The area of each circle is proportional to the percentage of sequences represented by that class within those wells, which are grouped together according to the concentration of sulfate These groups are designated HS, LS, and NS. The amount of sulfate in wells designated HS is > 0.2 mM, between $0.03 - 0.2$ mM in LS wells, and less than 0.03 mM in NS wells.

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CHAPTER 4

RESILIENCE OF MICROBIAL COMMUNITIES FOLLOWING LONG TERM EXPOSURE TO HIGH- AND LOW-SULFATE GROUNDWATER

4.1 Introduction

Microbial life thrives in nearly every environment on Earth, including subsurface sediments and groundwater. Many subterranean bacteria and archaea, which are thought to comprise nearly 95% of Earth's total biomass [Nielsen and Fisk, 2010], respire chemolithotrophically. Rather than breathing molecular oxygen, these microorganisms respire by reducing electron acceptors such as sulfate and ferric iron to sulfide and ferrous iron, respectively [Chapelle, 2001]. In this way, microbial metabolism directly controls the solubility of metals in groundwater as well as the chemical weathering of saturated sediments [Bethke, 2008].

While microbes catalyze many important biogeochemical reactions, the composition of microbial communities is in turn controlled by the availability of particular nutrients and substrates. Sulfate reduction, for example, cannot occur in sulfate-free groundwater, and the extent of iron reduction is controlled by the availability of enzymatically-reducible ferric iron. The presence or absence of these particular substrates is of particular importance to the fate and transport of metals in groundwater. Active iron reduction releases arsenic that has sorbed to aquifer sediments in the absence of sulfate [Kocar and Fendorf, 2009], while concurrent sulfate reduction can sequester arsenic through co-precipitation with iron sulfide minerals [Kirk et al., 2004]. Other studies show that because iron reduction generates considerably more hydrogen ions than sulfate reduction, the balance between the two processes largely determines the extent of chemical weathering of aquifer sediments [Park et al., 2009]. As a result, knowing the extent to which either process is active is essential to understanding aquifer biogeochemistry [Borch et al., 2010; Weiss and Cozzarelli, 2008].

Because the chemical composition of groundwater and aquifer sediment plays such a

critical role in determining the extent of microbial activity, there is considerable interest in understanding how microbial communities respond to changes in their environment. A recent survey of studies examining how microbial communities respond to external disturbances found the addition of inorganic nutrients such as nitrogen or phosphorus caused the largest perturbation in community composition [Allison and Martiny, 2008]. For example, studies of agricultural soil in Michigan have shown that the composition of the microbial community continues to be impacted by crop cultivation and fertilization even after fields have been left fallow for decades [Buckley and Schmidt, 2001]. An examination of denitrifying bacteria in salt marsh sediments, in contrast, found no change in the abundance or diversity of these organisms despite the biweekly application of nitrate over several decades [Bowen et al., 2011]. Few studies exist that examine the resilience of microbial communities in aquifers when faced with environmental change and the impact of those changes on the functioning of the community remain only poorly understood [Botton et al., 2006].

Using sediment traps to evaluate the composition of attached microbial community we showed that the availability of sulfate controls the distribution of functional groups of bacteria in the Mahomet aquifer of central Illinois (see Chapter 3). There, the relative abundance of 16S rRNA sequences related to known iron reducers such as *Geobacter* exceeds that of sequences related to sulfate reducers such as *Desulfobacter* in areas of the aquifer where groundwater contains less than 0.2 mM sulfate. When the amount of sulfate exceeds this amount, however, sequences from each functional group are detected in nearly equal abundance. In the current study, we examine the resilience of the composition of the bacterial community in response to changes in the concentration of sulfate.

To test this, sterile sediment traps were placed for one year within two wells, one with a high concentration of sulfate (1.5 mM) and the other a low concentration of sulfate (0.04 mM). After a year one trap from each well was placed in the other. These switched traps remained in the new wells alongside control traps, which were not switched, for another year. We hypothesized that the microbial community of the switched traps would change to become more similar to the community found in the characteristic high or low sulfate groundwater. Using molecular techniques and multivariate statistical analyses we found that the community composition did not change, however bacterial groups associated with

sulfate reduction were found to increase or decrease along with the concentration of sulfate.

4.2 Materials and Methods

4.2.1 Deployment of In Situ Microbial Samplers - "Bug Traps"

Two wells in the east-central Mahomet aquifer were selected for this study based on the sulfate concentration of the groundwater. The low sulfate well (hereafter designated $LoSO_4$) contained 0.04 mM sulfate (Table 4.1), placing it in the LS group of wells discussed in Chapter 3. The high sulfate well used in this study, hereafter referred to as $HiSO_4$, contained groundwater with a sulfate concentration of 1.5 mM, placing in in the HS group of wells. Groundwater flow in this part of the aquifer is slow, < 1 m yr^{-1} [Kempton et al., 1991], and repeated measurements of sulfate and other ions in nearby wells using passive diffusion samplers showed concentrations to be stable over a period of six months [Kyrias, 2010].

Sediment used for constructing the bug traps was obtained during the drilling of a well at a private residence located in McLean County, Illinois. Glacial outwash sand and gravel from the Banner formation of the Mahomet aquifer was collected prior to completion of the drilling operation. Containers used to store this sediment were filled completely with water to minimize contact with the atmosphere and stored on ice. Sediments were returned to the lab within several hours, where they were immediately washed in order to remove drilling mud and minimize re-oxidation of any ferrous iron sorbed to mineral surfaces. The washed sediment was then autoclaved at 121℃. Samplers were constructed by placing 40 g of sediment in a $4'' \times 8''$ AquaClear 20 mesh bag (Doctors Foster and Smith™, CD-120490, which were then re-autoclaved. After the second round of sterilization, individual samplers were placed inside sterile Whirl-Pak® bags. These bug traps were then stored at –45℃ until they were deployed in the field.

Four identical bug traps were deployed in each well (Figure 4.1). Before deploying a bug trap in a well to be colonized, stagnant groundwater was purged from each well by pumping at a rate of 8 l min^{-1} with a Grundfos® Redi-Flo II electric submersible pump. Bug traps were deployed only after pH, temperature and specific conductivity measurements of the

pumped groundwater had been stable for at least 10 minutes. Traps were lowered until level with the well screen, which was placed over a 5 to 10 foot range at the bottom of well. Traps were then left submerged in flowing groundwater to be colonized by indigenous microbes.

One of the bug traps from each well was removed after three months to compare the initial microbial community composition between the two wells. Two of the remaining three bug traps were removed from each well after being in place for 12 months. To prevent contact with atmospheric oxygen upon removal from the well, a plastic anaerobic glove bag was placed over the well casing, sealed with vinyl anaerobic tape (Coy Laboratory Products Inc, Grass Lake, MI), and purged with oxygen-free argon gas. Sediment from the first trap was placed in a sterile Whirl-Pak® bag and frozen on dry ice until returned to the lab, whereupon they were stored at –80℃ pending analysis. The second trap was placed into a sterilized glass Ball® mason jar filled with filtered, anaerobic groundwater which had been previously pumped from the well. The jar was then placed into a cooler filled with ice (approximately 4℃) and transported to the other well. In this manner, a trap from the $HiSO_4$ well was transferred to the $LoSO_4$ well and vice versa, while the third trap remained in its original well for the duration of the experiment. These traps were left in the wells for an additional 12 months after which they were retrieved and frozen as described above.

4.2.2 Microbial Community Analyses

Total DNA was extracted from sediment traps and filter membranes following the method of Tsai and Olson [1991] with some minor modifications. Cells were lysed by incubating individual filters or 4 g of sediment in 2 ml of lysis solution (15 mg ml^{-1} lysozyme, 0.15 M NaCl, 0.1 M Na_2EDTA, pH = 8.0) at 37℃ for 30 min. Detergent solution (2 ml; 0.1 M NaCl, 0.5 M Tris-Cl, 10% sodium dodecyl sulfate, pH = 8.0) was then added and incubated for 30 min, after which the samples were subjected to three freeze-thaw cycles using liquid nitrogen and a 55℃ water bath. Proteins were removed from the aqueous phase using equal volumes of phenol (pH = 7.8), phenol:chloroform:isoamyl alcohol (25:24:1, pH = 7.8) and chloroform:isoamyl alcohol (24:1). DNA was precipitated overnight by adding an equal volume of isopropanol followed by 10.5 M ammonium acetate to a final concentration of 2.5

M. The nucleic acid precipitate was centrifuged at $9,000 \times g$ for 30 min at 4℃, washed in 70% molecular grade ethanol, and resuspended in Tris-EDTA buffer (pH = 8.0). The amount and quality of DNA recovered was determined using agarose gel electrophoresis. Negative controls consisted of unused filters and sediment traps.

Bacterial primers 8F (5′ - AGAGTTTGATCCTGGCTCAG - 3′) and 787R (5′ - GGACTACCAGGGTATCTAAT - 3′) [Revetta et al., 2011] were used to amplify 16S rRNA genes. Amplification reactions contained 5 U of Ex Taq™ DNA polymerase (Takara Bio USA, Madison, WI), 5 µl of 10X concentrated Ex Taq™ Buffer, 4 µl of a 2.5 mM mixture of dNTPs, 3 µl each of forward and reverse primers (2.0 µM stock concentration), and 2 µl of template DNA (50 µl total volume). Amplification conditions for the bacterial assay included an initial denaturation step of 4 min at 94℃, followed by 35 cycles of 30 sec at 94℃, 30 sec at 56℃, and 1 min at 72℃, with a final extension step of 7 min at 72℃. Amplification products were screened using gel electrophoresis and GelStar™ dye (BioWhittaker Molecular Applications, Rockland, ME).

Community profiles were created from the extracted DNA using terminal restriction fragment length polymorphism (T-RFLP). This molecular technique distinguishes microbial populations within a sample by tagging a PCR-amplified gene with a fluorescent marker, then cutting the gene into fragments using a series of restriction enzymes; the size of a tagged fragment is characteristic of the strain from which the gene was derived [Liu et al., 1997]. To create the fragment profile, the 16S rRNA genes amplified from each well by PCR were digested in three separate reactions using two restriction enzymes (*Rsa*I and *Hha*I) that cut at different nucleotide sequences. The use of two digestions insures that the tagged fragments from phylogenetically distinct microbial populations differ in length [Marsh, 2005].

The 16S rRNA genes were also cloned into pCR4.1 TOPO TA vector following the manufacturer's instructions (Invitrogen™, Carlsbad, CA). Transformed cells were grown on Luria-Bertani agar plates containing ampicillin (100 mg ml^{-1}). Random colonies were screened for the presence of inserts of the correct size using M13 PCR primers and gel electrophoresis. Clones were sequenced using the BigDye® Terminator sequencing chemistry (Applied Biosystems, Foster City, CA) as described by Lu et al. [2008]. Raw sequence data was checked for quality and assembled into contigs using Sequencher® v4.10.1 (Gene Codes Corp, Ann

Arbor, MI), and then screened for chimeric assemblies using Bellerophon [Huber et al., 2004]. For the phylogenetic analyses bacterial and archaeal sequences were aligned using the algorithm implemented in the program Mothur [Schloss, 2009] against a high-quality reference alignment selected from the Greengenes 16S rRNA gene database [DeSantis et al., 2006]. Unique, chimera-free reference sequences were chosen from the 12 October 2010 release of Greengenes using ARB [Ludwig et al., 2004]. Cloned sequences from the Mahomet that aligned poorly to the reference database or contained ambiguous base calls were discarded. The phylogeny of archaeal and bacterial 16S rRNA gene sequences was classified in Mothur using the "gg_norm" taxonomic nomenclature.

The taxonomic richness of clones taken from each well was calculated using mothur [Schloss et al., 2009]. 16S rRNA gene sequences were clustered into operational taxonomic units (OTUs) based on an average nucleotide similarity at fixed cutoffs. Sequences with an average nucleotide similarity of 97% were binned together into a single OTU. Comparisons of community structure between wells based on phylogeny rather than OTUs were also calculated using the weighted implementation of the UniFrac distance metric [Lozupone et al., 2007; Lozupone and Knight, 2005].

The T-RFLP profiles created from each bug trap were compared quantitatively by using the Bray-Curtis similarity coefficient [Bray and Curtis, 1957] in the statistical package Primer-6 (Primer-E Ltd., Plymouth, UK). This metric is calculated between each pair of samples being compared by adding together the lesser height of each T-RF peak that the two communities and useful for comparing T-RFLP profiles [Rees et al., 2004]. Non-metric multidimensional scaling (MDS) was then used to plot differences in community structure as measured by the Bray-Curtis coefficient. MDS graphs are configured such that the distance between any two points is inversely proportional to their similarity. The overall similarity of bug traps originating in either the $HiSO_4$ or the $LoSO_4$ well was calculated using the analysis of similarity (ANOSIM) [Clarke, 1993]. Specifically, R-values (R_{ANOSIM}) were used to establish the dissimilarity of different paired-groups of microbial communities (e.g. communities from no sulfate vs. high sulfate groundwater). SIMPER (similarity percentage) was used to calculate the extent to which individual OTUs contribute to the dissimilarity groups sets and to rank the populations from most to least responsible for the differences

between groups [Rees et al., 2004; Clarke and Warwick, 2001].

4.3 Results

Analysis of similarity (ANOSIM) among the T-RFLP profiles obtained from our sediment traps shows bacterial communities initially incubated in the $LoSO_4$ well are more similar to each other on average than they are to communities originating in the $HiSO_4$ well (R_{ANOSIM} = 0.630, p = 0.0%) (Figure 4.2). Thus sediment traps that remained in the same well for the duration of the experiment, hereafter referred to as "static" traps, maintained a bacterial community composition more similar to that associated with the sediment trap with which they were originally incubated than with the trap that was transferred into that well.

UniFrac measurements of the phylogenetic distance shared by $HiSO_4$ and $LoSO_4$ communities provide further evidence that the switched communities remain more similar to those found in their original well than to those in the subsequent well. The bacteria attached to static sediment traps in the $HiSO_4$ well were more similar to those that colonized the trap transplanted from the $HiSO_4$ to the $LoSO_4$ well (UniFrac = 0.406) than to the trap that was transferred into the $HiSO_4$ well from the $LoSO_4$ well (UniFrac = 0.463). Similarly, the composition of the bacterial community found attached to the static traps in the LS well shares more phylogenetic history with the Lo-to-$HiSO_4$ trap (UniFrac = 0.509) than to the Hi-to-$LoSO_4$ trap (UniFrac = 0. 527).

16S rRNA sequences classified as δ-Proteobacteria, representing both iron- and sulfate-reducing taxa, are the most abundant taxa among the static and switched sediment traps in both the $HiSO_4$ and $LoSO_4$ wells (Figure 4.3). Sequences in this group comprise, on average, 35% of the total community attached to the $HiSO_4$ static traps and 21% of the community attached to $LoSO_4$ static traps. Clones classified as δ-Proteobacteria are of similar abundance in the switched traps, making up 33% of the total in the Hi-to-$LoSO_4$ trap and 36% in the Lo-to-$HiSO_4$ trap. Sequences classified as *Thermodesulfovibrio*, a genus of sulfate-reducing thermophilic bacteria of the class Nitrospira, are also abundant in both the static and transposed traps, although their abundance increases between the one-year and two-year samples for the $LoSO_4$ well and decreases over the same time frame for the

$HiSO_4$ well. *Thermodesulfovibrio* comprise 10% of the clones in the static $HiSO_4$ trap at 12 months but only 2% of the static trap in the same well after 24 months. Conversely, these sequences comprise $< 2\%$ of the total clones attached to the static $LoSO_4$ trap after one year but 9% of the total after two years. *Thermodesulfovibrio* clones are abundant in both transposed traps, representing 11% of the total clones in the Hi-to-$LoSO_4$ trap and 19% of the total in the Lo-to-$HiSO_4$ trap. Other abundant taxa include the β-Proteobacteria and the iron-reducing genera Geothrix. The former represent 13% of all sequences in both the $HiSO_4$ and $LoSO_4$ static traps, while the latter comprise 4% of the total community of each static trap. Both taxa are found in similar abundance in the transposed traps (Figure 4.3).

In each well, sequences classified in genera of known sulfate reducers in the δ-Proteobacteria (*Desulfobacter* or *Desulfobulbus*) are the most abundant functional group (Figure 4.4). These clones average 17% of all static sequences in the $HiSO_4$ well (21% after one year, 14% after two) compared 11% of static sequences in the $LoSO_4$ well (15% after one year, 8% after two). Sulfate reducers comprise 10% of all clones found on the Hi-to-$LoSO_4$ switched trap, and 15% of the Lo-to-$HiSO_4$ trap.

Sequences classified with genera known to be iron reducers were either members of the δ-Proteobacteria (*Geobacter*, *Desulfuromonas*, or *Anaeromyxobacter*) or in the genus *Geothrix* of the *Holophaga* (Figure 4.4). These sequences are of similar abundance in the static traps of each well, representing 11% of all sequences in the $HiSO_4$ traps (13% after one year, 9% after two) and 8% of sequences in the $LoSO_4$ wells (6% after one year, 10% after two). Among the switched traps, these putative iron reducers comprise 4% of the Hi-to-$LoSO_4$ trap and 8% of the Lo-to-$HiSO_4$ trap.

Genera related to known syntrophic δ-Proteobacteria, organisms which produce formate, acetate, and H_2 from long-chain fatty acids and alcohols [McInerney et al., 2008], are more abundant in traps in high sulfate groundwater compared to those in low sulfate groundwater (Figure 4.4). Static traps in the $HiSO_4$ well contain an average of 10% putative syntroph sequences classified as the genera *Syntrophus* or *Syntrophobacter* (14% after one year, 4% after two) and 16% of the the bacterial community attached to the Hi-to-$LoSO_4$ trap is comprised of apparent syntrophs. The $LoSO_4$ well averages only 1% putative syntrophs (2% after one year, $< 1\%$ after two) while the community attached to the Lo-to-$HiSO_4$ trap

contains 5% syntrophs.

4.4 Discussion

Once bacteria have colonized sediment in the Mahomet aquifer, they form a community that resists compositional change despite a 40-fold shift in the concentration of sulfate in groundwater. While previous studies found a discrete difference between the bacterial communities on sediment traps from $HiSO_4$ and $LoSO_4$ wells (Chapter 3), ANOSIM and UniFrac analysis of traps moved from one type of well to the other found little transitional change in community composition even after one year of addition incubation in the subsequent well (Figure 4.2). These switched traps instead retain a community structure that is more similar to that found in their original well. This corresponds with the findings of Bowen et al. [2011], who found that bacterial communities in saltmarsh sediments were similarly resistant to compositional change following the long-term addition of nitrate. These results suggest that once established, many populations within the bacterial community are resistant to changes in the geochemical composition of groundwater.

The relative abundance of some taxa, however, did appear to respond to the change in sulfate concentration (Figure 4.3). In particular, sequences most similar with known sulfate reducers, *Desulfobulbus* and *Desulfobacter*, became more abundant upon being switched from the $LoSO_4$ well to the $HiSO_4$ well (Figure 4.4). In the trap switched from $HiSO_4$ to $LoSO_4$, these same sequences became less abundant. While the proportion of these sulfate reducers decreases between year one and year two in the $LoSO_4$ well static traps, the proportion of sequences related to sulfate reducers in the Lo-to-$HiSO_4$ trap is nearly double that of the $LoSO_4$ static trap (15% vs 8%) sampled at the same time. Sulfate reducers in the $HiSO_4$ well similarly declined between year one and year two, dropping from 21% to 14% of the total bacterial community. For the trap switched from $HiSO_4$ to $LoSO_4$ the proportion of sulfate reducers showed an even greater decline to 10%. Thus while the overall structure of bacterial communities in these switched traps remained more similar to their parent well despite the difference in sulfate, the populations which would be expected to be most sensitive to this change, sulfate reducers, do indeed respond as predicted.

Unlike sulfate reducers, the abundance of iron reducers, the other major functional group in these samples, does not appear to be influenced by the change in the concentration of sulfate (Figure 4.4). This result is somewhat surprising, given that previous research has shown the relative abundance of iron reducers is greater when the concentration of sulfate is low (see Chapter 3). These traps, however, were incubated for 12 and 24 months compared to only 3 months for the traps discussed in Chapter 3. The relatively low abundance of iron reducers in the longer-term traps, then, could be a result of the gradual depletion of available ferric iron. Alternatively, it may be a result of mutualistic cooperation between iron reducers and sulfate reducers. Bioreactor studies of a natural microbial community amended with ferric iron and sulfate showed that following an initially-rapid rate of reduction, iron reducers slowed the production of ferrous iron over time until it reached a one-to-one stoichiometric balance with the production of sulfide [Bethke et al., 2011]. At that point, the amount of sulfide produced is sufficient to precipitate FeS minerals such as mackinawite without allowing ferrous iron to build up in solution, a result that benefits both groups of organisms. And while the abundance of iron reducers was less than that of sulfate reducers despite a greater amount of available energy, the rate of iron reduction in the presence of sulfate reduction still exceeded that of iron reduction without sulfate in a parallel experiment.

The relative abundance of *Thermodesulfovibrio* sequences appears to increase in the $LoSO_4$ static trap only after the $HiSO_4$ trap was transplanted to that well. These sequences are abundant in the $HiSO_4$ well and therefore this result suggests the abundance of *Thermodesulfovibrio* in the transplanted trap may influence the bacterial community of the $LoSO_4$ static trap. *Thermodesulfovibrio* comprised $< 2\%$ of all sequences in the $LoSO_4$ static trap after one year but nearly 10% of all sequences after an additional year of incubation with the trap switched over from the $HiSO_4$ well. This brings the relative abundance of *Thermodesulfovibrio* to nearly the same degree as found in the switched trap (11%). Populations such as *Thermodesulfovibrio* may be able to exist and even grow under $LoSO_4$ conditions, but are perhaps unable to do so without a large enough seed population. This result is similar to findings from bioaugmentation studies of natural microbial communities in an aquifer with the halorespiring bacterium *Dehalococcoides ethenogenes*. Even in contaminated aquifers rich where chlorinated solvents are plentiful, *Dehalococcoides* requires specific nutrient con-

ditions as well as a sufficiently large initial inoculum in order to compete with the native microbes [Becker, 2006].

These results show that, over the two-year timescale of this study, the bacterial community in an aquifer is not necessarily a direct mirror of its geochemical environment. Rather, a community retains the imprint of the forces that drove its initial formation and composition long after local conditions have changed. This finding is similar to what [Bowen et al., 2011] found in salt marsh sediments, even large changes in the availability of nutrients can be insufficient to drive compositional change among microbial communities. Even when environmental changes are great enough to change the structure of a community, as occurs when soils are cultivated by modern agricultural techniques [Buckley and Schmidt, 2001], the imprint of those changes lasts long after fertilization ceases. As appears to be the case with *Thermodesulfovibrio*, some populations can thrive in areas where they are not already abundant if added there in sufficient abundance. Thus rather than being "ecological species," defined by Cohan as a set of organisms that are functionally identical in all relevant ecological respects [Cohan, 2002], certain groups of bacteria may exhibit allopatric speciation in that they are endemic to a particular place [Cho and Tiedje, 2000; Whitaker, 2006; Whitaker et al., 2003]. Therefore instead of simply being comprised of whichever populations are ecologically favored in a particular niche, the active members of a microbial community are in part determined by its biogeographical and geological history over space and time.

This contrasts with the oft-cited explanation of the cosmopolitan nature of microbial communities, "everything is everywhere, but the environment selects" [de Wit and Bouvier, 2006; Baas Becking, 1934]. This hypothesis has come to imply that the diversity of microbial populations seen in most environments represents a well of genetic possibilities, where the most abundant populations are those best adapted to the conditions at the time of sampling. Our results suggest that while some populations clearly respond to environmental changes (i.e. sulfate reducers), the community composition as a whole must be viewed in the context of its natural history as well as its current environmental conditions. Our finding that despite 12 months of incubation in a well with a different geochemical composition, the switched traps remain more similar to their parent wells shows the importance of biogeographical context in interpreting microbial communities as products of a dynamic environment through

time and space. While a particular environment must be hospitable in order for any species to thrive, the abundance of some taxa, as appears to be the case here with *Thermodesulfovibrio*, may be as much of a result of historical events as environmental changes. Additional experiments that test the plasticity of microbial communities in aquifers over broad spatial and temporal scales, however, are necessary to quantify the role of either of these factors.

While the overall community structure appears resistant to the change in groundwater chemistry, the populations whose abundance does shift are not "functionally redundant" as is often suggested in ecological models of microbial activity [Allison and Martiny, 2008; Schimel, 2001]. For example, sulfate reducers cannot reduce ferric iron enzymatically, nor can iron reducers grow by reducing sulfate [Lovley et al., 2004]. While the extent to which an increase in the relative abundance of cloned sequences from a particular functional group corresponds to an increase in the rate of that particular biogeochemical process is poorly understood [Crawford et al., 2005; Torsvik et al., 2002], changes in the relative abundance of iron reducers and sulfate reducers in an aquifer could yield significant environmental effects. For example, iron reduction has been shown to drive the chemical weathering of alumino-silicate minerals in the Middendorf aquifer of South Carolina while sulfate reduction does not [Park et al., 2009]. And while iron reduction drives a net release of arsenic from aquifer sediments, concurrent sulfate reduction re-sequesters them as ferrous arsenosulfide minerals [Kirk et al., 2004]. This is of particular importance when considering the impact of geological sequestration of carbon dioxide, which is predicted to decrease the solution pH and thus increase the amount of energy available to iron reducers but not sulfate reducers [Kirk, 2011]. In this case, leakage of CO_2 into overlying aquifers could lead to an increase in iron reduction relative to sulfate reduction and the subsequent release of heavy metals into drinking water. We therefore suggest that changes in microbial community structure should be taken into account when calculating the impact of environmental change on ecosystem function. Further inquiry is needed in order to further elucidate the factors that control shifts in the abundance of these organisms, as well as the effect allopatric speciation may have on the biogeochemical function of microbial communities.

4.5 Tables

Table 4.1: Chemistry of groundwater in the high- and low-sulfate wells chosen for incubating the bug traps.

	$LoSO_4$	$HiSO_4$
Well Name	**Chm95B**	**Iro95A**
T (°C)	13.8	14.3
pH	7.4	7.3
$[\mathbf{SO_4^{2-}}]$ (mM)	0.04	1.50
$[\mathbf{Cl^-}]$ (mM)	0.03	0.08
$[\mathbf{HS^-}]$ (µM)	2.1	0.8
$[\mathbf{Fe^{2+}}]$ (µM)	12	16
DIC (mM)	7.9	–
DOC (mg l^{-1})	2.0	3.3

4.6 Figures

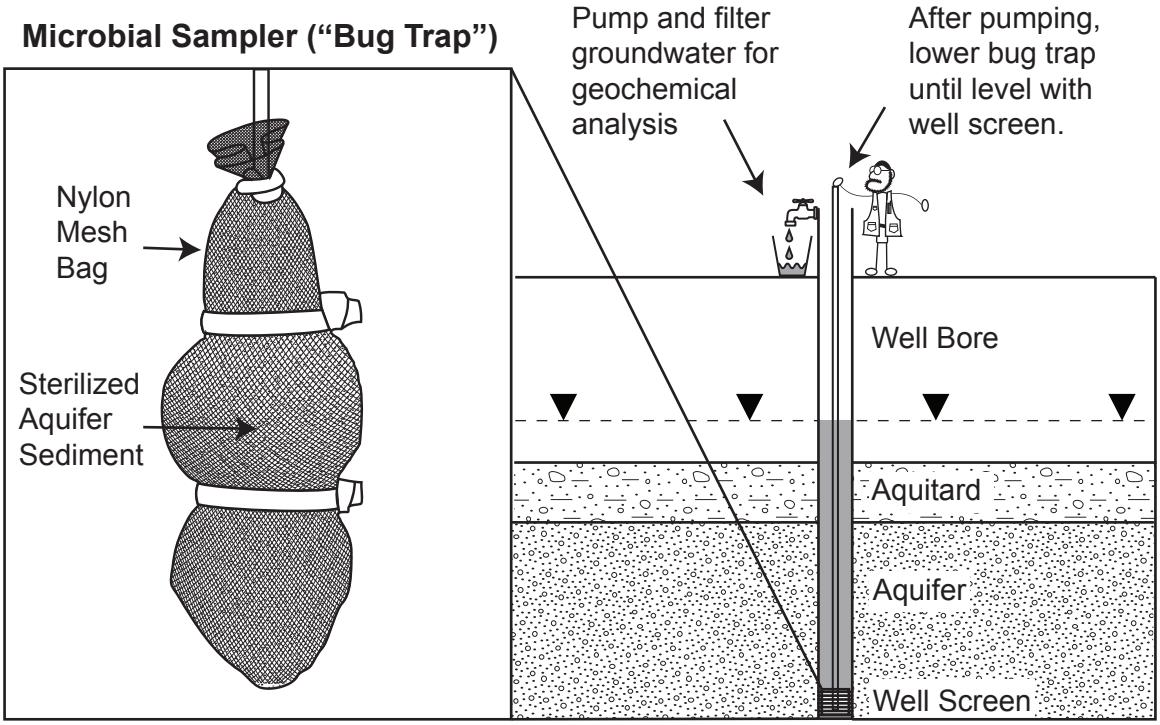

Figure 4.1: Diagram showing method for deploying in situ microbial samplers ("bug traps") within a well. Inset diagram shows how traps were constructed. Each trap consists of Mahomet aquifer sediment sterilized in an autoclave and placed inside a nylon mesh bag.

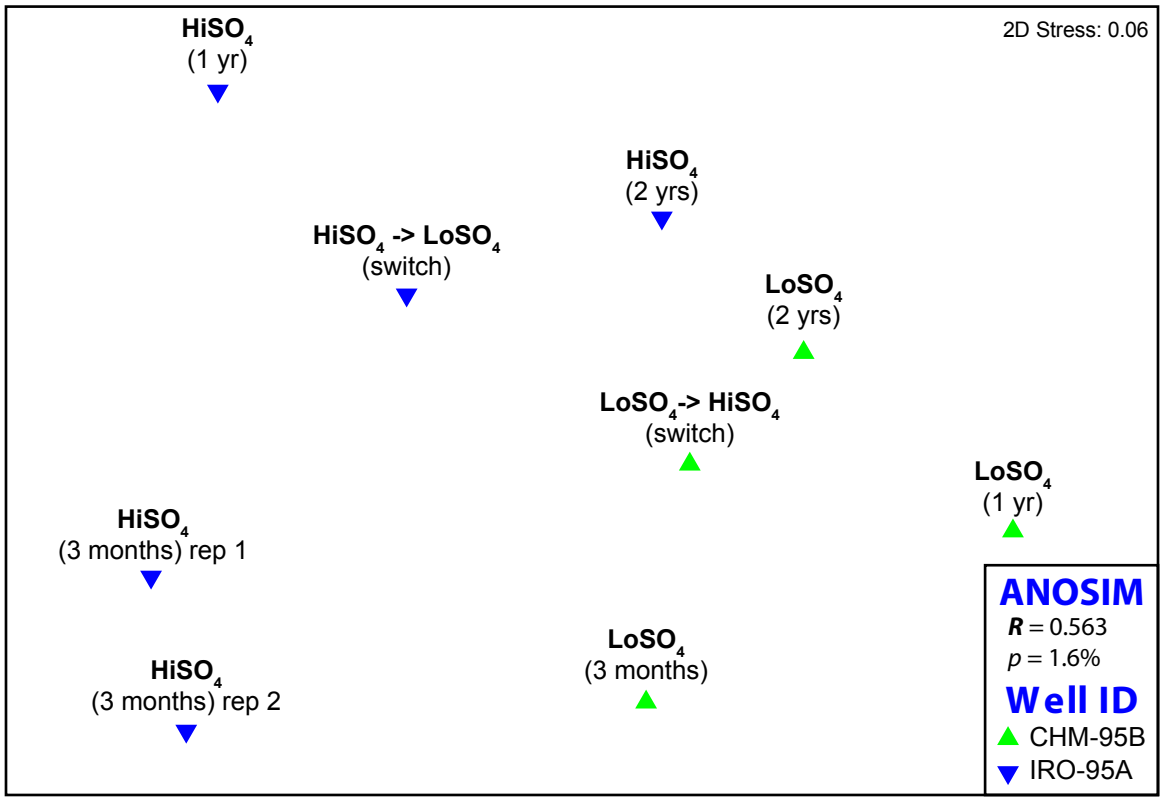

Figure 4.2: Multidimensional scaling (MDS) ordination of the Bray-Curtis coefficients of similarity for T-RFLP profiles of bacterial communities in the Mahomet aquifer. Diagram compares separate in situ microbial samplers (bug traps) from two wells (HS and LS) which were incubated for 3 months, 12 months, and 24 months. Samples indicated "switch" were moved from the well in which they were first incubated into the other.

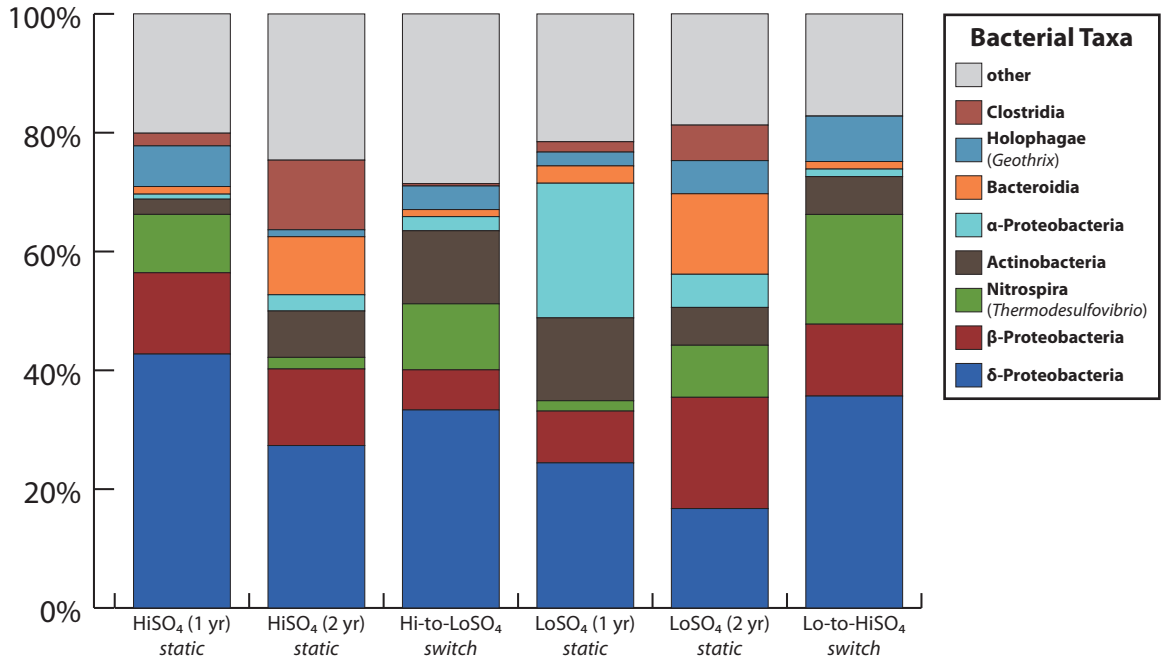

Figure 4.3: The taxonomy and relative distribution of bacterial populations attached to the sediment of in situ sediment traps. The relative abundance of taxa (out of 1,322 sequences analyzed) from traps which remained in the same well for the duration of the experiment have been averaged together. Sequences were classified to the genus level in Mothur [Schloss et al., 2009] using the gg_norm taxonomic nomenclature used by Greengenes[DeSantis et al., 2006].

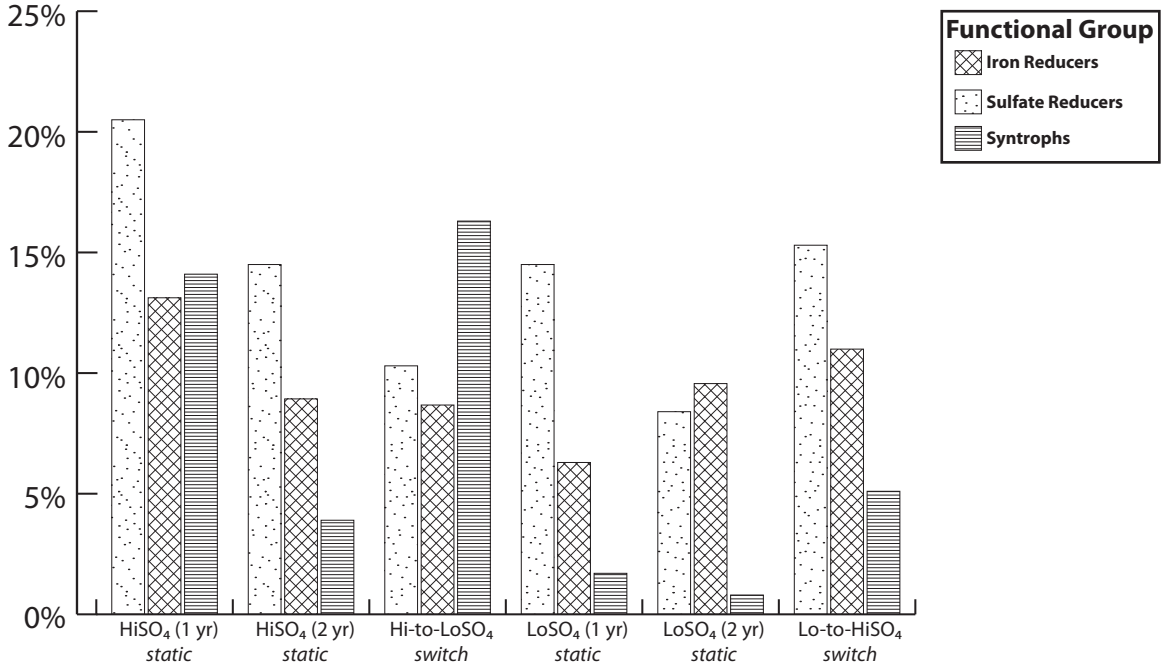

Figure 4.4: The relative abundance of sequences belonging to taxa correlated with the functional groups of sulfate-reducing bacteria, iron-reducing bacteria, and syntrophic bacteria. The sulfate reducers include clones classified as the genera *Desulfobacter* or *Desulfobulbus*. The group labeled iron reducers is comprised of sequences of the genera *Geobacter*, *Desulfuromonas*, *Anaeromyxobacter*, and *Geothrix*. The syntroph group contains sequences from the genera *Syntrophus* and *Syntrophobacter*.

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