



Attached and suspended microbial communities in a pristine confined aquifer

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[1] We compare the community of microbes attached to the sediments in a pristine confined aquifer to the free-floating community suspended in the groundwater there. We sampled the attached microbial community at 19 wells completed in the glacial Mahomet aquifer in east central Illinois using in situ samplers, and we sampled the suspended community by filtering microbes from groundwater. At each well, we profiled the two communities using terminal restriction fragment length polymorphism and compared the profiles we obtained with multivariate statistical analyses. Some populations at a well are detected both in the attached and suspended communities, but the shared populations represent, on average, only one third of each community; the remaining populations are detected exclusively in one community or the other. Clones closely related to the iron-reducing bacteria *Geobacter* and *Geothrix* represent more than 20% of the total attached community detected at many wells, but at no well do they make up more than 1% of the suspended community. To fully characterize the microbial community in an aquifer, it may be necessary to sample the attached as well as suspended communities.

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1. Introduction

[2] Microbes have been observed in the terrestrial subsurface since the invention of the microscope, but only in the last 30 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.

[3] 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 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].

[4] 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 and Das, 2002]. And some microbes may simply attach themselves to solid surfaces more securely than others.

[5] 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

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to the level of the 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. And 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.

[6] Several previous studies have compared attached and suspended communities in the field by 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; *Kölbl-Boelke et al.*, 1988; *Lehman et al.*, 2001] or by measuring uptake rates of a radio-labeled 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].

[7] Various nonculture-based techniques [*Marsh*, 2005; *Olsen et al.*, 1986; *Tedje 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.*, 2004, 2001], 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*, 2005; *Panno et al.*, 1994].

2. Materials and Methods

2.1. Sample Collection

[8] 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, 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 were placed in ice. Upon return to the laboratory, the sediment was immediately washed to remove drilling mud and to minimize the reoxidation of any ferrous iron sorbed to mineral surfaces. The washed sediment was then autoclaved at 121.1°C. We constructed the samplers by placing 110 g of wet sediment in a 4 × 8 inch AquaClear 20 mesh bag (Doctors Foster and Smith™, item CD-120490), which we secured with construction-grade nylon mason line (Lehigh®, item BNT1812YRL). Samplers were then stored at –45°C until use.

[9] 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°C pending analysis.

2.2. Genetic Analysis

[10] We extracted high-molecular weight genomic DNA from both samplers and filters with a bench top 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°C in a lysis solution (0.15 M NaCl and 0.1 M Na₂ ethylenediaminetetraacetic acid (EDTA) (pH = 8.0)) with 15 mg lysozyme per mL, followed by a second incubation in a solution of 0.1 M NaCl, 0.5 M Tris-Cl (pH = 8.0), and 10% sodium dodecyl sulfate. After freeze-thaw cycling between a 55°C 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 and 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.

[11] 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'-AGA GTT TGA TCC TGG CTC AG-3') with the universal reverse primer 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). Primers targeting the domain Archaea failed to produce amplification in our samples. Polymerase chain reaction (PCR) amplification was performed in 100 μ L reaction volumes (20 μ L TaqMaster™ PCR enhancer, 10 μ L TaqBuffer™ (500 mM KCl, 100 mM Tris-Cl (pH = 8.3), and 15 mM Mg(OAc)₂), 8 μ L of dNTPs (dATP, dTTP, dCTP, and dGTP all at 10 mM), 10 μ L each forward and reverse primer (4 μ M), 0.50 μ L Taq polymerase (Eppendorf MasterTaq®), 4 μ L of template genomic DNA per tube, and 37.5 μ L of HyPure™ Molecular Biology Grade water). Amplification began with a denaturing hot start of 4 min at 95°C and was followed by 30 cycles of the following: 30 s of denaturing at 94°C, 30 s for primer annealing at 50°C, and 90 s for extension at 72°C. The PCR was completed with a final extension step at 72°C for 5 min. 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.

[12] 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.

[13] 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].

2.3. Community Comparison

[14] 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 semiquantitative characterization of the diversity of a microbial community [Bent *et al.*, 2007; Blackwood *et al.*, 2007; Fierer *et al.*, 2007; Fierer and Jackson, 2006]. In this study, we are concerned not with determining diversity but with 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, 2008; 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, nonethe-

less, allow the importance of major populations to be compared among samples [Blackwood *et al.*, 2007; Dunbar *et al.*, 2000].

[15] 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 is 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 is equal to one for identical samples.

[16] After calculating the Bray-Curtis similarity coefficient for each pair of samples, we ranked the coefficients in decreasing order and created a plot using multidimensional scaling (MDS), a nonmetric configuration in which the distance between any two points on the plot is inversely proportional to their similarity. All Bray-Curtis similarity coefficients calculated in this study can be found in the auxiliary material.¹ The software then compresses the multidimensional plot into two dimensions, seeking the orientation that best represents the matrix of similarity coefficients [Kruskal, 1964]. Statistical analysis was performed using the Primer-6 software package (Primer-E Ltd., Plymouth, United Kingdom).

[17] We used the analysis of similarity (ANOSIM) method to quantify the differences at each well between the composition of the attached and suspended microbial communities. 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 that there is no difference between two groups by calculating *R* for 10⁶ 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].

[18] We used the similarity percentage (SIMPER) 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 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

¹Auxiliary material data sets are available at <ftp://ftp.agu.org/apend/wr/2007/wr006633>. Other auxiliary material files are in the HTML.

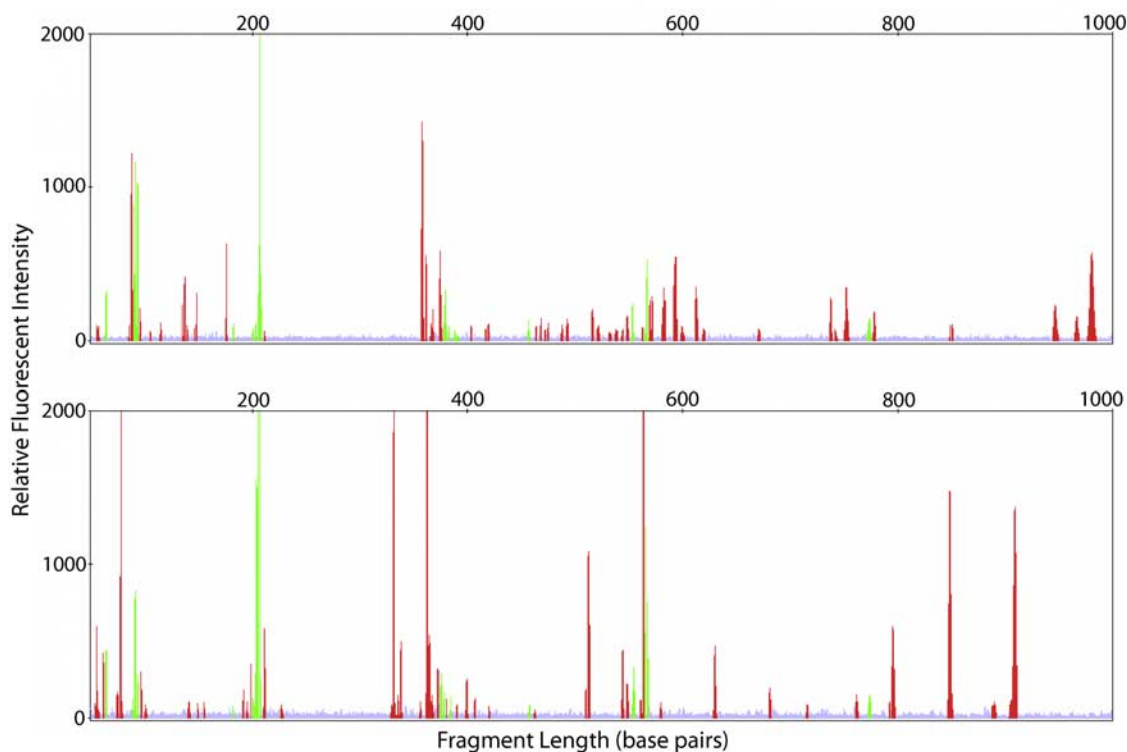


Figure 1. T-RFLP profiles of the (top) attached and (bottom) suspended communities from a single well (VER-94B). Green peaks represent T-RFs detected in both the attached and suspended communities, whereas red peaks show T-RFs detected in only one or the other.

attached community (T. M. Flynn et al., Relating groundwater geochemistry and microbial community composition in the Mahomet aquifer, manuscript in preparation, 2008) using the computer program Cleaver [Jarman, 2006]. By calculating the terminal fragment size of each clone in the library, we were able to classify the phylogeny of some of the dominant terminal fragments identified in the SIMPER analysis.

3. Results

[19] 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.

[20] 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 1). Figure 1 shows results for a single well but in a qualitative sense is representative of all. (Complete data for each of the T-RFLP profiles considered in this study can be found in the auxiliary material.) 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 $\pm 6\%$) of the populations at each well were detected in both communities. Shared T-RFs represent 36% ($\pm 20\%$) of the total peak height for the attached communities and 38% ($\pm 17\%$) 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.

[21] 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 significantly less than the average similarity within the attached (0.302) or suspended (0.299) communities from different wells. The MDS plot (Figure 2a), furthermore, shows systematic differences between the attached and suspended microbial communities. (Labeled MDS plots for all three restriction digests can be found in the auxiliary material.) The stress of ordination on these plots ranges from 0.13 to 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.

[22] 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

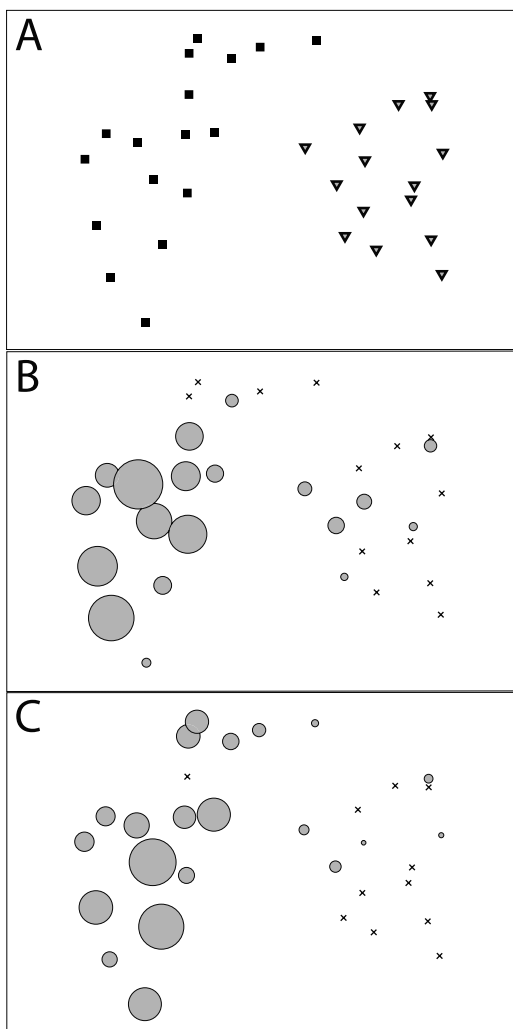


Figure 2. (a) Multidimensional scaling ordination of Bray-Curtis similarity coefficient rankings for attached (squares) and suspended (triangles) microbial communities. Stress, the amount of strain imposed by fitting a multidimensional ordination into two dimensions, is equal to 0.15. (b and c) Same ordination as Figure 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 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 2c are similarly representative of a clone belonging to the genus *Geothrix*. Communities in Figures 2b and 2c in which the *Geobacter* or *Geothrix* T-RF was not detected are indicated by a cross.

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 *Geobacter* and *Geothrix* 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 2b and 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 are 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.

4. Discussion

[23] 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.

[24] 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 excluded from sediment surfaces by competition from 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 or stable isotope probing 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.

[25] 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.

[26] For example, geochemical studies [Kelly *et al.*, 2005] 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. Microbiological study of the aquifer relying 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 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.

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