Using stable isotope probing to characterize differences between free-living and sediment-associated microorganisms in the subsurface

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ABSTRACT

Aquifers are subterranean reservoirs of freshwater with heterotrophic bacterial communities attached to the sediments and free-living in the groundwater. In the present study, mesocosms were used to assess factors controlling the diversity and activity of the subsurface bacterial community. The assimilation of ^{13}C, derived from ^{13}C-acetate, was monitored to determine whether the sediment-associated and free-living bacterial community would respond similarly to the presence of protozoan grazers. We observed a dynamic response in the sediment-associated bacterial community and none in the free-living community. The disparity in these observations highlights the importance of the sediment-associated bacterial community in the subsurface carbon cycle.

INTRODUCTION

The subsurface is a large habitat for microorganisms, yet the environment has generally been understudied because of the difficulties associated with accessing the subsurface. Groundwater is present in the pore space between subsurface sediments and can contain high nutrient concentrations (Valiela et al. 1990; Moore 1999). Furthermore, the chemical composition of groundwater changes during interactions with subsurface sediments and through mixing with other water sources (Charette and Sholkovitz 2006; Beck et al. 2007). Both groundwater and sediments are potential subsurface microbial habitats which have distinct bacterial communities (this study, Lehman et al. 2001; Flynn et al. 2008). The impact of these differences on carbon cycling in sediments versus groundwater systems is not well understood.

Bacterial cells, small eukaryotes, and viruses are all components of the subsurface microbial community (Goldscheider et al. 2006). Descriptions of prokaryotic (Shi et al.

1999; Griebler and Lueders 2009) and eukaryotic (Novarino et al. 1997; Valster et al. 2009) diversity in aquifers are based on microscopic observations, culturing experiments, and identifications from phospholipid fatty acids or small subunit rRNA gene libraries. There is spatial and temporal variability in prokaryotic diversity within both pristine and contaminated aquifers (Haack et al. 2004; López-Archilla et al. 2007), and microorganisms found in the subsurface are also found in other ecosystems (Griebler and Lueders 2009).

Previous research in groundwater microbial ecology indicates that the bacterial community is not passively transported through the subsurface but can participate in subsurface biogeochemical cycles (Ghiorse and Wilson 1988; Madsen and Ghiorse 1993). Bacterial cells grow in aquifer sediments (Chapelle et al. 1987) and there is temporal variability in bacterial biomass (Velasco Ayuso et al. 2009). Groundwater microorganisms also utilize a variety of organic substrates and the patterns in substrate utilization are spatially heterogeneous (Madsen and Ghiorse 1993; Pedersen et al. 2008; Velasco Ayuso et al. 2009). Finally, geochemical evidence of organic carbon consumption, denitrification, and production of carbon dioxide, methane or sulfide (Chapelle and Lovley 1990; Baker et al. 2000; Routh et al. 2001) as well as the presence of different functional genes (Griebler and Lueders 2009) indicate that a variety of bacterial metabolisms exist in the subsurface.

The use of isotopically-labeled compounds, or stable isotope probing, is one method that allows researchers to identify metabolically-active cells within a microbial community (Boschker et al. 1998; Dumont and Murrell 2005). Previous groundwater research has examined the assimilation of acetate within an aquifer contaminated with

hydrocarbons and found that only a subset of groundwater microorganisms in these systems are metabolically active vis-à-vis acetate assimilation (Pombo et al. 2002; Pombo et al. 2005). One limitation in using stable isotope probing is that metabolically-active cells which do not assimilate the labeled compound are incorrectly assigned to the "inactive" category. Nonetheless, even with these methodological limitations, the use of isotopically-labeled compounds has elucidated the role of specific microbial groups in substrate remineralization (see for example: Radajewski et al. 2002; Padmanabhan et al. 2003).

As methods for assessing microbial diversity have matured, microbiologists have been able to explore the factors in different ecosystems that control and structure bacterial community composition. Bacterial mortality due to protozoan grazers can have a major impact on bacterial community composition (Jürgens and Matz 2002; Nagaosa et al. 2008). The presence of grazers can also control bacterial growth in aquifer sediments (Mattison et al. 2002; Nagaosa et al. 2008), although the magnitude of this effect varies for different experimental systems (DeLeo and Baveye 1997). Finally, field studies have shown that the presence of grazers can increase the remineralization and mobilization of anthropogenic contaminants (Madsen et al. 1991; Kinner et al. 2002; Tso and Taghon 2006). Although grazers are acknowledged to be important in subsurface ecosystems, we still lack knowledge about the impact of grazers on metabolically-active bacterial cells.

The present project examined sediment-associated and free-living microorganisms in the subsurface. Mesocosms were used to mimic in situ conditions and we used stable isotope probing to characterize the microbial community involved in the assimilation of ^{13}C-acetate or its metabolic byproducts. Our results revealed that the free-living bacterial

84 community was passively transported through the subsurface. In contrast, the sediment-
85 associated microorganisms altered their community composition in the presence of
86 protozoan grazers.

87 ## MATERIALS AND METHODS

88 ### Experimental setup and sampling strategy

89 The mesocosms used in the present project were 25 cm high x 7 cm wide cylinders
90 based on a design by DeFlaun et al. (2001). The cylinders were filled with sediment
91 collected from the Waquoit Bay National Estuarine Research Reserve (Cape Cod, MA,
92 USA) which had been autoclaved for one hour, allowed to cool, and then autoclaved for
93 an additional two hours. The cylinders were setup in an unheated garage immediately
94 adjacent to the well used to sample the groundwater. Groundwater was pumped through
95 polyethylene tubing lined with fluorinated ethylene propylene from 2.4 m below the
96 surface using a peristaltic pump. Once at the surface, the groundwater traveled through
97 three meters of tubing protected from light in insulated sheaths until the water reached the
98 bottom of each cylinder. A temperature sensor was placed in-line immediately before the
99 groundwater was divided into the tubing used for each individual cylinder. The flow rate
100 through each cylinder was 30 ml hr^{-1} which resulted in an 8-hour residence time for the
101 groundwater within each cylinder. For half of the cylinders, protozoan grazers were
102 removed by filtering the groundwater through a 1 μm filter (Polycap 36 AS filter,
103 Whatman Inc. Florham Park, NJ). The other half of the cylinders received whole
104 groundwater with the microbial community intact. Groundwater flowed through the
105 sediment-filled cylinders for one month prior to the onset of the experiment.

Cylinders received either ^{12}C-acetate (Fisher, enzyme grade), uniformly-labeled ^{13}C-acetate (99% $^{13}CH_3$-^{13}COOH, Cambridge Isotope Laboratories, Andover MA), or no acetate. For the cylinders receiving acetate, a peristaltic pump pulled acetate from stock bottles and merged it with the flow of groundwater so that the concentration of acetate in the groundwater was 200 μM for the first 11 days of the experiment. On day 30 and day 38 of the experiment, concentrated acetate was injected directly into the base of each cylinder due to a problem with the peristaltic pump which had been injecting the acetate. The final concentration of the acetate in each cylinder immediately after these acetate injections was 198 μM.

The data presented include sediment and groundwater from six cylinders which received ^{13}C-acetate, four cylinders which received ^{12}C-acetate, and two cylinders which received no acetate (one exposed to whole groundwater and one exposed to 1 μm-filtered groundwater). During the experiment, groundwater was collected on the following days: day 0 (t0), day 3 (t1), day 19 (t2), day 30 (t3), day 37 (t4), and day 43 (t5). Each aliquot of groundwater integrates the previous three to six days of groundwater exiting the sediment-filled cylinders. Groundwater was filtered through combusted 0.2-μm Anodisc filters (Whatman) which were then stored at -80ºC until further processing. The experiment was terminated 46 days after the initial addition of acetate. At this point, the sediment was removed from the cylinders in four vertical sections, each of which was 5 cm high, and kept frozen at -80ºC until further processing.

Environmental data: groundwater

During the experiment, groundwater was collected to obtain cell abundances and organic carbon data. Measurements of salinity, pH, and oxygen concentration were taken

129 with a YSI 556 MPS handheld sensor (YSI Incorporated, Yellow Springs, OH). A HOBO
130 temperature probe (HOBO Probe v2, Onset, Bourne, MA) measured the temperature of
131 the groundwater pumped through the cylinders every 15 minutes. The groundwater used
132 to obtain the abundance of flagellates was fixed with 3.7 % formaldehyde (final
133 concentration), allowed to sit for 24 hours, and then filtered onto a 0.8 μm filter following
134 the methods of Sherr et al. (1993). For bacterial abundance, groundwater was fixed with
135 2% paraformaldehyde (final concentration), allowed to sit for one hour, and then frozen
136 at -80°C until analysis (Campbell 2001). DOC concentrations in groundwater were
137 measured with a Shimadzu TOC-V_{CSH} total organic carbon analyzer using sucrose as a
138 standard solution.

139 **Environmental data: sediments**

140 The concentration and carbon isotopic ratio of total organic carbon bound to the
141 sediments were obtained with the Europa 20-20 CF-IRMS interfaced with the Europa
142 ANCA-SL instrument. $\delta^{13}C$ values were reported relative to PeeDee belemnite using
143 standard notation: $\delta^{13}C$ (‰)= (R_{sample} / $R_{standard}$ - 1) *1000, where R is the ratio of the
144 heavy to light element. The $\delta^{13}C$ values were converted to atom %^{13}C for ease of
145 presentation.

146 **DNA extractions**

147 DNA was extracted from the sediments and the 0.2 μm Anodisc filters using the
148 UltraClean MegaPrep Soil DNA Kit (MoBio Laboratories, Inc. Carlsbad, CA) following
149 the manufacturer's protocol with the following modifications. The extracts were shaken
150 with solution S1 and the bead solution for 30 min at 65ºC at the beginning of the

extraction. The addition of solution S4 and the subsequent centrifugation step was repeated twice.

Ultracentrifugation

Ultracentrifugation was used to separate the ^{12}C- and ^{13}C-labeled DNA within DNA extracts from sediments from five cylinders (two from whole groundwater and three from 1 µm-filtered groundwater, four sections from each cylinder) and two groundwater samples (one from the whole groundwater treatment and one from the 1 µm-filtered groundwater treatment, with five time points for each). This resulted in ultracentrifugation of twenty DNA extracts from the sediments and ten DNA extracts from the groundwater. Extracted DNA was mixed with cesium chloride and spun in a Beckman Coulter Optima L-80 XP Ultracentrifuge (Fullerton, CA) following protocols modified from Freitag et al. (2006) as previously described (Longnecker et al. 2009). Briefly, DNA was spun at 140,000 x *g* for 66 hours at 20ºC using a VTi 65.2 vertical rotor. At the conclusion of the ultracentrifugation run, ten fractions were collected from each centrifuge tube and the refractive index was measured for each fraction. DNA was precipitated following a protocol adapted from Griffiths et al. (2000) and Freitag et al. (2006). Two volumes of a 30% w/v polyethylene glycol 6000/1.6 M sodium chloride solution were added to each fraction. Fractions were incubated at 4ºC for 72 hours, centrifuged at 20,000 x *g* for 15 minutes at 4ºC, and then washed three times with cold 70% ethanol. The DNA pellet was then dried and resuspended in 10 mM Tris.

Community fingerprinting

Terminal restriction fragment length polymorphism (T-RFLP) analysis was used to assess bacterial community composition. T-RFLP was conducted on DNA extracted directly from the sediments and groundwater, and on DNA collected from the ultracentrifugation fractions. DNA was amplified using the GoTaq Master Mix (Promega Corp. Madison WI) using FAM-labeled 27F and 519R. PCR conditions were an initial denaturation (95ºC for 5 minutes) followed by 35 cycles of denaturation (95ºC, 30 seconds), annealing (46ºC, 30 seconds), extension (72ºC, 90 seconds), and a final extension cycle of 72ºC for 5 minutes. Nested PCR was used to amplify DNA from the ultracentrifugation fractions. This involved an initial amplification with 15 cycles of the PCR program described above and the primers 27F and 1512uR. An aliquot from this PCR reaction was transferred into a new PCR reaction, and amplified for an additional 35 cycles using the same PCR program and the primers 27F and 519R. At least two additional negative controls from the first PCR reaction were run in the second reaction to detect possible contamination due to the increased number of PCR cycles.

PCR products were digested using 1 U of the restriction endonuclease *Hin*6I (Fermantas International, Inc. Burlington, Ontario) at 37ºC for 2 hours. After the restriction digest, DNA was analyzed on an Applied Biosystems 3730XL capillary sequencer as previously described (Longnecker et al. 2009). Chromatograms were analyzed using DAx Data Acquisition and Analysis software (Van Mierlo Software Consultancy Eindhoven, the Netherlands). The position of TRFs between samples was aligned using MATLAB (L. Finlay, J. Kitner, S.J. Giovannoni and E.B. Kujawinski, unpublished).

Cloning and sequencing of 16S rRNA genes in sediments

Two sediment samples were chosen for further analysis using clone libraries of almost full-length 16S rRNA genes: one from sediment exposed to whole groundwater and a second sample from sediment exposed to 1 μm-filtered groundwater. For both clone libraries, the sediment was from the bottom of cylinders that received ^{13}C-acetate. Briefly, DNA was amplified using 27F and 1492R. The resulting PCR products were cloned using the pCR4-TOPO vector (TOPO-TA, Invitrogen) according to the manufacturer's directions. DNA was extracted from the resulting colonies using a modified alkaline lysis protocol (Sambrook et al. 1989). The PCR-amplified inserts were then digested with 1U of the restriction endonucleases *Msp*I and *Hin*PI following the manufacturer's instructions (New England Biolabs). The clones were separated into different phylotypes based on the RFLP banding patterns.

At least one clone from each RFLP pattern was sequenced to 2x coverage by cycle sequencing using fluorescent dideoxy terminators. Internal primers were used to obtain the 2x coverage within the 16S rRNA gene. The primers used for sequencing were: M13F (5′- GTAAAACGACGGCCAG-3′), M13R (5′CAGGAAACAGCTATGAC-3′), 515F (5′GTGCCAGCMGCCGCGGTAA-3′), 1114F (5′GCAACGAGCGCAACC C-3′), 519R (5′GWATTACCGCGGCKGCTG-3′), and 907R (5′CCGTCAATTCMTTTGAGTTT-3′). Sequences were assembled using Sequencher (Gene Codes Corporation). Chimeras identified by Bellerophon (Huber et al. 2004) were removed from further analysis. GenBank sequence accession numbers are FJ719033-FJ719100; clones are preceded by 'p03' (sediment exposed to whole groundwater) or 'p04' (sediment exposed to 1 μm-filtered groundwater). The phylogenetic association of

each clone was determined using the small subunit rRNA taxonomy and alignment pipeline (STAP, Wu et al. 2008).

Statistical analysis

Non-metric multidimensional scaling (NMS) (Kruskal 1964; Mather 1976) was used to analyze variability in bacterial community composition. NMS is a multivariate statistical technique used to examine similarities, or differences, between samples by reducing the comparisons between samples from a multidimensional space to fewer dimensions, preferably two or three. Differences between individual samples were calculated based on the presence or absence of TRFs with the Bray-Curtis distance measure using the Fathom toolbox (David Jones, University of Miami – Rosenstiel, http://www.rsmas.miami.edu/personal/djones/matlab/matlab.html). The differences were then presented graphically in a multidimensional space; samples that are close together in the ordination are more similar to one another than samples located further apart. The statistics toolbox in MATLAB was used to run the NMS analyses. Additional code was written to assess the dimensionality of the data set by comparing 40 runs with real data to 50 runs with randomized data . Additional axes were added if the addition of the axis resulted in a significant improvement over the randomized data (at $p \leq 0.05$) and the reduction in stress was greater than 0.05. The p-values were calculated as the proportion of randomized runs with stress less than or equal to the observed stress which was calculated using Kruskal's stress formula 1; stress is a measure of goodness of fit used in NMS. The proportion of variation represented by each axis was assessed by using a Mantel test to calculate the coefficient of determination (r^2) between distance in the

ordination space and distance in the original space. All MATLAB code is available upon request.

One-way analysis of similarity (ANOSIM) was used to assess if groups visualized by NMS were statistically significant. MATLAB code for ANOSIM was also from the Fathom toolbox. The Bray-Curtis distance matrix calculated for the NMS was used for ANOSIM with the distances converted to ranked distances prior to ANOSIM calculations. The significance of each group was tested by 10,000 randomizations of the dataset, and p-values were calculated to determine the probability of no difference between groups. If the p-value was less than 0.05, we rejected the null hypothesis of no difference between groups.

The non-parametric Kruskal-Wallis test was also used to examine differences between samples which did not meet the requirements of normality or equal variance.

RESULTS

Groundwater chemical parameters and microbial abundances

There were minor changes in the bulk chemical properties of the groundwater during the experiment (Fig. 1). The salinity was always less than 0.1 (unitless, data not shown), oxygen averaged 8.4 mg L^{-1} and pH averaged 6.5. The groundwater temperature decreased from ~14ºC to 10ºC by the end of the experiment.

The concentration of dissolved organic carbon (DOC) in the groundwater prior to entering the sediment-filled cylinders averaged 75.1 μM (66.3 to 83.9 μM, 95% confidence interval, n = 4). In columns where no acetate was added, DOC increased to 91.6 μM in the whole groundwater (70.6 to 113.4 μM, 95% confidence interval, n = 11) and to 92.0 μM in the 1 μm-filtered groundwater (80.1 to 103.1 μM 95% confidence

interval, n = 15). In the sediment-filled cylinders which received additions of acetate, the DOC concentrations in the groundwater exiting the sediment-filled cylinders were significantly higher with average values of 110.8 μM (75.9 to 145.6 μM, 95% confidence interval, n = 27) in the 1 μm-filtered groundwater and 146.8 μM (95.4 to 198.2 μM, 95% confidence interval, n = 21) in the whole groundwater.

At the conclusion of the experiment, we measured the atom % ^{13}C bound to the sediments. This measurement includes both organic carbon abiotically bound or adsorbed to the sediment grains and organic carbon assimilated by the attached bacterial community. The % ^{13}C value from sediment sampled from a core with no carbon added was 1.08%, while the value from a core with ^{12}C carbon added was 1.13%. These values are close to 1.10% ^{13}C which is the natural abundance of ^{13}C. Larger amounts of the ^{13}C label were found in the bottom of the cylinders closest to the source of ^{13}C acetate. There was a general decrease in atom % ^{13}C as the water moved upwards through the sediment-filled cylinders (Fig. 2). Furthermore, a higher proportion of the ^{13}C label was found in sediments exposed to whole groundwater compared to sediments exposed to 1 μm-filtered groundwater (Kruskal-Wallis test, $p = 0.0178$, $n = 20$).

From groundwater collected at different time points throughout the experiment, the abundance of bacterial cells in the groundwater flowing into the sediment-filled cylinders was 2.1×10^4 cells ml^{-1} (95% confidence interval = 0.8 to 3.4×10^4 cells per ml^{-1}, n = 3). As was observed with the DOC data, the abundance of bacterial cells increased after the groundwater flowed through the sediment-filled cylinders. The abundance of heterotrophic bacterial cells in groundwater exiting the sediment-filled cylinders that

284 received acetate additions averaged 1.1 x10^5 cells ml^{-1} and 4.8 x 10^5 cells ml^{-1} in 1 μm-
285 filtered and whole groundwater, respectively.

286 The abundance of heterotrophic flagellates in the groundwater flowing into the
287 sediment-filled cylinders was <100 cells ml^{-1}. The protistan community in groundwater
288 was primarily comprised of flagellates between two and five μm long. Data on the
289 abundance of heterotrophic nanoflagellates in the groundwater exiting the sediment-filled
290 cylinders (Fig. 3) indicated that we were able to reduce the abundance of nanoflagellates
291 in the 1 μm-filtered groundwater five-fold relative to the whole groundwater treatments.

292 **Bacterial community composition of groundwater and sediment**

293 DNA was extracted from 54 samples and analyzed with T-RFLP analysis. Two of
294 the samples were from groundwater entering the sediment-filled cylinders; the remaining
295 samples were either from groundwater exiting the sediment-filled cylinders (n = 25) or
296 from the sediment within the cylinders (n = 27). ANOSIM was used to test for
297 differences in bacterial community composition (Table 1). There were statistically
298 significant differences in bacterial community composition between the incubations with
299 carbon added compared to those with no carbon added. There were also significant
300 differences in diversity between the sediment-associated bacterial community and the
301 free-living bacterial community, and in the bacterial community in treatments with whole
302 groundwater compared to those with 1 μm-filtered groundwater. Finally, the bacterial
303 community in groundwater entering the sediment-filled cylinders resembled the
304 groundwater bacterial community exiting the sediment-filled cylinders. However, there
305 were no significant differences in bacterial community composition across the four
306 different sediment sections removed from the cylinders (Table 1). Therefore, for the

307 remainder of the project, the sediment sections removed from each cylinder were treated
308 as pseudo-replicates from the same cylinder.

309 **Bacterial community composition following ultracentrifugation**

310 We defined ^{13}C-labeled DNA as DNA collected from densities ≥ 1.7258 g ml^{-1}
311 based on our previous results (Longnecker et al. 2009) and on the position of our ^{12}C- and
312 ^{13}C-labeled standard DNA. We observed faint PCR bands in regions defined as ^{13}C-DNA
313 from cylinders only exposed to ^{12}C-acetate. Since these cylinders had not been exposed to
314 ^{13}C-acetate, we would not expect DNA to be labeled with measureable amounts of ^{13}C.
315 Therefore, TRFs in the ^{12}C-only enrichments that occurred in the ^{13}C-rich region were
316 removed from further consideration in all samples in order to be conservative in
317 characterizing the bacterial community involved in acetate assimilation.

318 Based on analysis of the TRFs obtained after ultracentrifugation, the bacterial
319 community composition in the groundwater was significantly different from what was
320 observed in the sediment (ANOSIM, R = 0.2631 and p = 0.0001). There was no
321 significant difference in groundwater bacterial community composition between the
322 whole and 1 μm-filtered groundwater treatments (ANOSIM, p-value > 0.05).
323 Furthermore, there was no difference in groundwater bacterial community composition
324 between the ^{12}C-DNA region and the ^{13}C-DNA region following ultracentrifugation
325 (ANOSIM, p-value > 0.05).

326 Examination of the bacterial community composition in the sediments revealed a
327 different situation than was observed in the groundwater. The NMS calculation (Fig. 4)
328 resulted in an ordination with a final stress of 0.25 and r^2 = 0.70 with slightly more
329 variability on axis one than on axis two (r^2 on axis 1 = 0.33, r^2 on axis 2 = 0.23). Further

examination of the bacterial community in the sediments revealed the community composition of ^{12}C-DNA and the ^{13}C-labeled DNA was significantly different (ANOSIM, R = 0.2070, p = 0.0001), and there were significant differences in bacterial community composition between the whole and 1 μm-filtered treatments (ANOSIM, R = 0.2417, p = 0.0001).

Analysis of clone libraries

Two clone libraries from 16S rRNA genes amplified from the sediments were constructed to allow phylogenetic identification of a portion of the sediment-associated bacterial community. A total of 177 clones were analyzed with 90 clones from sediment exposed to whole water and 87 clones from sediment exposed to 1 μm-filtered groundwater. The clones were screened with RFLP, and 68 clones were fully sequenced (n = 29 and n = 39 from sediment exposed to whole water or 1 μm-filtered groundwater, respectively). With the limited number of sequences obtained in the present project, we opted to provide higher level taxonomic information rather than focusing on a detailed phylogenetic assessment. Phylogenetic identification revealed that the majority of the sequences were Proteobacteria (Table 2), with over 50% of the sequences originating from Betaproteobacteria. In the sediments exposed to 1 μm-filtered groundwater, two groups of Alphaproteobacteria, Bradyrhizobiales and Sphingomonadales, were also a large proportion of the clones obtained.

DISCUSSION

A static bacterial community in groundwater

In the present project, the composition of the groundwater bacterial community did not change in response to our experimental manipulations. We observed higher abundances of bacterial cells in groundwater exiting the sediment-filled cylinders, which indicates that the groundwater bacterial community was able to grow within our incubations. However, the groundwater bacterial community had a low response to the experimental manipulations based on two observations. First, filtration of the groundwater with a 1 µm filter did not alter the bacterial community composition in the groundwater. Second, the bacterial community in groundwater exiting the sediment-filled cylinders resembled the bacterial community entering the cylinders. Based on these two results, we conclude that subsurface microbial community assessments based solely upon groundwater samples are limited to an examination of the less responsive component of the microbial community. Indeed, the sediment-associated community may be the more biogeochemically-relevant community in subsurface systems where there are differences in community composition (this study, Lehman et al. 2001; Lehman et al. 2004; Flynn et al. 2008) and differences in metabolic capabilities associated with organic substrate remineralization (Kato 1984). However, while the groundwater bacterial community did not exhibit large changes during this experiment, groundwater is still an important vector for transporting the bacterial community and organic carbon through the subsurface.

A dynamic sediment-associated bacterial community

Proteobacteria dominated the bacterial community in the sediments in the present project. The dominance of Proteobacteria has also been observed in pristine aquifers (López-Archilla et al. 2007; Nagaosa et al. 2008; Blöthe and Roden 2009) and in biofilms grown in the presence of groundwater (Peacock et al. 2004). In addition, bottle incubations both with and without grazers determined that Proteobacteria were a large component of the bacterial community in groundwater (Longnecker et al. 2009). Given the diverse array of metabolisms possible within the Proteobacteria, we can only speculate about which metabolic processes the Proteobacteria could be utilizing within the present project. However, Proteobacteria have been implicated in sulfate reduction, denitrification, and iron-based metabolisms in other aquifers (López-Archilla et al. 2007; Blöthe and Roden 2009).

The presence of grazers in the groundwater entering the cylinders affected the development of the sediment-associated bacterial community. Grazers are known to alter bacterial diversity and activity (Jürgens and Matz 2002), in part by selectively grazing specific members of a bacterial community (Jezbera et al. 2005). Alternatively, the bacterial community may rely on nutrients released during protozoan grazing (Caron et al. 1988; Barbeau et al. 1996) or shifts in the composition of organic matter due to grazing activity (Kujawinski et al. 2004; Gruber et al. 2006). In the present study, the presence of grazers did not significantly alter the composition of organic matter in the groundwater exiting the sediment-filled cylinders (Longnecker and Kujawinski 2011). However, our data show that the presence of grazers was a key factor in controlling the

391 sediment-associated bacterial community constituents although determining which
392 processes are involved will require additional work.

393 We used the $\delta^{13}C$ measurements of the sediments as a means to quantify
394 differences in carbon cycling within our experiment. A higher proportion of the ^{13}C label
395 was bound to sediments when grazers were present. There are three possible explanations
396 for this observation. First, there could be increased consumption of acetate by the
397 sediment-associated microbial community in the presence of grazers. Second, the subset
398 of the bacterial community responsible for assimilation of the ^{13}C label could have been
399 resistant to grazing pressure within the sediments. Third, the metabolically-active
400 bacterial community incorporated higher amounts of carbon into the biofilm attached to
401 the sediments in the presence of grazers. This adds isotopically-labeled carbon to the
402 sediment grains and the biofilm could provide resistance to grazing pressure (Matz and
403 Kjelleberg 2005). However, whether the bacterial community was responding to changes
404 in the composition of organic matter, to increases in organic carbon, to nutrients released
405 due to grazing activity, or to some combination of all three processes, remains unknown.

406 **Conclusions**

407 The present project revealed distinct differences between sediment-associated and
408 groundwater bacterial communities. While the groundwater bacterial community did not
409 alter its composition during the present project, a distinct sediment-associated community
410 developed in the presence of protozoan grazers. Furthermore, the presence of protozoan
411 grazers increased the retention of labeled organic carbon in the sediments which indicates
412 that the presence of grazers can alter the retention of organic carbon in the subsurface.

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584 Wu D, Hartman A, Ward N and Eisen JA. 2008. An automated phylogenetic tree-based
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589

590 Table 1. Variability in bacterial community composition for DNA extracted from the
591 sediments and groundwater. ANOSIM was used to test for statistically significant
592 differences in bacterial community composition between groups. The following
593 comparisons are shown: 1) acetate amendment compared to no-carbon addition, 2) free-
594 living compared to sediment-associated bacterial community, 3) sediment-filled cylinders
595 exposed to whole versus 1 μm-filtered groundwater, and 4) the vertical location of the
596 sediment within the cylinders. "n.s." = the comparison was not significant.

Comparison	**n**	**ANOSIM**
No Carbon added	16	R = 0.1622
Acetate added	36	p = 0.0051
Groundwater	25	R = 0.3651
Sediment	27	p = 0.0001
Whole	24	R = 0.1014
1 μm-filtered	28	p = 0.0073
Top of core	6	n.s.
Upper middle	6	
Lower middle	6	
Bottom of core	9	

597

598 Table 2. Phylogenetic information from the 16S rDNA sequences from the two clone
599 libraries: one from sediment exposed to whole groundwater and one from sediment
600 exposed to 1 μm-filtered groundwater. Both samples were from cylinders with ^{13}C-
601 acetate added. The percentages do not add up to 100% due to rounding within each
602 phylogenetic group.

		% of clones	
Phylum	**Class / Order / Family**	**Whole groundwater**	**1 μm-filtered groundwater**
Acidobacteria	Solibacteres		1
Actinobacteria	Actinobacteridae	2	2
Bacteroidetes	Flexibacteraceae	4	
	Saprospiraceae	1	1
	Environmental sequences	1	
Cyanobacteria	Environmental sequences		1
OP11-5	Environmental sequences	2	
OP3	Environmental sequences	7	
Planctomycetes	Environmental sequences	1	1
Proteobacteria	Alphaproteobacteria		
	Bradyrhizobiales	2	12
	Caulobacterales	1	2
	Rhizobiales	9	
	Rhodobacterales		2
	Sphingomonadales	3	17
	Betaproteobacteria		
	Burkholderiales	53	36
	Methylophilales	2	
	Rhodocyclales	1	7
	Nitrosomonadales		8
	Gammaproteobacteria		
	Legionellales	4	
	Moraxellaceae		8
	Xanthomonadales		1
Verrucomicrobia	Verrucomicrobiae	4	

603

Figure legends

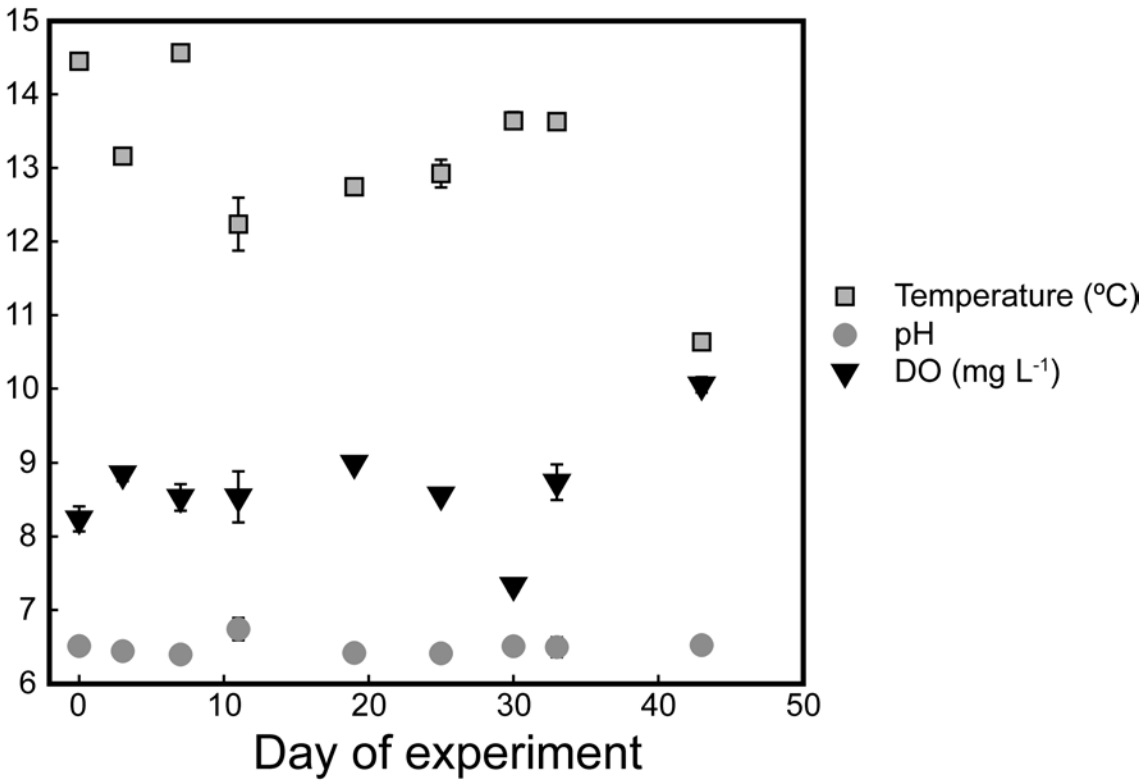
Fig. 1. Temperature, pH, and dissolved oxygen (DO) measurements made during the course of the experiment. Data are mean values of measurements taken from multiple cubitainers at each time point; for most samples, the error bars (± one standard deviation) are smaller than the symbol used in the figure.

Fig. 2. Atom % ^{13}C of organic carbon in the sediments removed from the cylinders at the conclusion of the experiment. Data from cylinders with ^{13}C-labeled acetate are shown here; data from control cylinders are discussed in the text. Data points have been jiggered on the y-axis for clarity of presentation. The points with error bars are duplicate subsamples of sediment.

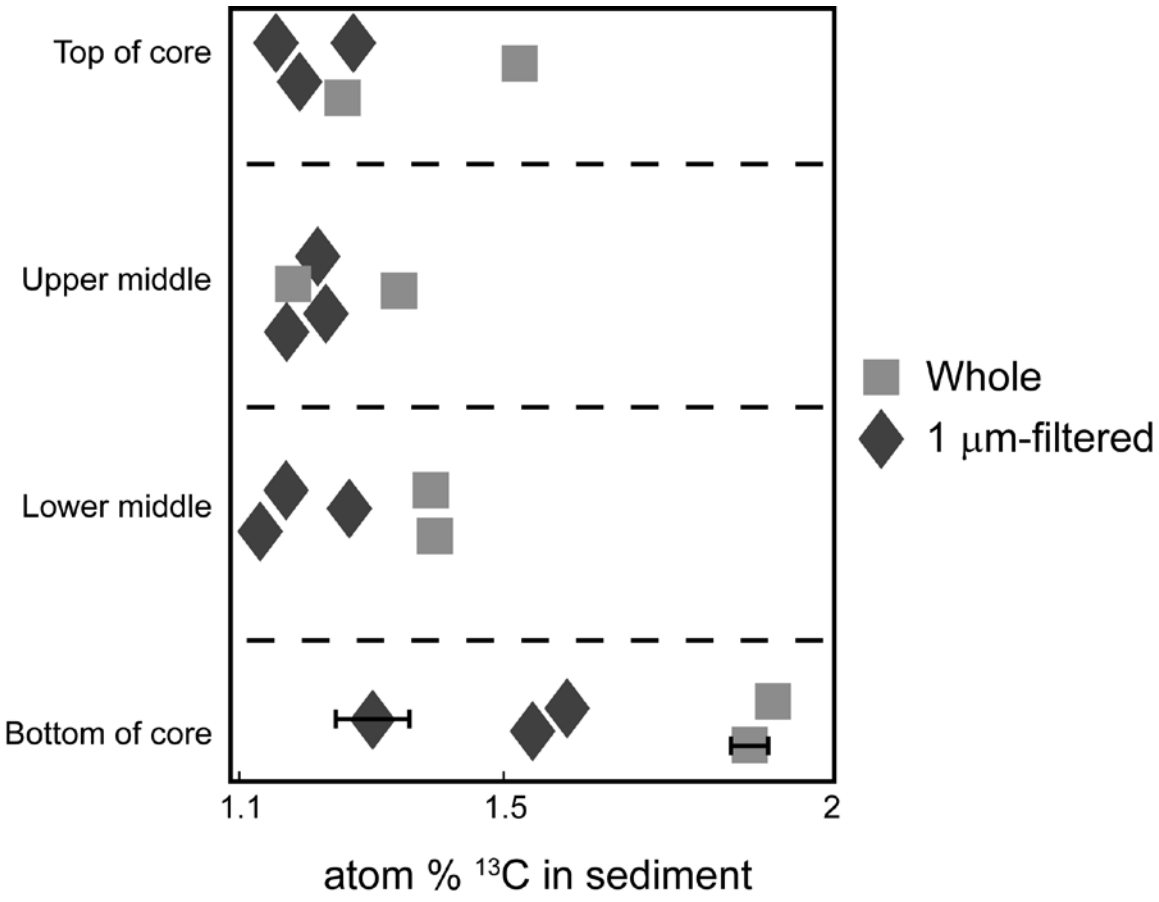
Fig. 3. Abundance of heterotrophic nanoflagellates (x 10^3 cells ml^{-1}) in the groundwater exiting the sediment-filled cylinders. The error bars are ± one standard deviation.

Fig. 4. NMS analysis of sediment-associated bacterial community composition after DNA was separated into ^{12}C- and ^{13}C-DNA using ultracentrifugation. The lines in the figure separating the treatments with and without grazers and the ^{12}C- and ^{13}C-DNA are based on statistically significant differences.

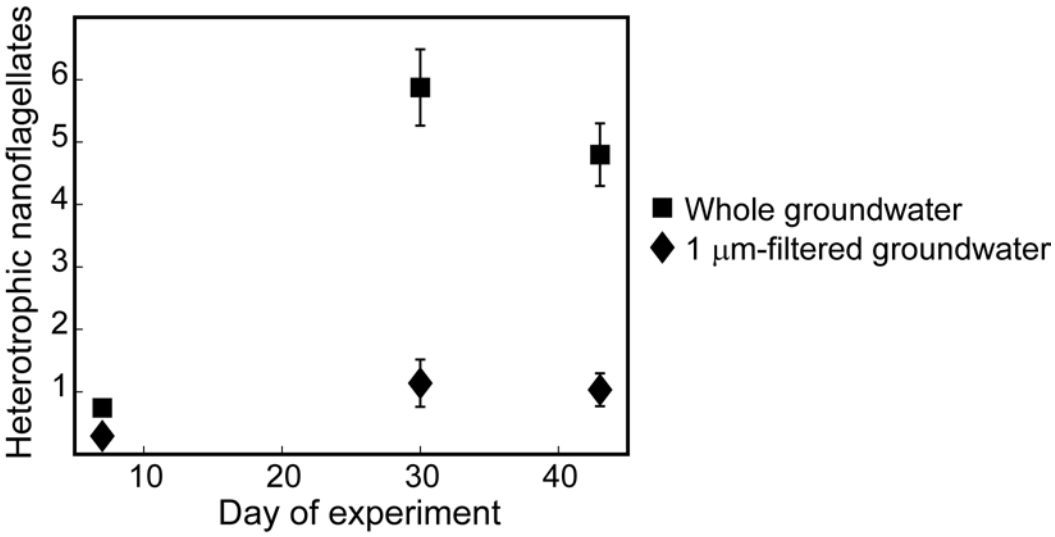
621 Longnecker and Kujawinski
622 Fig. 1
623
624



625
626 Longnecker and Kujawinski
627 Fig. 2
628



629
630 Longnecker and Kujawinski
631 Fig. 3
632



633
634 Longnecker and Kujawinski
635 Fig. 4