

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

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13

14

15 **ABSTRACT**

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

25 **INTRODUCTION**

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

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

38 1999; Griebler and Lueders 2009) and eukaryotic (Novarino et al. 1997; Valster et al.
39 2009) diversity in aquifers are based on microscopic observations, culturing experiments,
40 and identifications from phospholipid fatty acids or small subunit rRNA gene libraries.
41 There is spatial and temporal variability in prokaryotic diversity within both pristine and
42 contaminated aquifers (Haack et al. 2004; López-Archilla et al. 2007), and
43 microorganisms found in the subsurface are also found in other ecosystems (Griebler and
44 Lueders 2009).

45 Previous research in groundwater microbial ecology indicates that the bacterial
46 community is not passively transported through the subsurface but can participate in
47 subsurface biogeochemical cycles (Ghiorse and Wilson 1988; Madsen and Ghiorse
48 1993). Bacterial cells grow in aquifer sediments (Chapelle et al. 1987) and there is
49 temporal variability in bacterial biomass (Velasco Ayuso et al. 2009). Groundwater
50 microorganisms also utilize a variety of organic substrates and the patterns in substrate
51 utilization are spatially heterogeneous (Madsen and Ghiorse 1993; Pedersen et al. 2008;
52 Velasco Ayuso et al. 2009). Finally, geochemical evidence of organic carbon
53 consumption, denitrification, and production of carbon dioxide, methane or sulfide
54 (Chapelle and Lovley 1990; Baker et al. 2000; Routh et al. 2001) as well as the presence
55 of different functional genes (Griebler and Lueders 2009) indicate that a variety of
56 bacterial metabolisms exist in the subsurface.

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

61 hydrocarbons and found that only a subset of groundwater microorganisms in these
62 systems are metabolically active vis-à-vis acetate assimilation (Pombo et al. 2002; Pombo
63 et al. 2005). One limitation in using stable isotope probing is that metabolically-active
64 cells which do not assimilate the labeled compound are incorrectly assigned to the
65 “inactive” category. Nonetheless, even with these methodological limitations, the use of
66 isotopically-labeled compounds has elucidated the role of specific microbial groups in
67 substrate remineralization (see for example: Radajewski et al. 2002; Padmanabhan et al.
68 2003).

69 As methods for assessing microbial diversity have matured, microbiologists have
70 been able to explore the factors in different ecosystems that control and structure
71 bacterial community composition. Bacterial mortality due to protozoan grazers can have
72 a major impact on bacterial community composition (Jürgens and Matz 2002; Nagaosa et
73 al. 2008). The presence of grazers can also control bacterial growth in aquifer sediments
74 (Mattison et al. 2002; Nagaosa et al. 2008), although the magnitude of this effect varies
75 for different experimental systems (DeLeo and Baveye 1997). Finally, field studies have
76 shown that the presence of grazers can increase the remineralization and mobilization of
77 anthropogenic contaminants (Madsen et al. 1991; Kinner et al. 2002; Tso and Taghon
78 2006). Although grazers are acknowledged to be important in subsurface ecosystems, we
79 still lack knowledge about the impact of grazers on metabolically-active bacterial cells.

80 The present project examined sediment-associated and free-living microorganisms
81 in the subsurface. Mesocosms were used to mimic in situ conditions and we used stable
82 isotope probing to characterize the microbial community involved in the assimilation of
83 ¹³C-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 $1 \mu\text{m}$ 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.

106 Cylinders received either ^{12}C -acetate (Fisher, enzyme grade), uniformly-labeled
107 ^{13}C -acetate (99% $^{13}\text{CH}_3$ - $^{13}\text{COOH}$, Cambridge Isotope Laboratories, Andover MA), or no
108 acetate. For the cylinders receiving acetate, a peristaltic pump pulled acetate from stock
109 bottles and merged it with the flow of groundwater so that the concentration of acetate in
110 the groundwater was 200 μM for the first 11 days of the experiment. On day 30 and day
111 38 of the experiment, concentrated acetate was injected directly into the base of each
112 cylinder due to a problem with the peristaltic pump which had been injecting the acetate.
113 The final concentration of the acetate in each cylinder immediately after these acetate
114 injections was 198 μM .

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

126 **Environmental data: groundwater**

127 During the experiment, groundwater was collected to obtain cell abundances and
128 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. $\delta^{13}\text{C}$ values were reported relative to PeeDee belemnite using
143 standard notation: $\delta^{13}\text{C} (\text{‰}) = (R_{\text{sample}} / R_{\text{standard}} - 1) * 1000$, where R is the ratio of the
144 heavy to light element. The $\delta^{13}\text{C}$ 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 65°C at the beginning of the

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

153 **Ultracentrifugation**

154 Ultracentrifugation was used to separate the ¹²C- and ¹³C-labeled DNA within
155 DNA extracts from sediments from five cylinders (two from whole groundwater and
156 three from 1 μm-filtered groundwater, four sections from each cylinder) and two
157 groundwater samples (one from the whole groundwater treatment and one from the 1 μm-
158 filtered groundwater treatment, with five time points for each). This resulted in
159 ultracentrifugation of twenty DNA extracts from the sediments and ten DNA extracts
160 from the groundwater. Extracted DNA was mixed with cesium chloride and spun in a
161 Beckman Coulter Optima L-80 XP Ultracentrifuge (Fullerton, CA) following protocols
162 modified from Freitag et al. (2006) as previously described (Longnecker et al. 2009).
163 Briefly, DNA was spun at 140,000 x g for 66 hours at 20°C using a VTi 65.2 vertical
164 rotor. At the conclusion of the ultracentrifugation run, ten fractions were collected from
165 each centrifuge tube and the refractive index was measured for each fraction. DNA was
166 precipitated following a protocol adapted from Griffiths et al. (2000) and Freitag et al.
167 (2006). Two volumes of a 30% w/v polyethylene glycol 6000/1.6 M sodium chloride
168 solution were added to each fraction. Fractions were incubated at 4°C for 72 hours,
169 centrifuged at 20,000 x g for 15 minutes at 4°C, and then washed three times with cold
170 70% ethanol. The DNA pellet was then dried and resuspended in 10 mM Tris.

171 **Community fingerprinting**

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

186 PCR products were digested using 1 U of the restriction endonuclease *Hin6I*
187 (Fermantas International, Inc. Burlington, Ontario) at 37°C for 2 hours. After the
188 restriction digest, DNA was analyzed on an Applied Biosystems 3730XL capillary
189 sequencer as previously described (Longnecker et al. 2009). Chromatograms were
190 analyzed using DAX Data Acquisition and Analysis software (Van Mierlo Software
191 Consultancy Eindhoven, the Netherlands). The position of TRFs between samples was
192 aligned using MATLAB (L. Finlay, J. Kitner, S.J. Giovannoni and E.B. Kujawinski,
193 unpublished).

194 **Cloning and sequencing of 16S rRNA genes in sediments**

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

206 At least one clone from each RFLP pattern was sequenced to 2x coverage by cycle
207 sequencing using fluorescent dideoxy terminators. Internal primers were used to obtain
208 the 2x coverage within the 16S rRNA gene. The primers used for sequencing were:
209 M13F (5'- GTAAAACGACGGCCAG-3'), M13R (5'CAGGAAACAGCTATGAC-3'),
210 515F (5'GTGCCAGCMGCCGCGGTAA-3'), 1114F (5'GCAACGAGCGCAACC C-3'),
211 519R (5'GWATTACCGCGGCKGCTG-3'), and 907R
212 (5'CCGTCAATTCMTTGTGAGTTT-3'). Sequences were assembled using Sequencher
213 (Gene Codes Corporation). Chimeras identified by Bellerophon (Huber et al. 2004) were
214 removed from further analysis. GenBank sequence accession numbers are FJ719033-
215 FJ719100; clones are preceded by 'p03' (sediment exposed to whole groundwater) or
216 'p04' (sediment exposed to 1 µm-filtered groundwater). The phylogenetic association of

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

219 **Statistical analysis**

220 Non-metric multidimensional scaling (NMS) (Kruskal 1964; Mather 1976) was
221 used to analyze variability in bacterial community composition. NMS is a multivariate
222 statistical technique used to examine similarities, or differences, between samples by
223 reducing the comparisons between samples from a multidimensional space to fewer
224 dimensions, preferably two or three. Differences between individual samples were
225 calculated based on the presence or absence of TRFs with the Bray-Curtis distance
226 measure using the Fathom toolbox (David Jones, University of Miami – Rosenstiel,
227 <http://www.rsmas.miami.edu/personal/djones/matlab/matlab.html>). The differences were
228 then presented graphically in a multidimensional space; samples that are close together in
229 the ordination are more similar to one another than samples located further apart. The
230 statistics toolbox in MATLAB was used to run the NMS analyses. Additional code was
231 written to assess the dimensionality of the data set by comparing 40 runs with real data to
232 50 runs with randomized data . Additional axes were added if the addition of the axis
233 resulted in a significant improvement over the randomized data (at $p \leq 0.05$) and the
234 reduction in stress was greater than 0.05. The p-values were calculated as the proportion
235 of randomized runs with stress less than or equal to the observed stress which was
236 calculated using Kruskal’s stress formula 1; stress is a measure of goodness of fit used in
237 NMS. The proportion of variation represented by each axis was assessed by using a
238 Mantel test to calculate the coefficient of determination (r^2) between distance in the

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

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

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

251 **RESULTS**

252 **Groundwater chemical parameters and microbial abundances**

253 There were minor changes in the bulk chemical properties of the groundwater
254 during the experiment (Fig. 1). The salinity was always less than 0.1 (unitless, data not
255 shown), oxygen averaged 8.4 mg L⁻¹ and pH averaged 6.5. The groundwater temperature
256 decreased from ~14°C to 10°C by the end of the experiment.

257 The concentration of dissolved organic carbon (DOC) in the groundwater prior to
258 entering the sediment-filled cylinders averaged 75.1 µM (66.3 to 83.9 µM, 95%
259 confidence interval, n = 4). In columns where no acetate was added, DOC increased to
260 91.6 µM in the whole groundwater (70.6 to 113.4 µM, 95% confidence interval, n = 11)
261 and to 92.0 µM in the 1 µm-filtered groundwater (80.1 to 103.1 µM 95% confidence

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

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

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

284 received acetate additions averaged 1.1×10^5 cells ml^{-1} and 4.8×10^5 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 $\geq 1.7258 \text{ g ml}^{-1}$
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 $1 \mu\text{m}$ -filtered groundwater treatments (ANOSIM, $p\text{-value} > 0.05$).
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, $p\text{-value} > 0.05$).

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

330 examination of the bacterial community in the sediments revealed the community
331 composition of ^{12}C -DNA and the ^{13}C -labeled DNA was significantly different
332 (ANOSIM, $R = 0.2070$, $p = 0.0001$), and there were significant differences in bacterial
333 community composition between the whole and $1\ \mu\text{m}$ -filtered treatments (ANOSIM, $R =$
334 0.2417 , $p = 0.0001$).

335 **Analysis of clone libraries**

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

349

DISCUSSION

350 **A static bacterial community in groundwater**

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

369 **A dynamic sediment-associated bacterial community**

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

381 The presence of grazers in the groundwater entering the cylinders affected the
382 development of the sediment-associated bacterial community. Grazers are known to alter
383 bacterial diversity and activity (Jürgens and Matz 2002), in part by selectively grazing
384 specific members of a bacterial community (Jezbera et al. 2005). Alternatively, the
385 bacterial community may rely on nutrients released during protozoan grazing (Caron et
386 al. 1988; Barbeau et al. 1996) or shifts in the composition of organic matter due to
387 grazing activity (Kujawinski et al. 2004; Gruber et al. 2006). In the present study, the
388 presence of grazers did not significantly alter the composition of organic matter in the
389 groundwater exiting the sediment-filled cylinders (Longnecker and Kujawinski 2011).
390 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 $\delta^{13}\text{C}$ 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.

413

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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	
Upper middle	6	
Lower middle	6	n.s.
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.

Phylum	Class / Order / Family	% of clones	
		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

604 **Figure legends**

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

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

614 Fig. 3. Abundance of heterotrophic nanoflagellates ($\times 10^3$ cells ml^{-1}) in the groundwater
615 exiting the sediment-filled cylinders. The error bars are \pm one standard deviation.

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

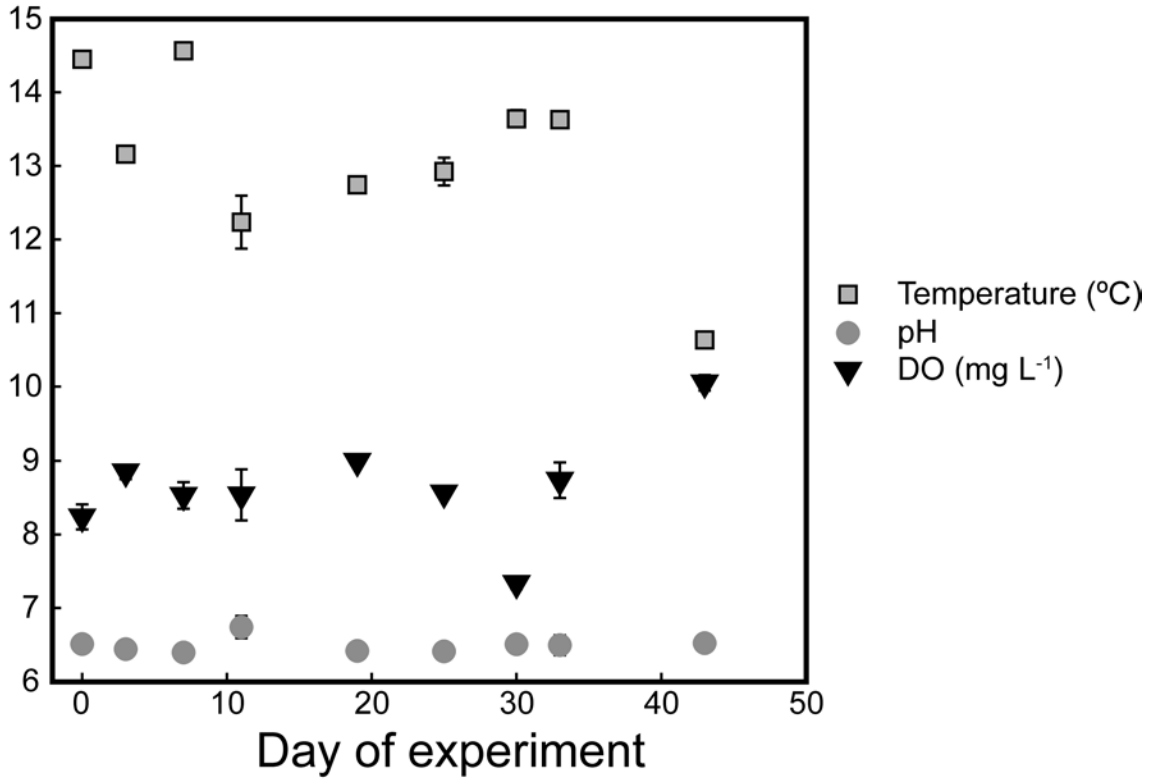
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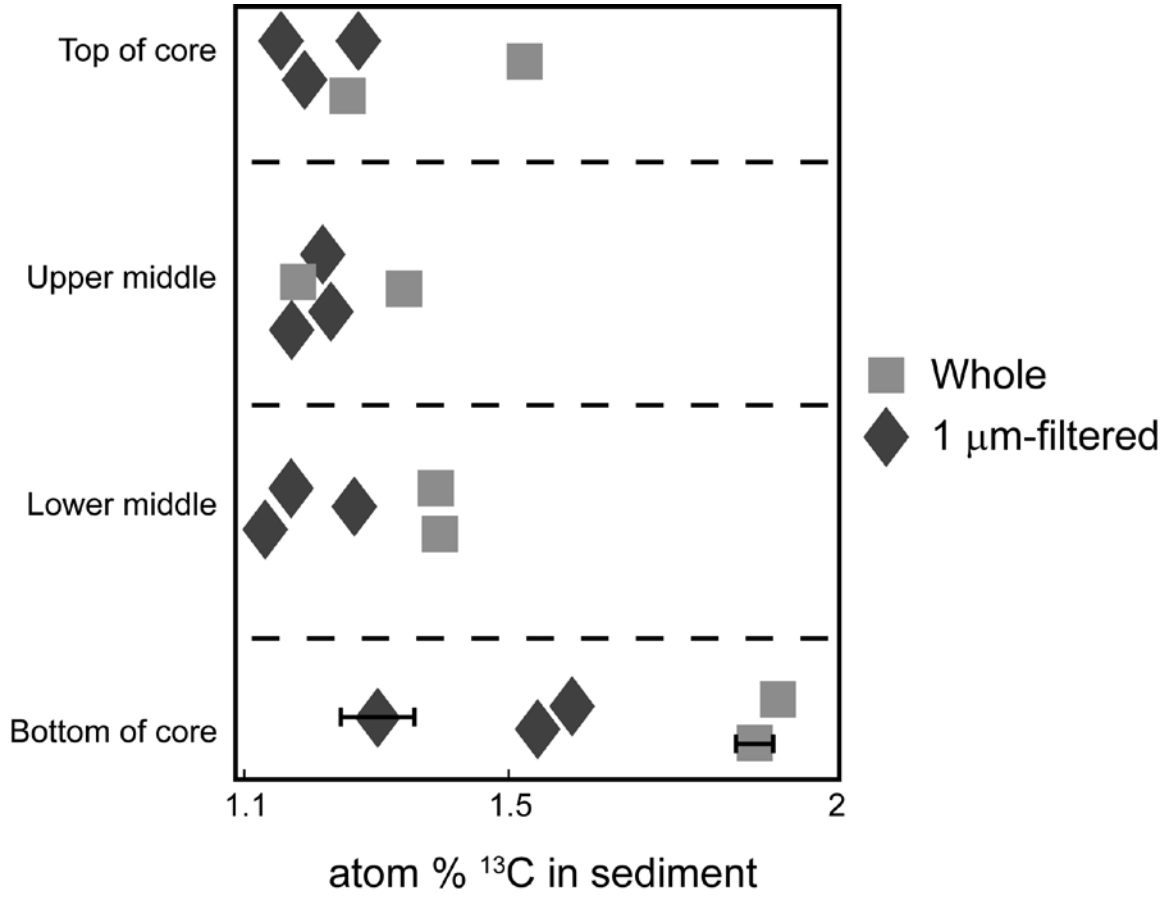
622 Fig. 1

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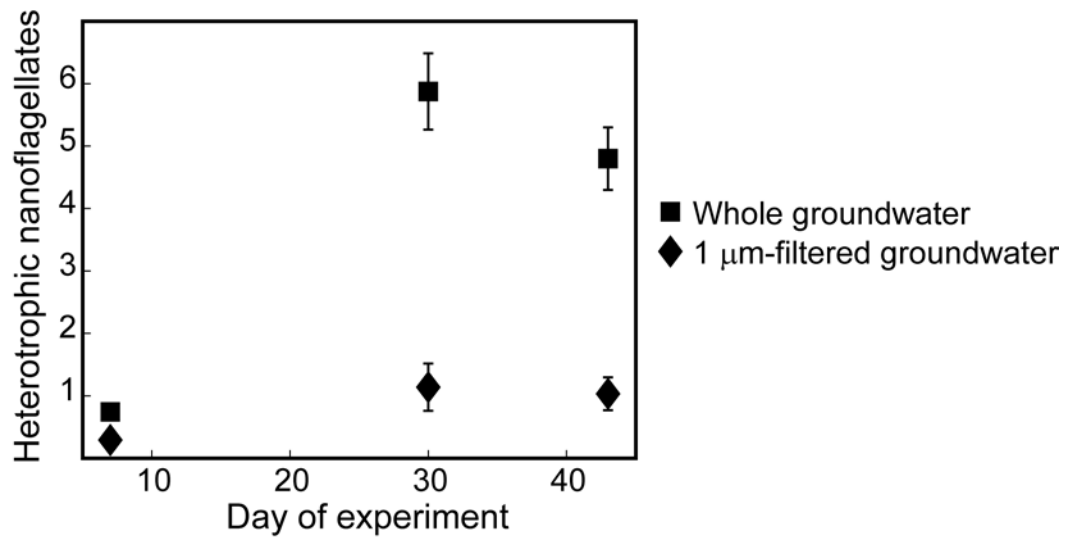


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627 Fig. 2
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Fig. 3



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635 Fig. 4

