

1 **Effect of carbon addition and predation on acetate-assimilating**  
2 **bacterial cells in groundwater**

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17

18 **Abstract**

19 Groundwater microbial community dynamics are poorly understood due to the  
20 challenges associated with accessing subsurface environments. In particular, microbial  
21 interactions and their impact on the subsurface carbon cycle remain unclear. In the  
22 present project, stable isotope probing with uniformly-labeled [<sup>13</sup>C]-acetate was used to  
23 identify metabolically-active and inactive bacterial populations based on their ability to  
24 assimilate acetate and/or its metabolites. Furthermore, we assessed whether substrate  
25 availability (bottom-up control) or grazing mortality (top-down control) played a greater  
26 role in shaping bacterial community composition by separately manipulating the organic  
27 carbon supply and the protozoan grazer population. A community fingerprinting  
28 technique, Terminal Restriction Fragment Length Polymorphism (T-RFLP), revealed that  
29 the bacterial community was not affected by changes in acetate availability but was  
30 significantly altered by the removal of protozoan grazers. In silico identification of  
31 terminal restriction fragments and 16S rDNA sequences from clone libraries revealed a  
32 bacterial community dominated by Proteobacteria, Firmicutes, and Bacteroidetes.  
33 Elucidation of the factors that structure the bacterial community will improve our  
34 understanding of the bacterial role in the carbon cycle of this important subterranean  
35 environment.

36 **Introduction**

37 Groundwater is important both as drinking water and as a source of freshwater flowing  
38 into coastal marine environments (Zektser & Everett, 2004; Mulligan & Charette, 2006).  
39 The groundwater microbial community is known to consist of prokaryotes,  
40 picoeukaryotes, and viruses (Ghiorse & Wilson, 1988; Fry *et al.*, 1997; Goldscheider *et*

41 *al.*, 2006). The eukaryotic community within aquifers is dominated by small (< 5 µm)  
42 flagellates although amoebae, ciliates, and fungi can also be present (Sinclair & Ghiorse,  
43 1987; Novarino *et al.*, 1997; Zarda *et al.*, 1998; Andrushchyn *et al.*, 2007; Brad *et al.*,  
44 2008). Research on groundwater bacterial diversity has primarily focused on  
45 contaminated aquifers (Zarda *et al.*, 1998; Ludvigsen *et al.*, 1999; Haack *et al.*, 2004;  
46 Lehman *et al.*, 2004; Bowman *et al.*, 2006) or on comparisons between contaminated and  
47 pristine aquifers (Shi *et al.*, 1999; Griebler *et al.*, 2002). An endemic groundwater  
48 bacterial community has not been identified, although the groundwater microbial  
49 community is distinct from the microbial diversity observed in soil and other aquatic  
50 habitats (Griebler & Lueders, 2009). A recent study of the microbial community in a  
51 pristine aquifer revealed a spatially variable community that was dominated by  
52 Proteobacteria and that included both Euryarchaeota and Crenarchaeota (López-Archilla  
53 *et al.*, 2007). While no study has examined temporal variability of bacterial diversity in  
54 pristine aquifers, the abundance and size of bacterial cells in aquifers can be temporally  
55 variable (Velasco Ayuso *et al.*, 2009).

56         An important factor regarding the groundwater bacterial community is the extent to  
57 which the microorganisms are involved in biogeochemical processes. However, the  
58 presence of a microorganism is not always indicative of its role in biogeochemical cycles  
59 and the proportion of metabolically-active bacterial cells within groundwater is unknown.  
60 In marine ecosystems, metabolically-active bacterial cells can comprise between 1 and  
61 80% of DAPI-stained cells (Karner & Fuhrman, 1997). Aquifers contain the same range  
62 of eutrophic and oligotrophic conditions as marine ecosystems and so it is likely that a  
63 similar range of metabolically-active cells will be observed in aquifers. Incubations with

64 labeled carbon substrates have revealed that some members of the groundwater bacterial  
65 community can incorporate  $^{13}\text{C}$ -labeled organic compounds (toluene, benzene, or acetate)  
66 into polar lipid fatty acids with concomitant appearance of  $^{13}\text{C}$ -labeled inorganic carbon  
67 (Pombo *et al.*, 2002; Kästner *et al.*, 2006) or methane (Pombo *et al.*, 2005). As a result,  
68 the bacterial community appears to consume and alter organic matter during transport  
69 through aquifers (DeFlaun *et al.*, 1997; Fuller *et al.*, 2000), suggesting a critical role for  
70 bacteria in the subsurface carbon cycle.

71 Bacterial community composition is generally determined by a balance between  
72 substrate availability (bottom-up control) and mortality due to grazing or viral lysis (top-  
73 down control). There are conflicting views as to whether top-down or bottom-up control  
74 prevails under different ecological conditions. Modeling results indicate top-down control  
75 is more important in both marine and freshwater eutrophic environments (Sanders *et al.*,  
76 1992; Thelaus *et al.*, 2008). Conversely, model and experimental results indicate the  
77 opposite in oligotrophic marine ecosystems (Gasol *et al.*, 2002). Due to substantial  
78 variations in organic carbon and inorganic nutrient concentrations within aquifers,  
79 groundwater ecosystems can range from oligotrophic to eutrophic conditions. This range  
80 further complicates broad assessments of whether top-down or bottom-up control plays  
81 the larger role in structuring bacterial community composition within groundwater  
82 (Corno & Jürgens, 2008). While there is spatial variability in nutrient concentrations  
83 within our study area (Charette & Sholkovitz, 2006), the groundwater used for the  
84 present project is comparable to a pristine oligotrophic aquifer due to its low nutrient and  
85 organic carbon concentrations.

86           The interaction between grazers and the bacterial community in groundwater is not  
87 clear. Protozoan grazers are found in both pristine and contaminated aquifers, although  
88 their abundances are lower in pristine aquifers (Sinclair *et al.*, 1993; Novarino *et al.*,  
89 1997). Grazing is known to occur in the subsurface and in flow-through columns  
90 designed to mimic in situ conditions (DeLeo & Baveye, 1997; Kinner *et al.*, 1997;  
91 Eisenmann *et al.*, 1998). In contaminated aquifers, interactions between grazers and their  
92 bacterial prey are linked to changes in the degradation of organic contaminants (Madsen  
93 *et al.*, 1991; Tso & Taghon, 2006). To our knowledge, only one study has examined the  
94 effect of grazers on bacterial community composition and its results were inconclusive  
95 because removing grazers either decreased or increased bacterial diversity depending on  
96 sampling depth within the aquifer (Nagaosa *et al.*, 2008).

97           Here we present the results of a study designed to examine patterns in the diversity  
98 of bacterial cells involved in acetate metabolism, and to further test factors which may be  
99 controlling microbial community structure in groundwater. There are differences in  
100 diversity between the free-living and sediment-associated bacterial community within  
101 groundwater (Lehman *et al.*, 2001; Lehman *et al.*, 2004). However, the present project  
102 focused on the free-living microbial community because of their ability to be transported  
103 to the coastal ocean at our study site (Cambareri & Eichner, 1998; Mulligan & Charette,  
104 2006). In contrast to many coastal aquifers, temporal and spatial changes in groundwater  
105 chemistry at this site have been fairly well-characterized by other investigators (Valiela *et*  
106 *al.*, 1990; Charette *et al.*, 2001; Charette *et al.*, 2005; Bone *et al.*, 2006) and thus our  
107 results can be placed within the appropriate geochemical context.

## 108 **Materials and Methods**

### 109 **Experimental setup**

110 Groundwater was sampled from the freshwater zone of the aquifer at the Waquoit  
111 Bay National Estuarine Research Reserve (Figure 1). Polyethylene tubing lined with  
112 fluorinated ethylene propylene was inserted 2.4 m into the ground, and groundwater was  
113 pumped to the surface using a peristaltic pump at  $100 \text{ ml min}^{-1}$ . A YSI 556 MPS  
114 handheld sensor (YSI Incorporated, Yellow Springs, OH) was used to characterize the  
115 groundwater used for the experiment. The initial conditions were: temperature = 10.6 -  
116 13.2°C; salinity < 0.1, pH = 6.4 - 6.6; and dissolved oxygen concentration = 8.5 - 10.2 mg  
117  $\text{L}^{-1}$ . The oligotrophic nature of our groundwater was confirmed by the low dissolved  
118 organic carbon concentrations (75  $\mu\text{M}$ , see Table 2), and low nitrate (6  $\mu\text{M}$ ) and  
119 phosphate (< 0.1  $\mu\text{M}$ ) concentrations obtained from discrete, aqueous samples analyzed  
120 with a Lachat Instruments QuickChem 8000 Nutrient Analyzer.

121 Polycarbonate bottles (2.5 L bottles) were filled with 1.8 L of water, leaving about  
122 three cm of headspace within the bottles. At the bottom of each bottle was 500 g of  
123 autoclaved playground sand similar in texture to the *in situ* aquifer sand; the sediment  
124 covered the bottom three cm of each 25 cm high bottle. The sediment was homogenized  
125 after autoclaving and prior to adding to the bottles. Half of the bottles received whole,  
126 unfiltered, groundwater and half of the bottles received groundwater filtered through a 1  
127  $\mu\text{m}$  Polycap™ 36 AS filter. The filter unit (Whatman Inc. Florham Park, NJ) had been  
128 soaked in 10% hydrochloric acid followed by a rinse with 2 L of Milli-Q and 1 L of  
129 unfiltered groundwater prior to use. Examination of microbial cells stained with DAPI  
130 from a parallel experiment conducted with the same groundwater indicates that the protist

131 community is primarily comprised of flagellates between two and five  $\mu\text{m}$  long which  
132 would be captured by a 1  $\mu\text{m}$  filter (data not shown).

133 The filled bottles were allowed to equilibrate in the dark for three days at room  
134 temperature (20°C). Experimental bottles were then separated into three additional  
135 treatments: no carbon addition, a single addition of carbon, and multiple carbon additions  
136 (Table 1). Carbon was added as unlabeled acetate (Fisher, enzyme grade) or [ $^{13}\text{C}$ ]-acetate  
137 (99%  $^{13}\text{CH}_3\text{-}^{13}\text{COOH}$  from Cambridge Isotope Laboratories, Andover MA). Final acetate  
138 concentration in the bottles was 200  $\mu\text{M}$ , which was added either as 200  $\mu\text{M}$  on day four,  
139 or as 40  $\mu\text{M}$  every other day for a total of 200  $\mu\text{M}$  of acetate by day 12 of the incubation  
140 period.

141 The incubations lasted a total of 13 to 15 days. At the conclusion of the experiment  
142 one ml of water from each bottle was set aside for flow cytometry, and then up to one  
143 liter from each bottle was filtered through either combusted 0.2  $\mu\text{m}$  Anodisc filters  
144 (Whatman International Ltd. Maidstone, England) or 0.22  $\mu\text{m}$  Sterivex filters (Millipore  
145 Corp. Billerica, MA). Filters were stored at -80°C until further processing. Water passed  
146 through the Anodisc filters (40 mL) was acidified using hydrochloric acid to pH~2 and  
147 stored at 4°C for dissolved organic carbon (DOC) analysis.

#### 148 **Flow Cytometry**

149 Water samples for flow cytometry were fixed with 0.2% w/v paraformaldehyde  
150 (final concentration), placed in the dark for at least 10 min at room temperature to harden  
151 cells, and stored at -80°C until sample processing. A Becton-Dickinson FACSCalibur  
152 flow cytometer was used for cell enumeration. Heterotrophic cells were enumerated after  
153 staining with a 1x working stock of SYBR Green I (Invitrogen, Carlsbad, CA) for 15 min

154 following a protocol modified from Marie et al. (1997). Flow rates for cell abundance  
155 calculations were determined by the addition of known concentrations of 1  $\mu\text{m}$   
156 Fluoresbrite YG microspheres (Polysciences, Warrington, PA) to each sample prior to  
157 loading on the flow cytometer. The coefficient of variability between triplicate runs on  
158 the flow cytometer to determine bacterial abundances was <7%.

159 **Dissolved organic carbon (DOC)**

160 DOC concentrations were measured with a Shimadzu TOC-V<sub>CSH</sub> total organic  
161 carbon analyzer using sucrose as a standard solution. DOC concentration was determined  
162 by subtracting the instrument blank area from the average peak area and dividing by the  
163 slope of the standard curve. Comparisons to low carbon water and deep-sea reference  
164 water provided by Prof. D. Hansell (University of Miami) were made daily. The  
165 coefficient of variability between triplicate injections was <1%.

166 **DNA extractions**

167 DNA was extracted from all filters using the UltraClean Soil DNA Kit (MoBio  
168 Laboratories, Inc. Carlsbad, CA), following the manufacturer's alternative protocol with  
169 the following additional modifications. The outer plastic shell of the Sterivex unit was  
170 broken. The filter was then removed and cut with flame-sterilized scissors prior to the  
171 DNA extraction. After addition of 200  $\mu\text{l}$  Solution IRS, samples were vortexed in a Mini-  
172 Beadbeater™ (BioSpec Products, Inc. Bartlesville, OK) at 4800 rpm for 5 minutes. The  
173 volume of solution S3 was reduced to 1.25 ml, and the addition of Solution S4 and the  
174 subsequent centrifugation step was repeated twice.



175 **Ultracentrifugation**

176           Extracted DNA was mixed with cesium chloride and spun in a Beckman Coulter  
177 Optima L-80 XP Ultracentrifuge (Fullerton, CA) following protocols modified from  
178 Freitag et al. (2006). DNA was mixed with 1x TE buffer (10 mM Tris and 1 mM EDTA)  
179 to obtain a final concentration of 500 ng of DNA in 800  $\mu\text{L}$ , and 4.25 mL of a cesium  
180 chloride solution (measured refractive index = 1.4143, corresponding to a calculated  
181 density of  $1.859 \text{ g ml}^{-1}$ ) was added to 4.9 mL OptiSeal tubes. DNA was spun at 140,000 x  
182 g for 66 hours at 20°C using a VTi 65.2 vertical rotor. Each batch of tubes included a  
183 standard to identify the proper separation of  $^{12}\text{C}$ - and  $^{13}\text{C}$ -labeled DNA. The standard was  
184 comprised of equal amounts of DNA extracted from *Halomonas halodurans* grown on  
185 unlabeled glucose or [ $^{13}\text{C}$ ] glucose (99%; Cambridge Isotope Laboratories).

186           At the conclusion of the ultracentrifugation run, a series of 250  $\mu\text{L}$  aliquots were  
187 removed from each tube using a pipette starting with the fraction at the top of the tube.  
188 Each fraction's refractive index was measured with an AR200 Digital Refractometer  
189 (Reichert, Inc. Depew, NY). The refractive index was converted to buoyant density using  
190 a linear regression calculated from tables translating refractive index to buoyant density.  
191 DNA was then precipitated following a protocol adapted from Griffiths et al. (2000) and  
192 Freitag et al. (2006). Two volumes of a 30% w/v polyethylene glycol 6000/1.6 M sodium  
193 chloride solution were added to each fraction. Fractions were incubated at 4°C for 24  
194 hours and then centrifuged at 20,000 x g for 15 minutes at 4°C. The supernatant was  
195 discarded and 1 ml of cold 70% ethanol was added to each fraction followed by  
196 centrifugation at 20,000 x g for 10 minutes at 4°C. This step was repeated twice more,  
197 discarding the supernatant each time for a total of three washings. The DNA pellet was

198 then dried and resuspended in 10 mM Tris. DNA from two adjacent fractions was then  
199 combined resulting in eight fractions from each sample.

200 Two factors were used to determine which fractions contained DNA labeled with  
201  $^{13}\text{C}$  and which contained  $^{12}\text{C}$ -DNA. First, we examined the separation of  $^{12}\text{C}$ -DNA and  
202  $^{13}\text{C}$ -DNA in our standard tube with quantitative PCR (see below for details). The position  
203 of the  $^{12}\text{C}$  and  $^{13}\text{C}$  peaks between the different ultracentrifugation runs varied  $<0.01 \text{ g ml}^{-1}$   
204  $^1$  within the cesium chloride gradient ( $n = 4$ , data not shown), implying that separation of  
205 DNA was reproducible between individual ultracentrifugation runs. Second, we  
206 examined variability in  $^{12}\text{C}$ -DNA buoyant density. DNA was extracted from a no carbon  
207 addition bottle, separated by ultracentrifugation, and processed in the same manner as the  
208  $^{13}\text{C}$ -substrate DNA. As expected, PCR product was only obtained from the upper,  $^{12}\text{C}$ -  
209 DNA, region of the cesium chloride gradient. The density of fractions which did not  
210 amplify with PCR were noted and used to constrain the  $^{13}\text{C}$ -DNA region of the cesium  
211 chloride gradient. Based on these two independent assessments, densities  $\geq 1.7258 \text{ g ml}^{-1}$   
212 contained  $^{13}\text{C}$ -labeled DNA.

### 213 **Quantitative PCR**

214 Quantitative PCR was used to amplify a section of the 16S rRNA gene with the  
215 ABsolute™ QPCR SYBR Green Mix (Thermo Fisher Scientific, Inc. Waltham, MA).  
216 Reaction conditions were 1x master mix and 200 nM of each primer: 27F and 519R  
217 (Operon Biotechnologies, Inc. Huntsville, AL). An enzyme activation cycle (95°C, 15  
218 minutes) was followed by 40 cycles of denaturation (95°C, 15 seconds), annealing (55°C,  
219 30 seconds), and extension (72°C, 30 seconds). This was followed by a melting curve  
220 program (95°C, 15 seconds, 55°C, 15 seconds) concluding at 95°C for 15 seconds.

221 **Community fingerprinting**

222 Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis was used  
223 to assess bacterial community composition in DNA extracted from the groundwater  
224 filling the sample bottles and DNA obtained from the ultracentrifugation fractions. DNA  
225 was amplified using the GoTaq Master Mix (Promega Corp. Madison WI). Reaction  
226 conditions were 2-10 ng of DNA, 700 nM FAM-labeled 27F and 700 nM 519R, and 1x  
227 GoTaq colorless master mix which contains 200  $\mu$ M dNTPs (final concentration) and 1.5  
228 mM MgCl<sub>2</sub> (final concentration). PCR conditions were an initial denaturation (95°C for 5  
229 minutes) followed by 35 cycles of denaturation (95°C, 30 seconds), annealing (46°C, 30  
230 seconds), extension (72°C, 90 seconds), and a final extension cycle of 72°C for 5 minutes.  
231 Due to the low recovery of DNA precipitated from the cesium chloride, nested PCR was  
232 used to amplify DNA from the ultracentrifugation fractions. This involved an initial  
233 amplification with 15 cycles of the PCR program described above followed by transfer  
234 into a new PCR reaction and amplification for an additional 35 cycles of the same PCR  
235 program. At least two additional negative controls from the first PCR reaction were run  
236 in the second reaction to detect possible contamination due to the increased number of  
237 cycles.

238 PCR products were digested using 1 U of the restriction endonuclease *Hin6I*  
239 (Fermantas International, Inc. Burlington, Ontario) in Tango Buffer at 37°C for 2 hours.  
240 The choice of the enzyme was based on the high number of possible restriction fragments  
241 obtained with a virtual digest at the Microbial Community Analysis web site (Shyu *et al.*,  
242 2007). After the restriction digest, DNA was precipitated with 0.3 vol 2 M lithium  
243 chloride and 2 vol 100% ethanol, centrifuged 15 min at 3220 x g, washed twice with 2

244 vol cold 70% ethanol, and dried. After mixing with Hi-Di formamide (Applied  
245 Biosystems) and MegaBACE™ ET900-R size standard (GE Healthcare,  
246 Buckinghamshire, UK), the terminal restriction fragments (TRFs) were analyzed on an  
247 Applied Biosystems 3730XL capillary sequencer.

248         Chromatograms were analyzed using DAX Data Acquisition and Analysis software  
249 Version 8.0 (Van Mierlo Software Consultancy Eindhoven, the Netherlands). The ladder  
250 added to each well included 37 fragments of known size ranging from 60 to 900 base  
251 pairs, and the position of these peaks was used to determine the length of TRFs within  
252 each well. The position of TRFs between samples was aligned using Matlab code written  
253 by Liam Finlay (Finlay, L., J. Kitner, S.J. Giovannoni and E.B. Kujawinski,  
254 unpublished). The program gathers fragment lengths from all TRFs and applies a user-  
255 defined error (0.75 for the present project) to align TRFs from different wells on the same  
256 T-RFLP plate or between multiple plates. Peaks above an experimentally determined  
257 threshold value (100 fluorescent units for the present project) were then converted into a  
258 presence/absence matrix, thereby not considering differences in relative peak heights  
259 between samples.

#### 260 **In silico identification of TRFs**

261         The web-based Microbial Community Analysis tool (MiCA, Department of  
262 Biological Sciences, University of Idaho, <http://mica.ibest.uidaho.edu/>) was used to  
263 obtain in silico identifications of the TRFs (Shyu *et al.*, 2007). The in silico analysis  
264 results in a list of 16S rRNA genes with restriction sites which would produce fragments  
265 the length of the user-provided TRFs. The web site allows the user to change the  
266 sensitivity of the analysis, and the following settings were used in the present project: one

267 mismatch within one base from the 5' end of the primer and a window size of 2 base  
268 pairs. We wrote code in Matlab to place the taxonomic identity of each sequence within  
269 the Hugenholtz taxonomic outline of 16S rRNA genes available from greengenes.lbl.gov  
270 (DeSantis *et al.*, 2006). A single TRF may match different 16S rRNA genes. In order to  
271 eliminate TRFs matching 16S rRNA genes within different taxonomic groups, we  
272 considered a hit to be valid only if more than 50% of the sequences matching the TRF  
273 were from the same taxonomic group.

#### 274 **Cloning and sequencing of 16S rRNA genes**

275 Two samples were chosen for further analysis using clone libraries followed by  
276 DNA sequencing: one from a bottle with whole water (p02) and a second sample from a  
277 1 µm-filtered bottle (p01). Both of the bottles had received a single addition of carbon.  
278 Almost full-length 16S rRNA genes were amplified and analyzed using conditions  
279 previously described (Longnecker & Reysenbach, 2001). Briefly, DNA was amplified  
280 using 27F and 1492R. The resulting PCR products were cloned using the pCR4-TOPO  
281 vector (TOPO-TA, Invitrogen) according to the manufacturer's directions. DNA was  
282 extracted from the resulting colonies using a modified alkaline lysis protocol (Sambrook  
283 *et al.*, 1989). Plasmid DNA was screened for appropriate-sized inserts with vector-  
284 specific primers. The PCR-amplified inserts were then digested with 1U of the restriction  
285 endonucleases *MspI* and *HinPI* following the manufacturer's instructions (New England  
286 Biolabs). The resulting products were separated by gel electrophoresis on a 3.5%  
287 NuSieve GTG agarose gel (Lonza) run in TBE buffer at 4°C. The clones were separated  
288 into different phylotypes based on the RFLP banding patterns.

289 At least one of each RFLP pattern was sequenced to 2x coverage by cycle  
290 sequencing using Applied Biosystems Big Dye Terminator v3.1. The coverage of each  
291 clone library was assessed using  $C = [1 - (n/N)] * 100$  where  $n$  = number of RFLP groups  
292 with a single clone and  $N$  = the total number of clones examined (Good, 1953).  
293 Sequences were assembled using Sequencher (Gene Codes Corporation), and the primer  
294 and vector were trimmed from each sequence. Chimeras identified by Bellerophon  
295 (Huber *et al.*, 2004) were removed from further analysis. GenBank sequence accession  
296 numbers are FJ602393-FJ602433; clones are preceded by 'p02' (whole water bottle) or  
297 'p01' (1  $\mu$ m-filtered bottle).

298 The phylogenetic association of each clone was determined using the small subunit  
299 rRNA taxonomy and alignment pipeline (STAP, Wu *et al.*, 2008). STAP gathers related  
300 16S rDNA sequences, aligns them to the target sequence, and then automatically  
301 generates phylogenetic trees to determine the taxonomic assignment for each DNA  
302 sequence.

### 303 **Indicator Species Analysis**

304 Indicator Species Analysis (ISA) was used to identify TRFs unique to different  
305 treatments (Dufrene & Legendre, 1997; McCune & Grace, 2002). ISA takes into account  
306 the relative abundance and the relative frequency of each TRF within a pre-defined  
307 group. The output from ISA is an indicator value for each TRF, with higher indicator  
308 values assigned to TRFs that are most representative of a pre-defined group. The highest  
309 indicator value possible is 100, obtained when a TRF is found within all samples of only  
310 one pre-defined group. The significance of the indicator value was evaluated by Monte  
311 Carlo simulations during which groups were randomized 1000 times and the indicator

312 value of the randomized data was compared to the real data. A cutoff of  $p < 0.05$  was used  
313 to determine if peaks were statistically significant indicators of the pre-defined groups.  
314 The use of relative abundance information in T-RFLP is problematic due to the potential  
315 for PCR bias (Suzuki & Giovannoni, 1996), therefore only the presence or absence of a  
316 TRF was used in the ISA calculations which reduced the number of TRFs with  
317 significant indicator values. While the use of ISA is not common in microbial ecology, it  
318 proved to be useful in identifying TRFs within different redox zones of tropical soil (Pett-  
319 Ridge & Firestone, 2005).

### 320 **Statistical analysis**

321 Nonmetric multidimensional scaling (NMS) (Kruskal, 1964; Mather, 1976) was  
322 used to analyze variability in bacterial community composition. NMS is a multivariate  
323 statistical technique that can be used to examine similarities, or differences, between  
324 samples by reducing the comparisons between samples from a multidimensional space to  
325 fewer dimensions, preferably two or three. Differences between samples were calculated  
326 based on the presence or absence of TRFs. The differences were then presented  
327 graphically in a multidimensional space; samples which are close together in the  
328 ordination are more similar than samples located further apart. Distances between  
329 samples were calculated with a distance measure using the Fathom toolbox (David Jones,  
330 University of Miami – Rosenstiel,  
331 <http://www.rsmas.miami.edu/personal/djones/matlab/matlab.html>). The distance measure  
332 used was either the Bray-Curtis distance measure or a relative Bray-Curtis distance  
333 measure as noted in the results section. The relative Bray-Curtis distance measure  
334 normalizes the distances by the total number of TRFs in each sample; this removes TRF

335 number as a factor in inter-sample variability. The statistics toolbox in Matlab was used  
336 to run the NMS analyses, and additional code was written to assess the dimensionality of  
337 the data set by comparing 40 runs with real data to 50 runs with randomized data.  
338 Additional axes were considered if the addition of the axis resulted in a significant  
339 improvement over the randomized data (at  $p \leq 0.05$ ) and the reduction in stress was  
340 greater than 0.05. The p-values were calculated as the proportion of randomized runs  
341 with stress less than or equal to the observed stress which was calculated using Kruskal's  
342 stress formula 1; stress is a measure of goodness of fit used in NMS. The proportion of  
343 variation represented by each axis was assessed with a Mantel test to calculate the  
344 coefficient of determination ( $r^2$ ) between distances in the ordination space and distance in  
345 the original space.

346 One-way analysis of similarity (ANOSIM) was used to assess if groups visualized  
347 by NMS were statistically significant. Matlab code for ANOSIM was also from the  
348 Fathom toolbox. The Bray-Curtis distance matrix calculated for the NMS was used for  
349 ANOSIM, and the distances were converted to ranked distances prior to ANOSIM  
350 calculations. The significance of each group was tested by 1000 randomizations of the  
351 dataset, and p-values were calculated to determine the probability of no difference  
352 between groups.

## 353 **Results**

### 354 **Bacterial abundance and DOC concentrations**

355 In the groundwater used to establish the experiment, the abundance of grazers was  
356 less than 100 cells  $\text{ml}^{-1}$ . By the conclusion of the experiment, both bacterial abundances  
357 and dissolved organic carbon concentrations were elevated relative to the initial



358 groundwater (Table 2). Measurements of the abundance of grazers present at the  
359 conclusion of the experiment were not conducted. Bacterial abundances in the water were  
360 highest in 1  $\mu\text{m}$ -filtered bottles even when no carbon was added to the bottles. Dissolved  
361 organic carbon concentrations were higher in bottles with whole water compared to  
362 bottles with 1  $\mu\text{m}$ -filtered water, and were higher in bottles which received multiple  
363 additions of carbon as opposed to a single addition of carbon. The increase in dissolved  
364 organic carbon in the bottles which did not receive added carbon was likely due to carbon  
365 leaching off the sediment added to each bottle, although we cannot discount the  
366 contribution of DOC exuded by bacterial cells.

#### 367 **Bacterial community analysis based on DNA extracted from filters**

368 Variability in bacterial community composition within the groundwater filling the  
369 bottles was assessed using NMS analysis of the T-RFLP data; we did not assess the  
370 bacterial community composition in the sand remaining at the bottom of each sampling  
371 bottle. The first step in this process was to compare similarities between samples based  
372 on the presence or absence of TRFs using the Bray-Curtis distance measure. NMS was  
373 then used to visualize differences between samples as shown in Figure 2. The NMS  
374 calculation resulted in an ordination with a final stress of 0.1734, and  $r^2 = 0.75$  with the  
375 variability split between the two axes ( $r^2$  on axis 1 = 0.38,  $r^2$  on axis 2 = 0.26). The  
376 bacterial community in bottles which received no carbon was not distinct from the  
377 bacterial community in bottles which received one or multiple carbon additions (Figure  
378 2A). All of the bottles received the same aliquot of autoclaved sediment at the beginning  
379 of the experiment. There were also no significant differences in bacterial community  
380 composition between bottles which received unlabeled acetate compared to bottles with

381 labeled acetate (Figure 2B). The presence of grazers significantly altered the composition  
382 of the bacterial community (ANOSIM,  $R = 0.48$ ,  $p = 0.0010$ ). The separation of the  
383 whole water and grazer-free bottles is visually evident by the clustering of whole water  
384 samples on the left side of axis one, and grazer-free samples on the right side of axis one  
385 (Figure 2C).

386         The in silico analysis at the MiCA web site produced over 36,000 matches to the  
387 TRFs we obtained from our samples. The results from the in silico analysis for the bottles  
388 which received a single addition of acetate compared to bottles which received multiple  
389 acetate additions were combined because of the lack of significant differences in  
390 community composition between those two treatments. Taxonomic assignments  
391 restricted to the phylum level identified between 30 and 48% of the TRFs from the  
392 present study. At the lower taxonomic levels (class, order, etc.), the number of TRFs  
393 identified dropped further because of an increase in the number of ambiguous  
394 identifications (data not shown). Based on results from the in silico analysis, the bacterial  
395 community in the sample bottles at the conclusion of the experiment was dominated by  
396 Firmicutes and Proteobacteria, with smaller contributions from Actinobacteria and  
397 Bacteroidetes (Table 3). The initial groundwater community was also dominated by  
398 Firmicutes and Proteobacteria (Table 3 – column 1), but this conclusion is based on lower  
399 numbers of TRFs due to the smaller number of samples processed from the initial  
400 groundwater.

401         The two clone libraries provided more detailed phylogenetic information about the  
402 bacterial diversity in a whole water bottle (clone library p02) compared to a 1  $\mu\text{m}$ -filtered  
403 bottle (clone library p01). A total of 192 clones were analyzed with 96 clones from each

404 clone library. After screening of the clone libraries using RFLP, 22 clones were fully  
405 sequenced from p01 while 28 clones were sequenced from p02. The clone library  
406 coverage of p01 was slightly higher than the coverage of p02 (91% and 87%,  
407 respectively). After removing chimeras, more than half of the sequences were from the  
408 Proteobacteria and the Bacteroidetes (Table 4) and most of the sequences were present in  
409 both clone libraries.

410       There were differences in phylogenetic diversity between the clone libraries and the  
411 in silico diversity assessment from the T-RFLP data. No Firmicutes were present in the  
412 clone libraries and only a single Actinobacteria sequence was observed. We used Probe  
413 Match at the Ribosomal Database Project (RDP, Cole *et al.*, 2009) to examine if the  
414 differences could be due to the presence of mismatches between our primers and the 16S  
415 rDNA sequences archived at RDP. There were 176,281 Firmicute sequences at RDP (as  
416 of December 2008), and the 519R primer we used for the T-RFLP matches 77%, 85%, or  
417 87% of those sequences if zero, one, or two mismatches are allowed. For the 1492R  
418 primer used for the clone libraries, only 2%, 10%, or 12% of the Firmicute sequences  
419 match if zero, one, or two mismatches are allowed. A similar situation was observed for  
420 the Actinobacteria where 65-75% of the 54,945 Actinobacteria sequences at RDP match  
421 the 519R primer, but only 3-17% of Actinobacteria sequences match the 1492R primer.  
422 Thus, we conclude that the clone libraries were likely biased against Firmicutes and  
423 Actinobacteria. This comparison between DNA sequences and primers presupposes that  
424 the sequences available from RDP are broadly representative of the DNA sequences  
425 which are added to online databases at an exponential rate (Benson *et al.*, 2009).

426 **Bacterial community composition following ultracentrifugation**

427 DNA extracts from seven bottles which received  $^{13}\text{C}$ -acetate were subjected to  
428 ultracentrifugation: two bottles from each of the carbon addition treatments listed in  
429 Table 1, except for the whole water bottles with multiple carbon additions where only  
430 one replicate was subjected to ultracentrifugation. A higher number of unique TRFs was  
431 obtained within the fractions from 1  $\mu\text{m}$ -filtered bottles compared to fractions from the  
432 whole water bottles (Table 3). Identifications from the in silico analysis of the  $^{13}\text{C}$ -labeled  
433 DNA fractions were predominantly Proteobacteria and Firmicutes (Table 3). Due to our  
434 conservative choices for the in silico analysis, only a portion of the TRFs were identified  
435 and therefore a component of the bacterial diversity remains unknown.

436 Patterns in the community composition of active ( $^{13}\text{C}$ -labeled) bacterial cells in  
437 whole and 1- $\mu\text{m}$  filtered treatments were assessed using NMS. A relative Bray-Curtis  
438 distance measure was used for this NMS analysis. This removes the bias from the  
439 observed differences in the number of TRFs between the whole and 1  $\mu\text{m}$ -filtered  
440 samples. The resulting NMS ordination had a final stress of 0.1289 and  $r^2 = 0.8395$  with  
441  $r^2$  on axis 1 = 0.6224 and  $r^2$  on axis 2 = 0.1582 (Figure 3). Similar to the observations for  
442 the DNA extracted from the filters, there was no significant difference in the composition  
443 of  $^{13}\text{C}$ -labeled DNA from the bottles which received a single addition of carbon  
444 compared to bottles which received multiple carbon additions (ANOSIM, p-value >  
445 0.05). However, there was a significant difference in the composition of active cells in  
446 the bottles with whole water compared to bottles with 1  $\mu\text{m}$ -filtered groundwater  
447 (ANOSIM,  $R = 0.2573$ ,  $p = 0.0234$ ).

448           The output from the Indicator Species Analysis provided three TRFs with  
449 significant indicator values: a Bradyrhizobiales, a Fibrobacterales, and one TRF which  
450 could not be identified. Significant indicator values were only obtained for TRFs  
451 primarily found within the <sup>13</sup>C-labeled DNA. The Fibrobacterales (indicator value = 80,  
452 p-value = 0.004) and the unidentified TRF (indicator value = 80, p-value = 0.016) were  
453 primarily found within the <sup>13</sup>C-DNA from 1 µm-filtered bottles. Bradyrhizobiales had  
454 high indicator values for <sup>13</sup>C-DNA in both whole (indicator value = 100, p-value =  
455 0.0090) and 1 µm-filtered bottles (indicator value = 72, p-value = 0.036), but not in the  
456 <sup>12</sup>C-DNA fractions. The indicator values for the Bradyrhizobiales are different between  
457 the whole water and 1 µm-filtered water because the analysis was run twice: once for the  
458 whole water bottles to compare <sup>12</sup>C- and <sup>13</sup>C-DNA, and a second time for the 1 µm-  
459 filtered bottles. Based on the higher indicator value, the Bradyrhizobiales is a better  
460 indicator of the <sup>13</sup>C-labeled community in the whole water bottles compared to the 1 µm-  
461 filtered bottles.

## 462 **Discussion**

463           Microorganisms in the subsurface represent a large component of bacterial biomass  
464 (Whitman *et al.*, 1998) and understanding the factors which control bacterial community  
465 composition and metabolic activity are critical to link the microbial community with  
466 subsurface biogeochemical cycles. We used a community fingerprinting technique, T-  
467 RFLP, to assess how variability in carbon supply and grazing affected the community  
468 structure of metabolically active bacterial cells in groundwater. Our use of T-RFLP was  
469 conservative in that we sought to explain patterns in bacterial community composition.  
470 Previous modeling studies and careful analysis of in situ samples has shown this to be a

471 valid use of T-RFLP data (Hartmann & Widmer, 2008; Zhang *et al.*, 2008). We also  
472 recognize that manipulating the groundwater can alter both bacterial diversity (Massana  
473 *et al.*, 2001; Agis *et al.*, 2007) and organic matter (Fuhrman & Bell, 1985). Thus, as with  
474 any experimental manipulation, there are compromises with respect to experimental  
475 design. In the present project we recognize that separating bacterial cells from grazers via  
476 filtration artificially altered the in situ community. An alternative design would be to add  
477 cultured flagellates to groundwater which has its own methodological concerns.

478         The active and inactive components of the bacterial community in our samples  
479 were separately identified using stable isotope probing. While stable isotope probing  
480 provides a window into the diversity and community composition of the metabolically  
481 active community (Neufeld *et al.*, 2007a), it is not without methodological problems  
482 (Neufeld *et al.*, 2007b). We attempted to minimize these issues with a number of  
483 constraints. For example, we relied on incubations without added carbon to confirm the  
484 location of <sup>13</sup>C-labeled DNA. Although this experiment was shorter than other studies  
485 that rely on stable isotope probing, transfer of labeled carbon within the microbial  
486 community was still likely during our incubations. As a result we do not claim that all the  
487 organisms identified within the “active” community are directly involved in acetate  
488 metabolism. Instead, we suggest that these organisms are participants in the broader  
489 carbon cycle within groundwater. Finally, the experiment relied on a single carbon source  
490 and our conclusion about the diversity of active cells is therefore only based on bacterial  
491 cells able to assimilate acetate and/or its metabolites. Acetate was chosen as a substrate in  
492 the present project to allow us to build on previous research in which acetate assimilation  
493 was used to characterize bacterial activity in a hydrocarbon-contaminated aquifer (Pombo

494 *et al.*, 2002; Pombo *et al.*, 2005). Inferences about other carbon sources will require  
495 additional research; however, our conclusions about the diversity of cells able to  
496 assimilate acetate are an important step towards identifying the bacterial community  
497 involved in the carbon cycle within groundwater.

498         Previous studies have shown that variability in the rate of substrate addition  
499 affected bacterial diversity (Carrero-Colón *et al.*, 2006), and that microorganisms able to  
500 respond rapidly to changes in substrate availability can out-compete microorganisms  
501 adapted to steady nutrient concentrations (Pernthaler *et al.*, 2001). In contrast, in the  
502 present study, variability in substrate supply (i.e. bottom-up control) did not affect  
503 bacterial community composition. Although bacterial abundance increased during the  
504 incubations suggesting that the bacterial community in the bottles was able to grow under  
505 our experimental conditions, there were no shifts in bacterial community composition  
506 which could be linked to substrate supply.

507         Experimental manipulation of the grazer population indicated that top-down control  
508 affected bacterial community composition in the present study. In aquatic ecosystems, the  
509 presence of grazers alters bacterial community composition (Suzuki, 1999; Jürgens &  
510 Matz, 2002; Beardsley *et al.*, 2003; Vázquez-Domínguez *et al.*, 2005), but the mechanism  
511 by which grazers select their prey and shape the bacterial community remains poorly  
512 understood (Boenigk & Arndt, 2002; Weisse, 2002). In a sedimentary aquifer, Nagaosa *et*  
513 *al.* (2008) found removal of grazers resulted in an increase in the number of operational  
514 taxonomic units identified within DNA collected 2 m below the surface, but the opposite  
515 situation was observed at 10 m depth. In the present study, we observed more restriction

516 fragments in  $^{13}\text{C}$ -DNA from grazer-free bottles compared to the number of restriction  
517 fragments found in  $^{13}\text{C}$ -DNA from the whole water bottles.

518         We consider two possible explanations for grazer control of bacterial diversity.  
519 First, grazers selectively consume more active bacterial cells (González *et al.*, 1990;  
520 Sherr *et al.*, 1992; González *et al.*, 1993; del Giorgio *et al.*, 1996). In our whole water  
521 incubations, the grazers could have removed the active bacterial cells resulting in the  
522 observed decrease in bacterial diversity. This removal of active bacterial cells was  
523 observed in our data as a lower number of TRFs when grazers were present.  
524 Alternatively, the increased diversity in the grazer-free incubations could reflect bacterial  
525 cells able to grow only when grazing pressure was relieved. Although the present project  
526 only considered the presence or absence of different bacterial phylogenetic groups, higher  
527 bacterial abundances were observed in the grazer-free incubations. Therefore data on the  
528 abundance of metabolically active bacterial cells will be necessary in order to assess  
529 which mechanism better explains the lower diversity in whole water incubations: growth  
530 in the absence of grazers or grazing of active cells.

531         The combination of the *in silico* analysis of the T-RFLP data and the clone libraries  
532 provided valuable information on the diversity of bacterial cells within the bottles.  
533 However, *in silico* analyses and clone libraries have different limitations when used to  
534 assess patterns in bacterial diversity. The *in silico* analyses ignore potential differences  
535 between the observed and predicted length of the restriction fragments and whether  
536 multiple organisms can have restriction fragments of the same length (Kitts, 2001; Thies,  
537 2007). To reduce these issues, we chose to be conservative and we focus here on the  
538 phylum level rather than present more detailed taxonomic information. Meanwhile, 16S



539 rDNA clone libraries offer less coverage of microbial diversity than is possible with  
540 pyrosequencing (Sogin *et al.*, 2006) or metagenomic approaches (Rusch *et al.*, 2007). We  
541 observed differences between the clone libraries and the in silico analysis which may be  
542 due to the use of two different reverse primers: one for the T-RFLP analysis and one for  
543 generating the PCR product used for the clone libraries. While both primers were  
544 designed to target Bacteria, the presence of mismatches between the primer and a target  
545 sequence decreases the likelihood a sequence will be amplified (Sipos *et al.*, 2007; Bru *et*  
546 *al.*, 2008). Furthermore, even when in silico analyses reveal a high level of specificity for  
547 a primer set, use of the primer set can result in amplification of a significant proportion of  
548 non-target sequences (Morales & Holben, 2009). The mismatches between target  
549 sequences and primers we observed using RDP's Probe Match likely biased our clone  
550 library against the Firmicutes and Actinobacteria identified from the in silico analysis of  
551 the T-RFLP data. Amplicon size, primer mismatch, cloning issues, and PCR-induced  
552 errors can all bias bacterial diversity assessments (Huber *et al.*, 2009). Therefore, due to  
553 the observed differences between the two methods used in the present study, we base our  
554 conclusions on the diversity of bacterial cells in our incubations on the combination of  
555 both datasets rather than relying solely on the T-RFLP data or the clone libraries.

556         The dominance of Proteobacteria in both the in silico analyses and the clone  
557 libraries is consistent with previous observations of Proteobacteria within aquifers  
558 (López-Archilla *et al.*, 2007; Blöthe & Roden, 2009). Proteobacteria have been observed  
559 in regions of sulfate reduction, denitrification, and iron-based metabolisms (López-  
560 Archilla *et al.*, 2007) suggesting this phylum may play a role in these biogeochemical  
561 transformations. Proteobacteria are capable of many different metabolisms, and the

562 possibility that they are involved in iron-based metabolisms is intriguing due to the  
563 presence of iron in both groundwater (Charette & Sholkovitz, 2006) and sediments  
564 (Charette *et al.*, 2005) sampled within 100 meters of our sampling location. Furthermore,  
565 most probable number (MPN) assays have enumerated bacterial cells participating in  
566 both iron oxidation and iron reduction in groundwater (Blöthe & Roden, 2009). In that  
567 same study, clone libraries generated from the groundwater and the MPN cultures were  
568 entirely Proteobacteria, with Betaproteobacteria representing more than 50% of the 16S  
569 rDNA sequences (Blöthe & Roden, 2009). While we cannot definitely state that the  
570 Proteobacteria identified within our bottle incubations were involved in iron-based  
571 metabolisms, evaluating microbial involvement in the subsurface iron cycle at our study  
572 site will be an interesting area of future research.

573         Two other phylogenetic groups, Saprospiraceae and Firmicutes, were dominant in  
574 our assessment of bacterial diversity. However, their importance is harder to assess  
575 because the Saprospiraceae were only found in the clone libraries while the Firmicutes  
576 were only identified with the *in silico* analysis of the T-RFLP data. To our knowledge,  
577 Saprospiraceae have not previously been observed in aquifers though they are found in  
578 freshwater lakes and ponds (Schauer & Hahn, 2005), in activated sludge (Kong *et al.*,  
579 2007; Xia *et al.*, 2008), and in an experiment examining carbon cycling in activated  
580 sludge (Ginige *et al.*, 2004). Firmicutes have also been identified within aquifers  
581 although they are generally more abundant in contaminated aquifers. Two studies have  
582 found a small proportion of Firmicutes within pristine aquifers (Miyoshi *et al.*, 2005;  
583 López-Archilla *et al.*, 2007). Within hydrocarbon-contaminated aquifers, Firmicutes can  
584 account for 30-60% of DNA sequences obtained from the bacterial community

585 (Richardson *et al.*, 2002; Gu *et al.*, 2004; Macbeth *et al.*, 2004; Bowman *et al.*, 2006).  
586 Due to the small number of studies on oligotrophic aquifers, we cannot make any  
587 inferences as to what causes such variability in the prevalence of the Firmicutes.  
588 However, Firmicutes were present in groundwater prior to the onset of the present  
589 project, and their presence within the Waquoit Bay aquifer indicates that much remains to  
590 be understood about bacterial diversity within non-hydrocarbon contaminated aquifers.  
591 To our knowledge, the presence of Firmicutes in groundwater has not been correlated  
592 with any single microbial metabolism, and therefore we cannot speculate on their role in  
593 the ecosystem.

594         Indicator Species Analysis provided two bacterial groups on which to focus future  
595 research efforts. A TRF identified as Fibrobacterales was diagnostic of the grazer-free  
596 bacterial community able to assimilate acetate. Fibrobacterales is a small phylum within  
597 the Bacteria and the few which have been described are responsible for degradation of  
598 cellulose within ruminants (Krause & Russell, 1996; Montgomery *et al.*, 1998). Since  
599 Fibrobacterales were found within the <sup>13</sup>C-DNA community, they do not appear to have  
600 been passively transported through the subsurface but were instead actively involved in  
601 the carbon cycle within the grazer-free incubations. The second TRF with a significant  
602 indicator value was a Bradyrhizobiales within the Alphaproteobacteria. This group is  
603 often associated with root nodules, and includes diverse metabolisms such as nitrogen-  
604 fixation, and both aerobic and anaerobic respiration (Kuykendall, 2005). Future work will  
605 be needed to assess how these two groups are involved in biogeochemical cycles within  
606 groundwater.

607 In conclusion, the present project revealed that the availability of carbon did not  
608 structure community composition in a groundwater bacterial community. Instead, the  
609 community composition of acetate-assimilating bacterial cells shifted in response to the  
610 removal of grazers in our experimental manipulations. Phylogenetic identifications from  
611 our incubation experiments included microbial groups known to be present in  
612 groundwater as well as the first identification of the Saprospiraceae which indicates that  
613 we lack a complete picture of bacterial diversity in groundwater. The present study  
614 further provides an important step in determining the factors which control the bacterial  
615 community and carbon cycling in groundwater. Further studies will be needed to assess  
616 whether these patterns are unique to the Waquoit Bay aquifer or extend to other aquifers.

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628 **References**

- 629 Agis M, Granda A & Dolan JR (2007) A cautionary note: Examples of possible microbial  
630 community dynamics in dilution grazing experiments. *J Exp Mar Biol Ecol* **341**: 176-183.
- 631 Andrushchyshyn OP, Wilson KP & Williams DD (2007) Ciliate communities in shallow  
632 groundwater: seasonal and spatial characteristics. *Freshw Biol* **52**: 1745-1761.
- 633 Beardsley C, Pernthaler J, Wosniok W & Amann R (2003) Are readily culturable bacteria  
634 in coastal North Sea waters suppressed by selective grazing mortality? *Appl Environ*  
635 *Microbiol* **69**: 2624-2630.
- 636 Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J & Sayers EW (2009) GenBank.  
637 *Nucleic Acids Res* **37**: D26-31.
- 638 Blöthe M & Roden EE (2009) Microbial iron redox cycling in a circumneutral-pH  
639 groundwater seep. *Appl Environ Microbiol* **75**: 468-473.
- 640 Boenigk J & Arndt H (2002) Bacterivory by heterotrophic flagellates: community  
641 structure and feeding strategies. *Antonie Leeuwenhoek* **81**: 465-480.
- 642 Bone SE, Gonnea ME & Charette MA (2006) Geochemical cycling of arsenic in a  
643 coastal aquifer. *Environ Sci Technol* **40**: 3273-3278.
- 644 Bowman KS, Moe WM, Rash BA, Bae H-S & Rainey FA (2006) Bacterial diversity of  
645 an acidic Louisiana groundwater contaminated by dense nonaqueous-phase liquid  
646 containing chloroethanes and other solvents. *FEMS Microbiol Ecol* **58**: 120-133.

647 Brad T, Braster M, van Breukelen BM, van Straalen NM & Roling WFM (2008)  
648 Eukaryotic diversity in an anaerobic aquifer polluted with landfill leachate. *Appl Environ*  
649 *Microbiol* **74**: 3959-3968.

650 Bru D, Martin-Laurent F & Philippot L (2008) Quantification of the detrimental effect of  
651 a single primer-template mismatch by real-time PCR using the 16S rRNA gene as an  
652 example. *Appl Environ Microbiol* **74**: 1660-1663.

653 Cambareri TC & Eichner EM (1998) Watershed delineation and ground water discharge  
654 to a coastal embayment. *Ground Water* **36**: 626-634.

655 Carrero-Colón M, Nakatsu CH & Konopka A (2006) Effect of nutrient periodicity on  
656 microbial community dynamics. *Appl Environ Microbiol* **72**: 3175-3183.

657 Charette MA & Sholkovitz ER (2006) Trace element cycling in a subterranean estuary:  
658 Part 2. Geochemistry of the pore water. *Geochim Cosmochim Acta* **70**: 811-826.

659 Charette MA, Buesseler KO & Andrews JE (2001) Utility of radium isotopes for  
660 evaluating the input and transport of groundwater-derived nitrogen to a Cape Cod  
661 estuary. *Limnol Oceanogr* **46**: 465-470.

662 Charette MA, Sholkovitz ER & Hansel CM (2005) Trace element cycling in a  
663 subterranean estuary: Part 1. Geochemistry of the permeable sediments. *Geochim*  
664 *Cosmochim Acta* **69**: 2095-2109.

665 Cole JR, Wang Q, Cardenas E, *et al.* (2009) The Ribosomal Database Project: improved  
666 alignments and new tools for rRNA analysis. *Nucleic Acids Res* **37**: D141-D145.

667 Corno G & Jürgens K (2008) Structural and functional patterns of bacterial communities  
668 in response to protist predation along an experimental productivity gradient. *Environ*  
669 *Microbiol* **10**: 2857-2871.

670 DeFlaun MF, Murray CJ, Holben W, *et al.* (1997) Preliminary observations on bacterial  
671 transport in a coastal plain aquifer. *FEMS Microbiol Rev* **20**: 473-487.

672 del Giorgio PA, Gasol JM, Vaqué D, Mura P, Agustí S & Duarte CM (1996)  
673 Bacterioplankton community structure: Protists control net production and the proportion  
674 of active bacteria in a coastal marine community. *Limnol Oceanogr* **41**: 1169-1179.

675 DeLeo PC & Baveye P (1997) Factors affecting protozoa predation of bacteria clogging  
676 laboratory aquifer microcosms. *Geomicrobiol J* **14**: 127-149.

677 DeSantis TZ, Hugenholtz P, Larsen N, *et al.* (2006) Greengenes, a chimera-checked 16S  
678 rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72**:  
679 5069-5072.

680 Dufrene M & Legendre P (1997) Species assemblages and indicator species: The need  
681 for a flexible asymmetrical approach. *Ecol Monogr* **67**: 345-366.

682 Eisenmann H, Harms H, Meckenstock R, Meyer EI & Zehnder AJB (1998) Grazing of a  
683 *Tetrahymena* sp. on adhered bacteria in percolated columns monitored by in situ  
684 hybridization with fluorescent oligonucleotide probes. *Appl Environ Microbiol* **64**: 1264-  
685 1269.

686 Freitag TE, Chang L & Prosser JI (2006) Changes in the community structure and  
687 activity of betaproteobacterial ammonia-oxidizing sediment bacteria along a freshwater-  
688 marine gradient. *Environ Microbiol* **8**: 684-696.

689 Fry NK, Fredrickson JK, Fishbain S, Wagner M & Stahl DA (1997) Population structure  
690 of microbial communities associated with two deep, anaerobic, alkaline aquifers. *Appl*  
691 *Environ Microbiol* **63**: 1498-1504.

692 Fuhrman JA & Bell TM (1985) Biological considerations in the measurement of  
693 dissolved free amino acids in seawater and implications for chemical and microbiological  
694 studies. *Mar Ecol Prog Ser* **25**: 13-21.

695 Fuller ME, Dong H, Mailloux BJ, Onstott TC & DeFlaun MF (2000) Examining bacterial  
696 transport in intact cores from Oyster, Virginia: Effect of sedimentary facies type on  
697 bacterial breakthrough and retention. *Water Resour Res* **36**: 2417-2431.

698 Gasol JM, Pedrós-Alió C & Vaqué D (2002) Regulation of bacterial assemblages in  
699 oligotrophic plankton systems: results from experimental and empirical approaches.  
700 *Antonie Leeuwenhoek* **81**: 435-452.

701 Ghiorse WC & Wilson JT (1988) Microbial ecology of the terrestrial subsurface.  
702 *Advances in Applied Microbiology* **33**: 107-172.

703 Ginige MP, Hugenholtz P, Daims H, Wagner M, Keller J & Blackall LL (2004) Use of  
704 stable-isotope probing, full-cycle rRNA analysis, and fluorescence in situ hybridization-  
705 microautoradiography to study a methanol-fed denitrifying microbial community. *Appl*  
706 *Environ Microbiol* **70**: 588-596.



707 Goldscheider N, Hunkeler D & Rossi P (2006) Review: Microbial biocenoses in pristine  
708 aquifers and an assessment of investigative methods. *Hydrogeol J* **14**: 926-941.

709 González JM, Sherr EB & Sherr BF (1990) Size-selective grazing on bacteria by natural  
710 assemblages of estuarine flagellates and ciliates. *Appl Environ Microbiol* **56**: 583-589.

711 González JM, Sherr EB & Sherr BF (1993) Differential feeding by marine flagellates on  
712 growing vs starving, and on motile vs non-motile, bacterial prey. *Mar Ecol Prog Ser* **102**:  
713 257-267.

714 Good IJ (1953) The population frequencies of species and the estimation of population  
715 parameters. *Biometrika* **40**: 237-264.

716 Griebler C & Lueders T (2009) Microbial biodiversity in groundwater ecosystems.  
717 *Freshw Biol* **54** 649-677.

718 Griebler C, Mindl B, Slezak D & Geiger-Kaiser M (2002) Distribution patterns of  
719 attached and suspended bacteria in pristine and contaminated shallow aquifers studied  
720 with an in situ sediment exposure microcosm. *Aquat Microb Ecol* **28**: 117-129.

721 Griffiths RI, Whiteley AS, O'Donnell AG & Bailey MJ (2000) Rapid method for  
722 coextraction of DNA and RNA from natural environments for analysis of ribosomal  
723 DNA- and rRNA-based microbial community composition. *Appl Environ Microbiol* **66**:  
724 5488-5491.

725 Gu AZ, Hedlund BP, Staley JT, Strand SE & Stensel HD (2004) Analysis and  
726 comparison of the microbial community structures of two enrichment cultures capable of  
727 reductively dechlorinating TCE and cis-DCE. *Environ Microbiol* **6**: 45-54.

728 Haack SK, Fogarty LR, West TG, *et al.* (2004) Spatial and temporal changes in microbial  
729 community structure associated with recharge-influenced chemical gradients in a  
730 contaminated aquifer. *Environ Microbiol* **6**: 438-448.

731 Hartmann M & Widmer F (2008) Reliability for detecting composition and changes of  
732 microbial communities by T-RFLP genetic profiling. *FEMS Microbiol Ecol* **63**: 249-260.

733 Huber JA, Morrison HG, Huse SM, Neal PR, Sogin ML & Welch DBM (2009) Effect of  
734 PCR amplicon size on assessments of clone library microbial diversity and community  
735 structure. *Environ Microbiol* **11**: 1292-1302.

736 Huber T, Faulkner G & Hugenholtz P (2004) Bellerophon: a program to detect chimeric  
737 sequences in multiple sequence alignments. *Bioinformatics* **20**: 2317-2319.

738 Jürgens K & Matz C (2002) Predation as a shaping force for the phenotypic and  
739 genotypic composition of planktonic bacteria. *Antonie Leeuwenhoek* **81**: 413-434.

740 Karner M & Fuhrman JA (1997) Determination of active marine bacterioplankton: a  
741 comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. *Appl*  
742 *Environ Microbiol* **63**: 1208-1213.

743 Kästner M, Fischer A, Nijenhuis I, *et al.* (2006) Assessment of microbial in situ activity  
744 in contaminated aquifers. *Eng Life Sci* **6**: 234-251.

745 Kinner NE, Harvey RW & Kazmierkiewicz-Tabaka M (1997) Effect of flagellates on  
746 free-living bacterial abundance in an organically contaminated aquifer. *FEMS Microbiol*  
747 *Rev* **20**: 249-259.

748 Kitts CL (2001) Terminal restriction fragment patterns: a tool for comparing microbial  
749 communities and assessing community dynamics. *Curr Issues Intest Microbiol* **2**: 17-25.

750 Kong Y, Xia Y, Nielsen JL & Nielsen PH (2007) Structure and function of the microbial  
751 community in a full-scale enhanced biological phosphorus removal plant. *Microbiology*  
752 **153**: 4061-4073.

753 Krause DO & Russell JB (1996) How many ruminal bacteria are there? *J Dairy Sci* **79**:  
754 1467-1475.

755 Kruskal JB (1964) Multidimensional scaling by optimizing goodness of fit to a nonmetric  
756 hypothesis. *Psychometrika* **29**: 1-27.

757 Kuykendall LD (2005) Genus Bradyrhizobium. *The Alpha-, Beta-, Delta- and*  
758 *Epsilonproteobacteria, The Proteobacteria, Part C, Bergey's Manual of Systematic*  
759 *Bacteriology*, Vol. 2 (Brenner, Krieg, Staley & Garrity, eds.), pp. 438-443. Springer, New  
760 York.

761 Lehman RM, O'Connell SP, Banta A, Fredrickson JK, Reysenbach A-L, Kieft TL &  
762 Colwell FS (2004) Microbiological comparison of core and groundwater samples  
763 collected from a fractured basalt aquifer with that of dialysis chambers incubated in situ.  
764 *Geomicrobiol J* **21**: 169-182.

765 Lehman RM, Roberto FF, Earley D, *et al.* (2001) Attached and unattached bacterial  
766 communities in a 120-meter corehole in an acidic, crystalline rock aquifer. *Appl Environ*  
767 *Microbiol* **67**: 2095-2106.

768 Longnecker K & Reysenbach A-L (2001) Expansion of the geographic distribution of a  
769 novel lineage of  $\epsilon$ -Proteobacteria to a hydrothermal vent site on the Southern East Pacific  
770 Rise. *FEMS Microbiol Ecol* **35**: 287-293.

771 López-Archilla AI, Moreira D, Velasco S & López-García P (2007) Archaeal and  
772 bacterial community composition of a pristine coastal aquifer in Doñana National Park,  
773 Spain. *Aquat Microb Ecol* **47**: 123-139.

774 Ludvigsen L, Albrechtsen HJ, Ringelberg DB, Ekelund F & Christensen TH (1999)  
775 Distribution and composition of microbial populations in a landfill leachate contaminated  
776 aquifer (Grindsted, Denmark). *Microb Ecol* **37**: 197-207.

777 Macbeth TW, Cummings DE, Spring S, Petzke LM & Sorenson KS, Jr. (2004) Molecular  
778 characterization of a dechlorinating community resulting from in situ biostimulation in a  
779 trichloroethene-contaminated deep, fractured basalt aquifer and comparison to a  
780 derivative laboratory culture. *Appl Environ Microbiol* **70**: 7329-7341.

781 Madsen EL, Sinclair JL & Ghiorse WC (1991) In situ biodegradation: microbiological  
782 patterns in a contaminated aquifer. *Science* **252**: 830-833.

783 Marie D, Partensky F, Jacquet S & Vaultot D (1997) Enumeration and cell cycle analysis  
784 of natural populations of marine picoplankton by flow cytometry using the nucleic acid  
785 stain SYBR Green I. *Appl Environ Microbiol* **63**: 186-193.

786 Massana R, Pedrós-Alió C, Casamayor EO & Gasol JM (2001) Changes in marine  
787 bacterioplankton phylogenetic composition during incubations designed to measure  
788 biogeochemically significant parameters. *Limnol Oceanogr* **46**: 1181-1188.

789 Mather PM (1976) *Computational methods of multivariate analysis in physical*  
790 *geography*. J. Wiley & Sons, London.

791 McCune BM & Grace JB (2002) *Analysis of Ecological Communities*. MjM Software  
792 Design, Gleneden Beach, Oregon.

793 Miyoshi T, Iwatsuki T & Naganuma T (2005) Phylogenetic characterization of 16S  
794 rRNA gene clones from deep-groundwater microorganisms that pass through 0.2-  
795 micrometer-pore-size filters. *Appl Environ Microbiol* **71**: 1084-1088.

796 Montgomery L, Flesher B & Stahl D (1998) Transfer of *Bacteroides succinogenes*  
797 (Hungate) to *Fibrobacter* gen. nov. as *Fibrobacter succinogenes* comb. nov. and  
798 description of *Fibrobacter intestinalis* sp. nov. *Int J Syst Bacteriol* **38**: 430-435.

799 Morales SE & Holben WE (2009) Empirical testing of 16S rRNA gene PCR primer pairs  
800 reveals variance in target specificity and efficacy not suggested by in silico analysis. *Appl*  
801 *Environ Microbiol* **75**: 2677-2683.

802 Mulligan AE & Charette MA (2006) Intercomparison of submarine groundwater  
803 discharge estimates from a sandy unconfined aquifer. *J Hydrol* **327**: 411-425.

804 Nagaosa K, Maruyama T, Welikala N, *et al.* (2008) Active bacterial populations and  
805 grazing impact revealed by an in situ experiment in a shallow aquifer. *Geomicrobiol J* **25**:  
806 131 - 141.

807 Neufeld JD, Wagner M & Murrell JC (2007a) Who eats what, where and when? Isotope-  
808 labelling experiments are coming of age. *ISME J* **1**: 103-110.

809 Neufeld JD, Dumont MG, Vohra J & Murrell JC (2007b) Methodological considerations  
810 for the use of stable isotope probing in microbial ecology. *Microb Ecol* **53**: 435-442.

811 Novarino G, Warren A, Butler H, *et al.* (1997) Protistan communities in aquifers: a  
812 review. *FEMS Microbiol Rev* **20**: 261-275.

813 Pernthaler A, Pernthaler J, Eilers H & Amann R (2001) Growth patterns of two marine  
814 isolates: Adaptations to substrate patchiness. *Appl Environ Microbiol* **67**: 4077-4083.

815 Pett-Ridge J & Firestone MK (2005) Redox fluctuation structures microbial communities  
816 in a wet tropical soil. *Appl Environ Microbiol* **71**: 6998-7007.

817 Pombo SA, Pelz O, Schroth MH & Zeyer J (2002) Field-scale <sup>13</sup>C-labeling of  
818 phospholipid fatty acids (PLFA) and dissolved inorganic carbon: tracing acetate  
819 assimilation and mineralization in a petroleum hydrocarbon-contaminated aquifer. *FEMS*  
820 *Microbiol Ecol* **41**: 259-267.

821 Pombo SA, Kleikemper J, Schroth MH & Zeyer J (2005) Field-scale isotopic labeling of  
822 phospholipid fatty acids from acetate-degrading sulfate-reducing bacteria. *FEMS*  
823 *Microbiol Ecol* **51**: 197-207.

824 Richardson RE, Bhupathiraju VK, Song DL, Goulet TA & Alvarez-Cohen L (2002)  
825 Phylogenetic characterization of microbial communities that reductively dechlorinate  
826 TCE based upon a combination of molecular techniques. *Environ Sci Technol* **36**: 2652-  
827 2662.

828 Rusch DB, Halpern AL, Sutton G, *et al.* (2007) The Sorcerer II Global Ocean Sampling  
829 expedition: Northwest Atlantic through Eastern Tropical Pacific. *PLoS Biology* **5**: e77.

830 Sambrook J, Fritsch EF & Maniatus T (1989) *Molecular Cloning: A Laboratory Manual*.  
831 Cold Spring Harbor Laboratory Press, Plainview, NY.

832 Sanders RW, Caron DA & Berninger U-G (1992) Relationships between bacteria and  
833 heterotrophic nanoplankton in marine and fresh waters: an inter-ecosystem comparison.  
834 *Mar Ecol Prog Ser* **86**: 1-14.

835 Schauer M & Hahn MW (2005) Diversity and phylogenetic affiliations of  
836 morphologically conspicuous large filamentous bacteria occurring in the pelagic zones of  
837 a broad spectrum of freshwater habitats. *Appl Environ Microbiol* **71**: 1931-1940.

838 Sherr BF, Sherr EB & McDaniel J (1992) Effect of protistan grazing on the frequency of  
839 dividing cells in bacterioplankton assemblages. *Appl Environ Microbiol* **58**: 2381-2385.

840 Shi Y, Zwolinski MD, Schreiber ME, Bahr JM, Sewell GW & Hickey WJ (1999)  
841 Molecular analysis of microbial community structures in pristine and contaminated  
842 aquifers: field and laboratory microcosm experiments. *Appl Environ Microbiol* **65**: 2143-  
843 2150.

844 Shyu C, Soule T, Bent S, Foster J & Forney L (2007) MiCA: A web-based tool for the  
845 analysis of microbial communities based on terminal-restriction fragment length  
846 polymorphisms of 16S and 18S rRNA genes. *Microb Ecol* **53**: 562-570.

847 Sinclair JL & Ghiorse WC (1987) Distribution of protozoa in subsurface sediments of a  
848 pristine groundwater study site in Oklahoma. *Appl Environ Microbiol* **53**: 1157-1163.

849 Sinclair JL, Kampbell DH, Cook ML & Wilson JT (1993) Protozoa in subsurface  
850 sediments from sites contaminated with aviation gasoline or jet fuel. *Appl Environ*  
851 *Microbiol* **59**: 467-472.

852 Sipos R, Székely AJ, Palatinszky M, Révész S, Márialigeti K & Nikolausz M (2007)  
853 Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA  
854 gene-targeting bacterial community analysis. *FEMS Microbiol Ecol* **60**: 341-350.

855 Sogin ML, Morrison HG, Huber JA, *et al.* (2006) Microbial diversity in the deep sea and  
856 the underexplored "rare biosphere". *Proc Natl Acad Sci USA* 0605127103.

857 Suzuki MT (1999) Effect of protistan bacterivory on coastal bacterioplankton diversity.  
858 *Aquat Microb Ecol* **20**: 261-272.

859 Suzuki MT & Giovannoni SJ (1996) Bias caused by template annealing in the  
860 amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microbiol* **62**: 625-  
861 630.

862 Thelaus J, Haecky P, Forsman M & Andersson A (2008) Predation pressure on bacteria  
863 increases along aquatic productivity gradients. *Aquat Microb Ecol* **52**: 45-55.



864 Thies JE (2007) Soil microbial community analysis using terminal restriction fragment  
865 length polymorphisms. *Soil Sci Soc Am J* **71**: 579-591.

866 Tso S-F & Taghon G (2006) Protozoan grazing increases mineralization of naphthalene  
867 in marine sediment. *Microb Ecol* **51**: 460-469.

868 Valiela I, Costa J, Foreman K, Teal JM, Howes B & Aubrey D (1990) Transport of  
869 groundwater-borne nutrients from watersheds and their effects on coastal waters.  
870 *Biogeochemistry* **10**: 177-197.

871 Vázquez-Domínguez E, Casamayor EO, Català P & Lebaron P (2005) Different marine  
872 heterotrophic nanoflagellates affect differentially the composition of enriched bacterial  
873 communities. *Microb Ecol* **49**: 474-485.

874 Velasco Ayuso S, Guerrero MC, Montes C & López-Archilla AI (2009) Spatiotemporal  
875 distribution of microbial communities in a coastal, sandy aquifer system (Doñana, SW  
876 Spain). *Geobiology* **7**: 66-81.

877 Weisse T (2002) The significance of inter- and intraspecific variation in bacterivorous  
878 and herbivorous protists. *Antonie Leeuwenhoek* **81**: 327-341.

879 Whitman WB, Coleman DC & Wiebe WJ (1998) Prokaryotes: The unseen majority. *Proc*  
880 *Natl Acad Sci USA* **95**: 6578-6583.

881 Wu D, Hartman A, Ward N & Eisen JA (2008) An Automated Phylogenetic Tree-Based  
882 Small Subunit rRNA Taxonomy and Alignment Pipeline (STAP). *PLoS ONE* **3**: e2566.

883 Xia Y, Kong Y, Thomsen TR & Halkjaer Nielsen P (2008) Identification and  
884 ecophysiological characterization of epiphytic protein-hydrolyzing *Saprospiraceae*  
885 ("*Candidatus* Epiflobacter" spp.) in activated sludge. *Appl Environ Microbiol* **74**: 2229-  
886 2238.

887 Zarda B, Mattison G, Hess A, Hahn D, Hohener P & Zeyer J (1998) Analysis of bacterial  
888 and protozoan communities in an aquifer contaminated with monoaromatic  
889 hydrocarbons. *FEMS Microbiol Ecol* **27**: 141-152.

890 Zektser IS & Everett LG (2004) Groundwater resources of the world and their use.  
891 United Nations Educational, Scientific and Cultural Organization, IHP-VI, Series on  
892 Groundwater No. 6.

893 Zhang R, Thiagarajan V & Qian P-Y (2008) Evaluation of terminal-restriction fragment  
894 length polymorphism analysis in contrasting marine environments. *FEMS Microbiol Ecol*  
895 **65**: 169-178.

896  
897  
898

899 Table 1. Experimental bottles were divided based on differences in carbon addition and  
 900 whether the groundwater was filtered to remove grazers. All of the carbon addition  
 901 bottles received the same total concentration of carbon either as a single addition of 200  
 902  $\mu\text{M}$  carbon (single addition), or five additions of 40  $\mu\text{M}$  carbon (multiple addition). For  
 903 each carbon addition treatment, two of the bottles received unlabeled acetate and the  
 904 remaining bottles received [ $^{13}\text{C}$ ]acetate.

<b>Carbon</b>	<b>Filtered</b>	<b># of bottles</b>
none	Whole	2
none	1 $\mu\text{m}$ -filtered	2
Single addition	Whole	4
Single addition	1 $\mu\text{m}$ -filtered	5
Multiple addition	Whole	4
Multiple addition	1 $\mu\text{m}$ -filtered	5

905

906

907 Table 2. Summary of ancillary data collected from selected bottles at the conclusion of  
 908 the experiment. Values are means  $\pm$  standard deviations. Only single measurements are  
 909 available for the no carbon added bottles.

	<b>Initial</b>	<b>Whole</b>	<b>1 <math>\mu</math>m-filtered</b>
<hr/>			
<i>Bacterial abundance</i> (x 10 <sup>4</sup> cells ml <sup>-1</sup> )	1.61 $\pm$ 0.02		
Multiple carbon addition		28.5 $\pm$ 13.4	73.5 $\pm$ 37.5
Single carbon addition		19.6 $\pm$ 18.9	27 $\pm$ 11.3
No carbon addition		18	57
<hr/>			
<i>Dissolved organic carbon</i> ( $\mu$ M)	75 $\pm$ 4		
Multiple carbon addition		198 $\pm$ 61	139 $\pm$ 4
Single carbon addition		138 $\pm$ 3	107 $\pm$ 10
No carbon addition		145	94
<hr/>			

910

911 **Table 3.** The number of terminal restriction fragments (TRFs) for the different phylogenetic groups identified using the in silico  
912 analysis. Not all of the TRFs were identified, and the number of TRFs putatively identified was further reduced as described in the  
913 methods. ‘Unclassified sequences’ are environmental sequences which have been deposited in GenBank, but have not received any  
914 further taxonomic assignment. Data in the table are from DNA extracted from filters or DNA removed from the cesium chloride  
915 gradient following ultracentrifugation. Identification based on DNA removed from the cesium chloride gradient is further split  
916 between the whole water bottles and the 1 µm-filtered bottles, there is a subsequent division based on whether or not the TRF was  
917 found within the <sup>12</sup>C- or the <sup>13</sup>C-region of the cesium chloride gradient.

918

919

Phylum	Identification of TRFs in DNA extracted from filters		Identification of TRFs in fractions following ultracentrifugation			
	Prior to experiment	Final diversity within bottles	Whole water		1 µm-filtered water	
			<sup>12</sup> C-DNA	<sup>13</sup> C-DNA	<sup>12</sup> C-DNA	<sup>13</sup> C-DNA
Proteobacteria	11	65	9	12	22	14
Firmicutes	12	119	9	15	25	19
Actinobacteria	3	29	2	2	5	8
Bacteroidetes	5	18	2	5	6	7
Chlorobi		1				
Fibrobacteres		3				1
Acidobacteria		6		1	1	1
OP3		1				
Aminanaerobia		1				
Planctomycetes		1				
Gemmatimonadetes		1				1
Haloanaerobiales	1	1				
OP9_JS1		1				
Unclassified sequences	3	19	1	4	4	5
# of TRFs with good taxonomic assignments	35	266	23	39	63	56
Total # of TRFs	89	548	77	109	186	166

920

921

922 Table 4. Summary of phylogenetic information from the 16S rDNA sequences from the  
 923 clone libraries: one from a bottle with whole water and one from a bottle with 1 µm-  
 924 filtered water.

Phylum	Class Order (-ales) / Family (-aceae) / Genus	% of clones	
		Whole water	1 µm-filtered water
Actinobacteria	Microbacteriaceae   Aureo-Microbacterium		1
Proteobacteria	Alphaproteobacteria		
	Rhizobiales   Rhizobiaceae   Rhizobium/Agrobacterium	4	
	Sphingomonadales   Novosphingobium	4	
	Bradyrhizobiales		
	Methylobacteriaceae		4
	Unclassified		7
	Caulobacterales	6	
	Consistiales   Caedibacteraceae		5
	Betaproteobacteria		
	Burkholderiales		
	Comamonadaceae	29	16
	Oxalobacteraceae	3	14
	Ralstoniaceae   Cupriavidus/Wautersia	3	
	Ralstoniaceae   Unclassified		19
	Gammaproteobacteria		
Moraxellaceae   Acinetobacter	6		
Epsilonproteobacteria			
Helicobacteraceae   Wolinella	4		
Bacteroidetes	Saprospiraceae   Unclassified	36	34
	Flavobacteriales   Unclassified	3	

925

926

927 **Figure legends**

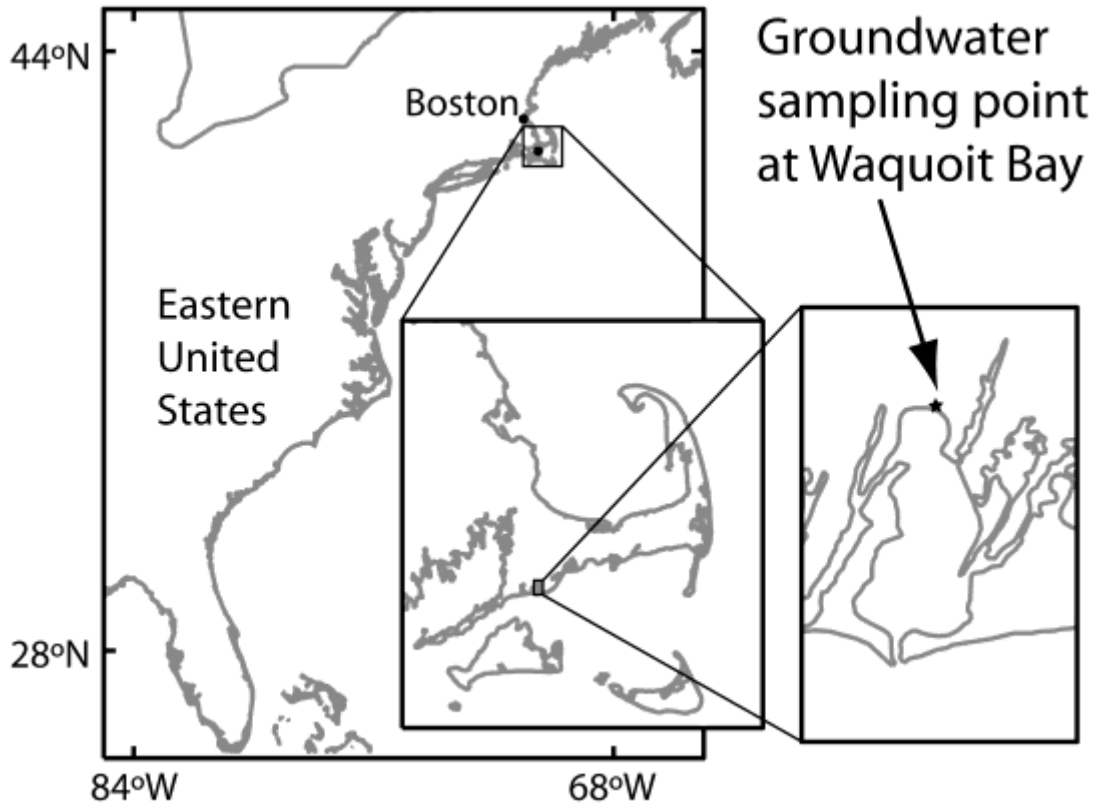
928 Figure 1. Map of groundwater sampling region. Groundwater was pumped from a well  
929 installed at the Waquoit Bay National Estuarine Research Reserve. The well was within  
930 the freshwater region of the aquifer above the zone where freshwater and saltwater mix  
931 within the subterranean estuary (Charette *et al.*, 2005).

932 Figure 2. Non-metric multidimensional scaling (NMS) analysis showing the differences  
933 in bacterial community composition between samples based on T-RFLP analysis of DNA  
934 extracted from filters prior to ultracentrifugation. Each point within the figure is a single  
935 sample. Points that are closer together are more similar, while points that are located  
936 further apart display greater differences. All three panels of the figure contain the same  
937 NMS results with the samples coded differently to highlight (A) the rate of carbon  
938 addition, (B) the type of carbon added, and (C) filtration to remove grazers.

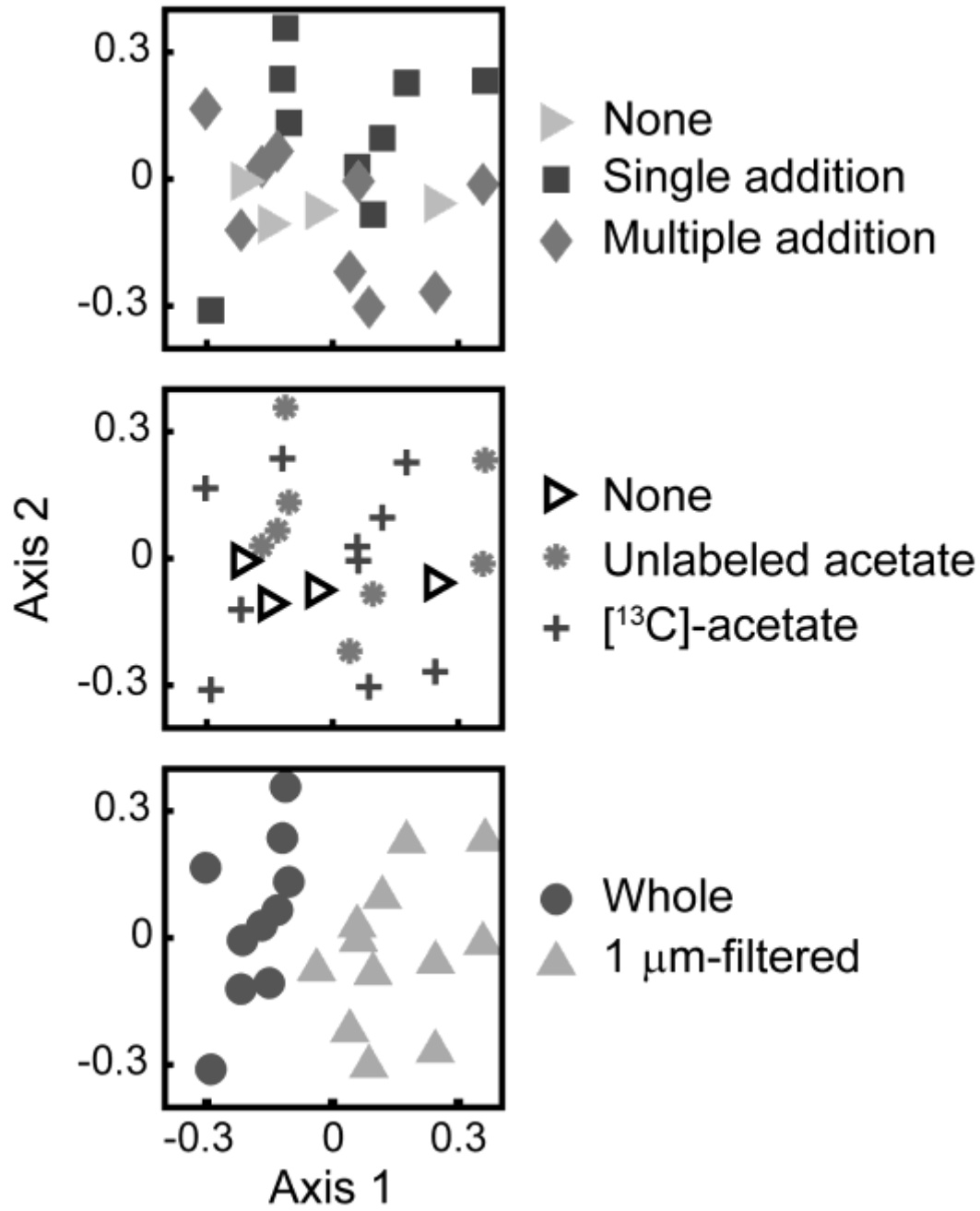
939 Figure 3. Patterns in the community composition of <sup>13</sup>C-labeled DNA ('active' bacterial  
940 cells based on their ability to assimilate acetate and/or its metabolites) in the incubations  
941 based on the NMS analysis. (A) and (B) contain the same NMS results with (A) coded to  
942 high the effect of carbon addition and (B) revealing the differences between the whole  
943 and 1 µm-filtered bottles.



Longnecker et al.  
Figure 1



Longnecker et al.  
Figure 2



Longnecker et al.  
Figure 3

