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1 **Influence of depth and time on diversity of free-living microbial community in**
2 **the variably saturated zone of a granitic aquifer.**

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22

23 Abstract

24 We investigated the temporal and vertical changes in the microbial communities related to
25 hydrological variations an aquifer (Brittany, France). Five water samplings were carried out,
26 spanning 3 hydrological cycles in the variably and the permanently saturated zones. Seasonal
27 variations in the major anion concentrations (NO_3^- , SO_4^{2-} and Cl^-) indicated that different
28 physical processes occurred during the recharge process in the 2 zones. The variably saturated
29 zone is mainly dominated by diffusion and advection processes from the soil whereas the
30 permanently saturated zone is controlled by moderate advective transfer from the variably-
31 saturated zone. Bacterial diversity was investigated by flow cytometry, 16S rRNA and *narG*
32 genes analyses. Part of this diversity was new in that 6 of the 27 16S rRNA gene sequence
33 phlotypes were unknown even at the class or phylum level. The *narG* gene analysis did not
34 reveal any clear variation in time or depth within the nitrate reducers' community. In contrast,
35 16S rRNA gene analyses showed modifications of community composition that could be
36 related to the hydrologic and chemical contrast between the 2 zones. It was concluded that the
37 physical processes of water transfer could influence bacterial diversity at the soil aquifer
38 interface.

39

40 Keywords: hydrology, microbial community, 16S rRNA gene, T-RFLP, water table
41 fluctuation zone

42

43 **Introduction**

44 Aquifer quality is considered as a major point in water protection and directives management.
45 Beside these policies, the understanding of ecosystem health is crucial (Griebler et al. 2010).
46 If stream water quality has been investigated and monitored for a while, aquifer quality has
47 been neglected across the world (e.g. Korbel & Hose 2011). Aquifers are not anymore
48 considered as a water resource but also as a living ecosystem which controls the water quality
49 (e.g. Griebler et al. 2010). Investigations of groundwater microbial diversity and ecosystem
50 have increased in the last few years (e.g. Griebler & Lueders 2009, Bougon et al. 2009).
51 However our understanding of the microbial diversity involved in key biogeochemical
52 processes for groundwater functioning is still limited. The knowledge of groundwater habitats
53 is mainly framed under the characterization of hydrological processes including fluxes,
54 chemistry and geological heterogeneity (Madsen and Ghiorse 1993).
55 Microbial community (diversity and abundance) in groundwater seems to be related to
56 complex relationships between organic contaminants, nutrients and hydrogeology (Chapelle
57 2000, Christensen et al. 2001, Griebler & Lueders 2009, Pearce et al. 2011). Despite our
58 understanding about the impact of contamination on microbial diversity (e.g. Röling et al.
59 2001), little is known about the impact of physical processes as hydrologic changes on the
60 microbial diversity in groundwater. Studies on porous media revealed that bacterial
61 distribution was mostly affected by passive transport processes (e.g., advection, diffusion or
62 filtration) (Balkwill et al. 1998, Huysman and Verstracte 1993, Trevors et al. 1990) and
63 several models have focused on understanding and predicting microbial transport in natural
64 porous and high nutrient media (e.g. Ginn et al. 2002, Harvey et al. 1993, Murphy and Ginn
65 2000, Rockhold et al. 2004). Few attempts have been made to characterize microbial
66 distribution and activities in unsaturated or variably saturated systems. More research seems

67 to be required to better understand microbial responses to hydrological changes (Fisk et al.
68 2003).

69 Within aquifers, knowledge of the physical processes occurring in the fluctuation zone is also
70 important to our understanding of biological and biochemical processes. This zone of water
71 table fluctuation is unique with highly variable saturation conditions controlling the
72 availability of nutrients to microbial organisms (Legout et al. 2007). Although considered
73 mainly as a transfer zone it also exhibits biogeochemical reactions such as the various
74 denitrification processes (Dunn et al. 2005, Legout et al. 2005). Although the recharge
75 process has been widely investigated (e.g. de Vries and Simmers, 2002), few studies have
76 focused on the processes governing water flow and solutes transfer in the fluctuation zone of
77 the water table. The different processes recently identified in this zone include: rapid vertical
78 fluxes from soil to water table (Legout et al. 2007), lateral fluxes along the hillslope below the
79 soil (Derby and Knighton 2001, Haria and Shand 2004, Rawlins et al. 1997) and cyclic
80 chemistry control by groundwater fluctuations (Fretwell et al. 2005). Hydrological conditions
81 in the variably saturated zone present both seasonal variations and a vertical contrast with the
82 permanently-saturated zone. With a seasonal rhythm, conditions change from a permanently-
83 saturated zone in depth to a variably-saturated medium, close to the soil. The vertical contrast
84 between the two zones is characterized by a water table and chemical characteristics
85 seasonally close to the soil in the variably-saturated zone and more constant chemical
86 characteristics in the deeper permanently-saturated zone. Groundwater ecosystems are
87 generally characterized by low concentration of dissolved organic carbon and by the lack of
88 light. These factors are amongst the most differences with the soil ecosystem (Gibert 1994,
89 Griebler and Lueders 2009). The investigation of these 2 zones presents an ecological interest
90 by presenting differing environmental conditions but also potential seasonal connections.

91 In this study we (i) analyse the temporal and vertical changes in the microbial communities
92 (i.e. distribution and structure) related to hydrological variations in the water table fluctuation
93 zone of a granitic aquifer and immediately below. The studied aquifer is characterized by
94 nitrate concentrations (Legout et al. 2005) exceeding regularly the upper limit of 50 mg.L⁻¹
95 recommended by EU groundwater directive. Legout *et al.* (2005) also demonstrated a
96 denitrification potential in the fluctuation zone of this particular catchment. In this context,
97 the work presented herein aims (ii) to address a possible link between the nitrate
98 concentration in groundwater and changes in nitrate reducing community. To do so,
99 molecular tools allowing rapid profiling of microbial community were used. To assess the
100 'global' diversity and 'nitrate reducing' diversity, the 16S rRNA and *narG* genes were used as
101 targets respectively. The *narG* gene encoding a subunit of the nitrate reductase that catalyses
102 the reduction of nitrate to nitrite is found in many bacteria (e.g. Firmicutes, Actinomycetes
103 and Proteobacteria) and Archaea (Chèneby et al. 2003, Packman et al. 2004). We hereafter
104 define 'nitrate-reducing bacteria' as those bacteria possessing the capacity, within their
105 genome, to transform nitrate to nitrite. As a first approximation, we assumed that 'nitrate-
106 reducing bacteria' could be assessed by analysing *narG* gene diversity, although other genes
107 harbour the ability to perform this function.

108 The analysis of temporal and spatial changes in the microbial communities is assessed herein
109 from water samplings using a pan-flute device. This device allows investigating the
110 unsaturated zone by intercepting water at 6 m, 8m, 10 m and 15 m depth by different
111 piezometers. The analysis of the communities of free-living bacteria was coupled with a
112 characterization of chemical and hydrological properties over 2.4 years, during 3 hydrological
113 cycles at each depth.

114

115 **Material and methods**

116

117 **Site description**

118 The Kerrien catchment covers an area of 0.12 km², is located in an agricultural site, mainly
119 devoted to pasture and dairy cows, in south-west Brittany (47°57'N-4°8'W, 2347.35N –
120 117.5E, see Fig. S1 in Supplementary material). The outlet is about 10 km from the sea
121 (Legout 2006, Martin 2003, Martin et al. 2004, Molénat et al. 2007). This catchment serves as
122 a national research observatory for environmental investigations (INSU-CNRS and INRA
123 observatory, http://www.inra.fr/ore_agrhys) which has been intensively monitored for
124 hydrochemistry for more than 10 yrs. The different compartments of the aquifer and their
125 contribution to catchment functioning has been highlighted in previous research (Martin 2003,
126 Martin et al. 2004, Molénat et al. 2007, Pierson-Wickmann et al. 2009) along with solute
127 transfer in the unsaturated zone (Legout et al. 2007).

128 The climate is oceanic with a mean annual precipitation of 1167 +/- 195 mm. The bedrock is
129 fissured, fractured granite, overlain by weathered material within which the shallow
130 groundwater accumulates and fluctuates (Legout et al. 2007). The weathered granite lies
131 between 0.7 and 1.2 m below the soil surface. The soils are mainly sandy loams. The depth of
132 the water-table ranges from 2 to 8 m below the soil surface. A plot with six piezometers at 3
133 to 15 m depth (3, 5, 6, 8, 10 and 15 m) was set up in the weathered material. These
134 piezometers consisted of PVC tubes with a 1-m-thick perforated screen at one end, which
135 allowed the collection of water from a 1-m-thick groundwater layer. Whilst only one
136 piezometer set was investigated for microbiology, three other similar plots are located along
137 the slope of the catchment and a further line of three other plots are located 100 m to the West
138 as a replicate. The chemical concentrations have been monitored for 10years and variations of
139 the vertical chemical gradients have been observed along the slope as well as spatially. This

140 monitoring (Legout et al. 1997, Rouxel et al. 2011) provides a good characterization of the
141 chemical variations with depth and the adequacy of the sampling design.

142

143 **Analytical methods**

144 The piezometric level in this plot in the Kerrien catchment was continuously recorded from
145 mid-December 2004 until mid-April 2007 (2.4 years). Five water chemical measurements and
146 bacterial samplings were carried out in the different piezometers along the vertical soil-
147 groundwater profile during this 2.4 yr period. Three campaigns corresponded to high water
148 (05/25/05; 03/13/06 and 01/25/07) and two to low water levels (01/18/06 and 11/22/06).

149 Physico-chemical parameters such as pH, temperature, redox potential, dissolved oxygen and
150 electrical conductivity were measured *in situ*. The water samples were filtered in the field
151 through 0.22 µm cellulose-acetate filters (Sartorius Minisart), and stored for less than 24 h in
152 the dark below 4°C before being analyzed for major anions (Cl^- , SO_4^{2-} and NO_3^-) by ionic
153 chromatography (Dionex DX120) in the CAREN-Geosciences Rennes laboratory facilities.

154 The level of uncertainty for the major anion concentrations was below 4 %. The total
155 dissolved carbon (TDC) and dissolved inorganic carbon (DIC) were analyzed with a
156 Shimadzu TOC 5050A (quantification limits: 0.85 mg L^{-1} ; detection limits: 0.08 mg L^{-1}). The
157 dissolved organic carbon (DOC) was calculated from TDC and DIC (i.e. $\text{DOC}=\text{TDC}-\text{DIC}$).
158 The organic carbon was not measured during the 3rd and 5th campaigns due to technical
159 problems.

160

161 **Groundwater sampling**

162 Groundwater samples were collected at the Kerrien Site from the piezometers at 6, 8, 10 and
163 15 m depth. This pan-flute device has the advantage to allow the recharge process, seasonal
164 variation and vertical gradient to be monitored, and to allow easy water sampling. For the

165 latter, an ISMA® sampler was used during the first two field campaigns but was replaced in
166 subsequent campaigns by a Grundfos MP1 immersed pump to ensure better efficiency. The
167 ISMA sampler was rinsed three times with deionized water and once with groundwater
168 between each well sampling. The Grundfos pump was rinsed with groundwater. Two liters
169 were collected from each piezometer. The fourth field sampling campaign was characterized
170 by a lack of water in the 6 m depth piezometer. The microbial sampling protocol was adapted
171 from a marine sampling strategy (Massana 2004). Each 2 litre sample was subjected to
172 cascade filtration using three thresholds i.e., 10µm (Isopore™ - TCTP, membrane filters),
173 5µm (Isopore™ - TMTP, membrane filters) and 0.22µm (Sterivex™ -GV, Millipore) *via* a
174 peristaltic pump. This step was necessary to concentrate the microbial biomass before nucleic
175 acid extraction and was done immediately after sampling in the field. The filters were placed
176 in Petri dishes and stored at 0°C in a refrigerant container, and then at -20°C in the laboratory
177 prior to DNA extraction. DNA was extracted in the 96 hours following the sampling and then
178 stored at - 80°C.

179 The potential effect of well structure (tube and gravel surrounding the tube), on water
180 biochemistry and bacterial diversity, was estimated by sampling before and after purging the
181 borehole (i.e. replicates, see below for details). This purge consisted of removing a water
182 volume equivalent to three times the well volume i.e. a minimum of 90 liters, with a Grundfos
183 MP1 immersed pump. The pump was made of inert materials to avoid any contamination of
184 the pumped liquid. For a given well, we expected the local heterogeneity being the highest
185 before and after purge. Thus, for each piezometer we decided to perform 2 true-sampling
186 replicate one before and one after purge.

187

188 **Bacterial counts**

189 The bacteria were counted by flow cytometry on a FACSort (Becton Dickinson). Two
190 milliliters of water placed in a sterile cryovial were fixed with 7 μ L of glutaraldehyde in the
191 field and stored in the refrigerant container during the sampling period. Back in the
192 laboratory, the cryovials were dipped in liquid nitrogen and stored at -80°C before analyses.
193 This was done similarly for all the samples.

194

195 **DNA Extraction**

196 DNA was extracted from the filters (see above, 'groundwater sampling') by physical
197 disruption of cells by bead-beating at a frequency of 30 oscillations per sec for 27s (Retsch
198 MM400, Germany) and by using the DNeasy Plant miniKit (Qiagen) according to the
199 manufacturer's protocol. This method was chosen from preliminary tests using different
200 strategies as beat-beating frequencies and durations, standard CTAB extraction method and
201 Dneasy Plant mini kit (results not shown).

202

203 **Primers**

204 A set of *narG* sequences of bacteria and Archaea, available from the GenBank database, were
205 aligned and scanned for conserved regions that could provide suitable primer targets. Two
206 degenerated primers, narGr (5'-TTTTTCSTACCAGGTCGC-3') and narGf (5'-
207 TAYGTCCGGCAGGAAAA-3'), were selected from the analysis to amplify a specific 840 bp
208 fragment of the *narG* gene (Bougon et al. 2009).

209 The universal bacterial primers (Orphan et al. 2000, modified by Vandenkoornhuysen et al.
210 2007), Eub-519F (5'-GTTTCAGCMGCMGCCGCGGT) and Eub-1390R (5'-
211 GTTTGACGGGCGGTGTGT) were used to amplify a 871 bp internal fragment of the 16S
212 rRNA gene.

213

214 **16S rRNA and *narG* genes amplifications**

215 PCRs amplifications were performed for each 16S rRNA and *narG* genes. The PCRs
216 amplifications were performed in a total volume of 50 µl containing 0.2 to 0.3 µg of purified
217 DNA, 250 µM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP;
218 Promega, Madison WI), 1 µL of proof reading *Taq* DNA polymerase (5U/µl - Applied
219 Biosystems), 1x *Taq* DNA polymerase buffer containing 1.5 mM magnesium chloride
220 (Applied Biosystems) and 2 µM of Eub-519F and Eub-1390R for the 16S rRNA gene
221 amplification or 1 µM narGr and narGf for the *narG* gene amplification. For the 16SrRNA
222 gene amplification, the cycling regime was an initial step at 94°C for 2 min 30s, followed by
223 35 cycles at 94°C for 30 s, 52°C for 1 min, and 72°C for 50 s. The amplification ended by a
224 final extension step at 72°C for 10min. For the *narG* gene amplification, the cycling regime
225 was an initial step at 94°C for 1 min 30 s; followed by 36 cycles at 94°C for 30 s, 51°C minus
226 0.1°C per cycle for 1 min 15 s, and 72°C for 1 min 30 s. The program ended by a final
227 extension step at 72°C for 10 min. All the amplifications were done in an MJ Research PTC-
228 100™ thermocycler. Amplifications were checked by electrophoresis (4 µl of PCR product in
229 1% agarose in 0.5×TBE buffer).

230

231 **Terminal-Restriction fragment length polymorphisms (T-RFLPs)**

232 Environmental DNA extracts were amplified with fluorescently labelled primers i.e. Eub-
233 519F (5'-[6FAM] GTTTCAGCMGCMGCCGCGGT) and Eub-1390R (5'-[5Hex]
234 GTTTGACGGGCGGTGTGT), or narGf (5'- [6FAM] TAYGTCTGGGCAGGAAAA-3') and
235 narGr (5'-[5Hex] TTTTCSTACCAGGTTCGC-3'). The specificity of amplification was
236 checked and PCR products were purified using the High Pure PCR product purification kit
237 (Roche) according to the manufacturer's protocol. Two aliquots of the purified amplicons
238 were digested, one with 2 U *Hinf*I and the other with *Sty*I restriction enzymes (Promega), at

239 37°C for 2 h in a low adsorption microtube (Axygen) to limit the loss of T-RFs during the
240 different steps of the protocol. The quantity of digested DNA per reaction corresponded to 1/3
241 of a PCR reaction. The digested products were precipitated with 2 volumes of absolute
242 alcohol and 1/10 volume of 3M Acetate buffer pH 5.4. The mixture was then placed
243 overnight at -20°C, and centrifuged at 16400 g for 20 min at 4°C. The pellets were washed in
244 200 µL of 70% alcohol, centrifuged at 16400g for 10 min, and incubated for 20 min at 37°C.
245 The dried pellets were resuspended in 10 µL of ultrapure water and placed for 2 to 4 h in
246 darkness on melting ice.

247 The quantity of T-RFs to load on the sequencer was defined in preliminary tests to accurately
248 size the fragments (no signal saturation mainly). Aliquots of the digested products (1 µl) were
249 mixed with 9 µl of deionized formamide and ROX labelled GS400HD internal size standard.
250 T-RFs were separated and sized using an automated sequencer ABI Prism® 3130xl DNA
251 analyser (Applied Biosystems). The size of the T-RFs was estimated in reference to the
252 internal standard (Genemapper version 3.7 software, Applied Biosystems) which allowed
253 accurate sizing of the T-RF lengths. Because of the use of the GS 400HD internal standard,
254 the accuracy of the sizing of the T-RFs was good in between 50 bp and 400 bp. Thus only the
255 fragments within this interval were used for the data analyses. The sequencer detected all
256 fluorescent DNA fragments and background noise. The samples/formamide mixture and
257 partial digestions may generate signals that do not correspond to a true terminal restricting
258 fragment (T-RF). These potential artefacts were avoided by performing 3 replicates (i.e.
259 independent PCRs and independent digestions). These true replicates eliminated all
260 inconsistent peaks and increased the number of observed T-RFs per T-RFLP profile by
261 retaining any very small but consistently present peaks. Thus there was no need to apply a
262 minimal peak cut-off height. Background noise was determined by comparing the replicates
263 of the profiles of the peaks (Vandenkoornhuysen et al. 2003). By using this strategy, between 1

264 to 35 TRF and 1 to 27 TRF were detected respectively using *Hinf*I and *Sty*I. It should also be
265 underlined that the reproducibility was excellent in between replicates (i.e. maximum
266 divergence observed in terms of presence/absence was 9.5 %). However, we have noticed
267 important differences in the peak intensities. Even more problematic, we observed differences
268 in peak intensities even for identical samples ran in different capillaries. Thus, only
269 presence/absence of a given T-RF was analysed.

270

271 **Clone library construction and sequencing**

272 The purified PCR products from 16S rRNA gene amplifications were cloned. The first step
273 was to add a non-complemented A to the amplicons using an A-overhanging post-
274 amplification procedure. One unit of *Taq* DNA polymerase (Invitrogen), 1x concentrated *Taq*
275 DNA polymerase buffer (Invitrogen) and 0.1 mM of dATP was added to the purified
276 products, and incubated for 15 min at 72°C. The ligation mixture containing 1.5 µL of 3'A-
277 tailed PCR products, 1.0 µL of pGEM-T vector, 5.0 µL of rapid pGEM-T buffer and 1 µL of
278 T4-DNA ligase (pGEM-T cloning kit, Promega) in a final volume of 10 µL was incubated
279 overnight at 4°C. Transformation of *E. coli* DH5α competent cells (Invitrogen) was carried
280 out following the manufacturer's instructions. The positive clones were screened by PCR
281 amplifications targeting sequences of the vector with the primers T-7 and SP-6. Cloned DNA
282 fragments (871 bp) were sequenced for the 2 strands on an automated sequencer ABI Prism®
283 3130xl DNA analyser (Applied Biosystems). This cloned library was built to highlight
284 whether or not an original microbial diversity exists in the studied aquifers. The purpose of
285 this analysis was thus not to compare bacterial communities among samples.

286

287 **Sequence data analyses**

288 All the contigs were produced using Sequencher 4.7 (GeneCodes) and all the nucleotides for
289 each sequence were checked by the software and corrected manually when required. After
290 cloning and sequencing, chimeras were eliminated by analyzing the sequences with the
291 chimera check program on the Ribosomal RNA Database website. A multiple alignment of
292 the checked 16S rRNA gene sequences was performed using ClustalX 1.81 (Thompson et al.
293 1997) and refined by eye. Each sequence was compared with the NCBI sequences using the
294 Basic Local Alignment Search Tool (BLASTn).

295 Three techniques were used to calculate the phylogenetic relationships between 48 known
296 bacterial sequences (i.e. the highest BLAST hits, closest phylogenetic neighbours and
297 additional sequences belonging to the different bacterial phyla) and the 107 environmental
298 sequences. These were (i) phenetic reconstruction using the Kimura 2-parameters (K2P)
299 distance model and clustering using the neighbor joining (NJ) algorithm (Saitou and Nei
300 1987) with Clustal X 1.81; (ii) maximum parsimony (MP) reconstruction (Swofford 1993)
301 done by a heuristic tree search with tree bisection and reconnection (TBR) as swapping
302 algorithm and the use of 500 replicates of random addition of sequences to find the shortest
303 tree and (iii) maximum likelihood (ML) reconstruction using the GTR+I+G distance model,
304 selected from modeltest analyses (Posada and Crandall 1998). The MP and ML Phylogeny
305 were calculated using PAUP 4.0 β 10 (Sinauer associates). Bootstrap support at the nodes was
306 estimated from 500 pseudo-replicates for MP, 100 for ML and 1000 replicates for the NJ
307 phylogenies. The chosen outgroup was an uncultured Crenarchaeota (AJ347774).

308

309 **Patterns of microbial community analyses**

310 The information provided by the different T-RFs was combined by applying a multivariate
311 approach based on principal component analysis (PCA). Non-centred PCAs were calculated
312 because they maintain the mean differences between variables (i.e. frequency of observation

313 of each T-RF, Vandenkoornhuyse et al. 2003). The statistical reliability of the data was
314 determined from the PCAs by computing confidence ellipses and stars joining the gravity
315 centre to each dot of the considered modality in the vectorial space (Sokal and Rohlf 1995).
316 The PCA analyses were implemented with the ADE-4 software package (Thioulouse et al.
317 1997).

318

319 **Nucleotide sequence accession numbers.**

320 The 16S rRNA gene sequences recovered in this study were deposited in the GenBank
321 nucleotide sequence database under the accession numbers **FJ429673 to FJ429781**.

322

323 **Community comparisons**

324 Genetic diversity (hereafter meaning species richness) was estimated using the Shannon index
325 H' . The proportion of species i in relation to the total number of species (P_i) was calculated,
326 and then multiplied by the logarithm of this proportion ($\log_2 P_i$). The resulting product was
327 summed across species, and multiplied by -1:

$$328 \quad (1) \quad H' = - \sum_{i=1}^n P_i \times \log_2 P_i;$$

329

330 where P_i is the frequency of a given T-RF. This synthetic index was used to compare the
331 species richness of the nitrate-reducing communities in relation to the different forcing
332 variables.

333 The Morisita-Horn index provides a similarity measurement based on presence-absence data
334 only (Magurran 1988). This index is not influenced by sample size or richness (Wolda, 1980).
335 The Morisita-Horn index was calculated as follows:

$$336 \quad (2) \quad C_m H = \frac{2 \sum_{i=1}^n (a_{ni} \times b_{ni})(d_a + d_b)}{(aN \times bN)}$$

337

338 where CmH is the Morisita-Horn index of similarity, N is the total number of species for
339 communities A and B; aN and bN are the total number of individuals of all species collected
340 from community A and B, respectively; ani and bni are the number of individuals of the i^{th}
341 species collected from community A and B, respectively. The denominator incorporates two
342 terms which are defined as:

343 (3)
$$da = \frac{\sum_{i=1}^S an_i^2}{aN^2}$$
 and
$$db = \frac{\sum_{i=1}^S bn_i^2}{bN^2}$$

344

345 This index was used to compare sample pairs such as two campaigns for a given depth and
346 two depths for a given campaign. The index was calculated using EstimateS software
347 (Colwell 2005). The Morisita-Horn index ranges from 0 (no congruence between samples) to
348 1 (full congruence). As species abundance is taken into account, the index of communities
349 containing the same species but in different abundances will be less than 1 (Heijs *et al.*, 2006;
350 Juottonen *et al.*, 2005).

351

352 **Results**

353

354 **Hydrogeology/Hydrochemistry**

355 Hydrogeology

356 The water-table fluctuation depth ranged from 2.1 to 6.1 m below the soil surface (Fig. 1)
357 during the 2.4 years of monitoring. The hydrological year 2004/2005 was relatively dry. The
358 highest water-table level was 4.2 m during December 2004. The recharge period during the
359 following year (2005-2006), was relatively long with a slow water table increase during
360 winter and a final peak during spring (highest level 2.9 m during March). In comparison, the
361 recharge process was greater during winter of the final hydrological year (2006-2007), the
362 level of the high water table ranging from 3.8 to 2.1 m from January to March. Comparison

363 with long-term chronicles revealed that the first two monitoring years could be considered as
364 relatively dry (results not shown).

365 Groundwater chemistry

366 The groundwater was characterized by constant temperature, slight acidity (Tab. 1), low total
367 organic carbon concentrations and high nitrate concentrations (Tab. 1). For the sake of clarity,
368 we mainly used two depth measurements as a reference to the variably-saturated zone (6 m
369 depth) and the permanently-saturated zone (15 m depth). Two chemical trends were apparent
370 within the data set: a relatively stable chemical composition in the permanent aquifer (15 m
371 depth) and time-related chemical variability in the water-table fluctuation zone (6 m depth)
372 (Fig. 1, Tab. 1). Such evolution is characteristic of this catchment. It was observed for several
373 years along the piezometer plots replicates placed in the hillslope (Legout et al. 1997; Rouxel
374 et al. 2011).

375 Groundwater samples at 6 m depth showed variations in chloride concentrations ranging from
376 17.7 mgL^{-1} (12/20/2006) to 47.2 mgL^{-1} (01/27/2005) (Fig. 1). These variations were also
377 correlated with nitrate ($\Delta=31.2 \text{ mgL}^{-1}$), sulphate ($\Delta=43.2 \text{ mgL}^{-1}$) and dissolved oxygen ($\Delta=5$
378 mgL^{-1}) concentrations (Tab. 1), and were closely linked to the hydrological fluctuations. The
379 highest chloride, nitrate and sulphate concentrations were observed during the low water
380 period (the whole 2004-2005 cycle) and the lowest concentrations were observed at the
381 beginning of the recharge period (January-February 2006 and January 2007).

382 A slight vertical gradient was noted for oxygen, with relatively higher concentrations at 6 and
383 8 m depths (Tab. 1). The hydrological cycle was also characterized by higher oxygen
384 concentrations at all depths during the periods of high water table (Ker3 and Ker5).

385

386 **Purge effect**

387 The bacterial cell counts were estimated by flow cytometry. The number of cells ranged from
388 $2.3 \cdot 10^5$ to $2.5 \cdot 10^6$ per mL and did not show any clear gradient with depth (Fig. S2 in
389 Supplementary material). Comparison of the cell counts before and after purging the borehole
390 revealed modifications related to purging i.e. a decrease in bacterial cells at 6, 8 and 10 m.
391 However, only slight differences were apparent between T-RFLP analyses of the community
392 structure of free-living bacteria before and after the purges (Fig. S3 in Supplementary
393 material). Homology in diversity signatures among sample pairs ranged 78% to 98%. Thus,
394 ellipses drawn on the PCA vectorial space overlapped considerably indicating no significant
395 statistical differences. Heterogeneity between true replicates was so low that our results
396 suggested that the community of free-living bacteria analysed in each sample could be
397 considered as representative of the groundwater community. It also confirms the reliability of
398 the sampling design.

399

400 **Phylogenetic analyses**

401 A sequence analysis of a fragment of the 16S rRNA genes was performed to better understand
402 the phylogenetic diversity of microorganisms in groundwater. The aim of this analysis was
403 not to compare communities. Accordingly, the sequencing depth was not the same in all the
404 samples. Twenty seven different phylotypes were obtained (Fig. 2). The resulting rarefaction
405 curves (not shown) indicated that the number of phylotypes was probably under-estimated.
406 Strikingly, 6 of these 27 phylogenetic species did not belong to a known class or phylum
407 (phylotypes 1, 2, 3, 6, 9, 11; Fig. 2). Most of the observed phylotypes in the Proteobacteria
408 phylum were also new. Only 6 of the 27 phylotypes (phylotypes 5, 14, 16, 17, 20, 24; Fig. 2)
409 displayed a high level of homology with known bacteria. The diversity at the Kerrien soil-
410 aquifer interface seemed to be original and possibly specific to this environment. This could
411 result from (i) the use of new primers (Vandenkoornhuysse et al. 2007) not widely used up to

412 date and a related bias in the knowledge , (ii) the very limited number of studies of microbial
413 diversity published for this particular environment. The diversity of soil microorganisms is
414 now better understood. If the detected microorganisms had colonized the aquifer habitat after
415 percolation from the soil surface, a more classical bacterial diversity would be expected. The
416 bacterial communities at 6 m contained mainly α -, β -, and γ - proteobacteria phylotypes which
417 are closely related to *Pseudomonas* and to a *Rickettsiella* known to be abundant in soils. None
418 of the results obtained during sub-sampling of the 16S rRNA gene sequences contradicted the
419 hypothesis that bacterial community composition should be modified with depth or that the
420 microbial community at 6 m would be more closely connected to the soil community than
421 microbial communities from deeper samples.

422

423 **Comparisons of microbial communities based on 16S rRNA gene diversity signatures**

424 T-RFLP analyses provide an interesting approach for assessing the microbial diversity and
425 dynamics of microbial communities that occur temporally or spatially in response to
426 environmental perturbations (Liu et al. 1997). The 2 enzymes chosen to digest the 16S rRNAs
427 amplicons generated 720 different T-RFs. This high level of detected polymorphism suggests
428 the presence of considerable diversity. The experimental design was defined to address the
429 hypothesis of possible differentiation of the microbial community in connection to depth and
430 time.

431 Time (Fig. 3) affected the community in both the long and short term. As regards the
432 sampling date, the bacterial community compositions of the closest samplings (i.e. Ker4/Ker5
433 and Ker2/Ker3) showed greater similarity than those of the more distant (Ker1/Ker5). This
434 can be seen in the proximity of the Ker4 /Ker5 campaigns and in the strong difference
435 between Ker1 and Ker5 at 15 m (Fig. 3) and indicates a time-dependent community structure.
436 This effect is also apparent in the similarity index (Tab. 2a). Apart from some values obtained

437 during the Ker1 campaign, this index increased over time in the Ker3/Ker4 and Ker4/Ker5
438 campaigns. Maximum similarity (Tab. 2a) was found between Ker4 and Ker5 at 15 m, as also
439 shown in Figure 3. The greatest diversity (Tab.3) was observed during the high water periods.
440 The Shannon index ranged from 4.4 to 5.1 at Ker3 and from 4.3 to 5.8 at Ker5 with the
441 highest values occurring at 15 m. The PCAs and the similarity indexes indicate that the
442 communities evolved towards more homogeneous structures during the monitoring period.
443 The diversity results indicate that short term variations in community structure occurred
444 during the high water periods.
445 The T-RFLP diversity signatures generated for each depth revealed different community
446 structures, those from 6 m and 8 m depth showing greater similarity than those from 10 m and
447 15 m. The highest similarity indexes were obtained between 8/10 and 15 m in each campaign.
448 For the Ker2 and Ker4 campaigns, the index rose to 60% similarity between the 8 m and 15 m
449 depths (Tab. 2b). Two different communities i.e. one at 6 m and the other at 8/10/15 m could
450 be distinguished from the similarity indexes.

451

452 ***narG* gene diversity**

453 The non-centred PCA did not indicate any clear structuring of the nitrate reducer community
454 in relation to depth (Fig. 4). However, some modifications of the nitrate reducer community
455 were independent of depth (i.e. the difference between the Ker1 & Ker2 communities and
456 those of Ker4 & Ker5, Fig 4). These results accord with the 16S rRNA gene analysis which
457 indicated dynamic changes in the microbial community over time.

458

459 **Recharge hydrological conditions**

460 In this section, we investigated the nature of physical water transfer mechanisms and the
461 effects of these physical hydraulic recharge processes on chemical concentrations in order to

462 provide a clear framework to the investigation of the relationships between microbial
463 communities and hydrological conditions. Chemical composition, as regards nutrient and
464 oxygen concentrations, was relatively homogeneous, with time and depth, throughout the
465 entire groundwater section. No variation in the physico-chemical parameters (dissolved
466 oxygen and pH) was detected between the water table fluctuation zone (3-8 m) and the
467 permanently saturated zone (8-15 m) (Tab. 1). As mentioned above, we used the 6 and 15 m
468 depth as representative of the variably- and permanently-saturated zones. Slightly lower
469 oxygen concentrations were found at 15 m depth (Tab. 1) but the redox conditions remained
470 relatively oxidant whatever the depth and time. The Kerrien subsurface is characterized by a
471 homogeneous redox environment. In contrast it is apparent from the chloride concentration
472 that hydraulic processes are important and modify the anion concentration (Fig. 1). The
473 nitrate and sulphate concentrations also exhibit variations but these variations have no deep
474 impact on biological activity. Nitrates are always available and no reducing conditions
475 develop. A seasonal variation in Cl^- was found at 15 m but was limited compared to that at
476 6 m. The high Cl^- variations observed at 6 m were strongly related to water table fluctuations.
477 During periods when the water table was low, the chloride concentrations at 6 m were higher
478 than at any other depth whereas during periods when the water table was high, the chloride
479 concentrations at 6 m were lower than at any other depth (Fig. 1). The observed variations in
480 chloride concentration can be explained by the recharge processes. Different water
481 compartments i.e. mobile porosity and immobile porosity are mobilized during recharge
482 (Legout et al. 2007). Mobile porosity transfers solutes down to the water-table by advection
483 through the effect of gravity in the mobile pore volume. Immobile or matrix porosity
484 exchanges solutes with the mobile pore volume by diffusion. The mobile/immobile concept of
485 water fluxes allows interpretations of the observed chemical variations.

486 (i) At 6 m depth, at the beginning of the hydrological cycle, the immobile porosity contains
487 water with high chloride concentrations brought about by evapotranspiration during summer.
488 As the water-table rises, waters containing a high proportion of rainwater are transferred by
489 mobile porosity. This leads to saturation of the bulk volume of the water-table fluctuation
490 zone, and low solute concentrations. Solute mixing processes then occur by diffusion between
491 matrix porosity and mobile porosity, producing concentrations similar to those at 15 m. The
492 entire process is highly time-dependent. At 6 m depth, there is a seasonal variation from a
493 diffusive-dominant process during summer to an advective-dominant process during recharge
494 period. Whilst the first period is characteristics of matrix water, the second one is
495 characteristic of precipitation and soil water rapidly transferred from the soil.

496 (ii) At 15 m depth, seasonal variation is much more limited and mainly related to vertical
497 transfer from the variably saturated zone. Although this variation is also time-dependent, it is
498 mainly due to vertical transfer from the variably saturated zone to the permanently saturated
499 zone. This description provides an interesting framework to investigate the effects of physical
500 transfer on microbial communities since the redox parameters are mainly constant from
501 surface to depth and nitrates are always available whatever the conditions. In the 2 zones, 2
502 physical recharge processes occur. From diffusion to advection from soil in the variably-
503 saturated zone, and moderate advective transfer from the variably- to the permanently-
504 saturated zone are the main drivers of the environmental changes in the two superposed
505 ecosystems. The effects of seasonal changes in the hydrological conditions at 6 and 15 m
506 depth on the microbial communities are investigated below.

507

508 **DISCUSSION**

509 **1. Bacterial diversity characterization**

510 The number of bacteria in the water samples was close to other measures in groundwater
511 environments (Pedersen 1997) with values ranging from $2 \cdot 10^5$ to $2 \cdot 10^6$ cells per milliliter (see
512 Fig. S2 in Supplementary material). These numbers are clearly less than those found in soil
513 environments and correspond only to unattached bacteria. It has been estimated that 10^{10}
514 prokaryote microorganisms can coexist in one gram of soil (Torsvik et al. 1990)
515 corresponding to total community of attached and pelagic bacteria. The difference observed
516 can be explained by (i) the absence of the attached microorganisms in our samples and by (ii)
517 the low total organic carbon content of groundwater samples, as in the Kerrien aquifer. The
518 bacterial diversity was surprisingly high in that at least 27 different phylotypes were found
519 (Fig. 2) which probably represented a subset of the real diversity in this environment.
520 Saturation was not reached on the rarefaction curves. Our results are in agreement with other
521 studies mainly performed on porous aquifers where β and γ Proteobacteria predominated
522 (Bordenave et al. 2007, Detmers et al. 2004, Dojka et al. 1998, Hess et al. 1997, Reardon et
523 al. 2004, Rodríguez-Martínez et al. 2006). It might be possible that Archaea were present in
524 our environmental DNAs since Archaea have recently been shown to predominate over their
525 better known bacterial counterparts during ammonia oxidation (the first step of nitrification)
526 in pristine aquifers (Leininger et al. 2006). Contrary to expectations based on the literature
527 (LaMontagne et al. 2003), a greater diversity of free-living bacteria was observed at 10 and
528 15 m than at 6 m. In fact, LaMontagne et al. (2003) who showed a decline in bacterial species
529 richness with depth, compared bacterial communities in the subsurface soil and the vadose
530 zone (1-4 m). In our study, the subsurface soil was not taken into account and the variably
531 saturated zone (3-8 m) was compared with the permanently saturated zone (8-15 m).

532 **2. Relationships between nitrate reducing community and nitrate concentrations**

533 A heterotrophic lifestyle is mundane in micro-organisms in nature. Heterotrophic nitrate
534 consumption was demonstrated in a similar environment in a previous study (Legout et al.

535 2007). The main electron acceptor in the Kerrien aquifer is nitrate which is available
536 irrespective of depth and time (Tab. 1).

537 The absence of a clear differentiation of the nitrate-reducing community with depth cannot be
538 attributed to the sampling approach. No difference was found before and after the purge (i.e.
539 true replicates) of the pan-flute piezometers (Fig S3 in Supplementary material). The stability
540 of nitrate reducing communities can be attributed to the chemical concentration of minerals,
541 in particular the high nitrate concentration and very low amount of carbon which remained
542 homogeneous throughout the water column. We suggest that this homogenous oligotrophic
543 status is the main driver of this community stability. It is in agreement with other findings
544 (Rivett et al. 2008). Along with that, we observed a contrasting pattern of divergence in the
545 nitrate reducer community between different sampling campaigns only in the first sampling at
546 6 m. This sample was characterized by a low $\text{NO}_3^-/\text{Cl}^-$ ratio (1.4) compared to the other
547 campaigns (~3). Chloride is known to be a conservative element and is only influenced by
548 physical processes such as evaporation or dilution (i.e. mixing). The absence of a concomitant
549 decrease in chloride implied that the 'low' nitrate concentration was due to nitrate reducer
550 activity. The T-RFLP analysis only indicates the diversity of free-living bacteria and not their
551 activity.

552 **3. Influence of seasonal variations of physical recharge processes on community** 553 **structure**

554 The water pumped from a well is not representative of all the groundwater present around the
555 well, but only of the mobile water. Water which is more strongly bound to rock, especially
556 within the micropores (De Marsily 1994), may constitute an active part of the groundwater,
557 from a biological point of view, as observed in similar sites (Ayraud et al. 2008, Legout et al.
558 2005, Tarits et al. 2006). The relative contribution of mobile *vs* immobile water from a given
559 geological layer is dependent on the hydraulic conductivity of this latter. Consequently, the

560 bacteria in water samples from highly permeable layers may over-represent the free-living
561 bacteria (Goldscheider et al. 2006). However the diversity within our true replicates (i.e. water
562 sample before and after pumping for a given piezometer) indicate that the purge had no effect
563 on community structure (Fig. S3 in Supplementary material). From this it is concluded that
564 the free-living bacteria in water might be a proxy of the bacteria in aquifers.

565 The relationships between microbial populations and their environment are often complex and
566 non-linear (Palumbo et al. 2004). In the case of the Kerrien aquifer, the distribution of
567 bacterial diversity is related to environmental characteristics expressed as seasonal variations,
568 as shown in Figure 4. Since the physico-chemical parameters (i.e. pH, dissolved oxygen)
569 remain stable through time especially at 15 m, the composition and structure of the bacterial
570 community in the subsurface groundwater should thus be driven mainly by hydrological
571 conditions, i.e. physical recharge processes described above. The effects of large seasonal
572 variability at 6 m depth and limited variability at 15 m depth are presented below. The effect
573 of the vertical contrast between the variably- and permanently-saturated zones is investigated
574 in section 4.

575 ***Effect of seasonal physical processes on time-related bacterial diversity (6 m depth):*** An
576 increase in microbial diversity was observed in the fluctuation zone during high water table
577 periods (Tab.3). During low water table periods, the decline in chloride concentration was
578 concomitant with a decrease in diversity. The chloride concentration at 6 m depth was
579 strongly related to diffusion/mixing and dependent on microporosity, as explained above.
580 Chloride diffusion might also be concomitant with bacterial diffusion. During subsurface
581 bacterial transport, bacteria can passively migrate with the ambient groundwater but they also
582 can attach to or detach from the solid matrix and flow or swim along a chemical gradient,
583 (Fenchel and Finlay 2004). In oligotrophic environments, chemotaxis can be a very important
584 transport mechanism (Ginn et al. 2002). This active process is however oriented and

585 chemotaxis is generally related to food availability which could be relevant when moving
586 from reduced conditions of micro-porosity to oxygen-rich macro-porosity. The cyclic
587 hydrological stresses are expected to induce a seasonal variation through advective transport,
588 which allows chemotaxis when the water content is sufficiently high to create a continuous
589 water film (Schaefer et al. 1995), and to limit transport conditions governed by capillary
590 forces when the water content is low (Huysman and Verstracte 1993). This variation may
591 explain the short-term changes in diversity apparent from the T-RFLP diversity signatures of
592 the 16S rRNA gene amplicons. The increase in diversity during high water periods might be
593 related to an introduction of bacteria from the soil. As explained above, during this period
594 there is a large introduction of precipitation water which has been rapidly transferred from the
595 soil to the variably-saturated zone.

596 *Effect of seasonal physical processes on depth-related bacterial diversity (15 m depth):* The
597 physical processes are different in the deeper zone. The weathered granite is constantly
598 saturated with water and therefore the bacteria are not subjected to cyclic hydrological stress.
599 The periods of high water are characterized by increased diversity and a ‘blending’ of the
600 bacterial communities along the water column. Comparisons of the communities at 6 m and
601 15 m during the periods of high water in the different campaigns (Ker3 and Ker5 samples)
602 revealed greater homogeneity than during the periods of low water when discrimination
603 between 6 m and 15 m was easier (Tab. 2b, Tab. 3). This variation in diversity can be
604 explained by a mixing of non-native and native species. Indeed, almost the same number of
605 bacterial cells was found at 15 m and 6 m (Fig. S2 in Supplementary material). A connection
606 between the bacterial communities at 6 m and 15 m results from the occurrence of vertical
607 fluxes of solutes and bacteria during the recharge process. The long distance passive dispersal
608 of bacteria is facilitated by their small size.

609

610 **4. Structuring of the microbial communities in the variably- and permanently- saturated**
611 **zones (influence of depth)**

612 We here discuss the microbial structuring in the hydrologically contrasted zones showing
613 highly variable conditions and stable conditions. Stable conditions are generally associated
614 with an increase in diversity, whereas disturbed environments lead to the outgrowth of
615 dominant adapted populations (Haack et al. 2004, Torsvik et al. 2002), which can result in an
616 ‘epidemic’ population structure [...] *when a successful individual arises and increases*
617 *rapidly to produce an epidemic clone* [...] (Maynard-Smith et al. 1993). In fact,
618 environmental changes or perturbations might lead to higher infraspecific diversity and allow
619 the selection of more rapidly adapted genotypes, and the appearance of an ‘epidemic’
620 population structure (e.g. Vandenkoornhuyse et al. 2001).

621 The results obtained for the vadose zone are in accordance with the hypothesis of increased
622 bacterial community diversity under stable conditions. The water table fluctuation zone
623 displays a specific community and a lower diversity (Fig. 3; Tab. 3). A few meters lower, the
624 communities at 15 m depth present higher diversity related to seasonal mixing with the more
625 specific bacteria from the variably saturated zone.

626 This compartmentalization with depth over time is apparent as a clear structuring of the
627 bacterial communities (Fig. 3). Microbial interactions may also contribute to the community
628 structure but little is known about the species interactions that occur within microbial
629 communities during transport processes (Balkwill et al. 1998). Our results do not confirm a
630 potential interaction during transport but instead emphasize the potential ‘mixing’ of bacterial
631 communities from two different environments.

632

633 The estimates of bacterial diversity and comparisons of community composition along a depth
634 profile during three successive hydrological cycles provided clear evidence of bacterial

635 community structuring. Despite the relative similarity of the physico-chemical conditions
636 over time, the bacterial communities could be differentiated whatever the depth. This suggests
637 that bacterial community structure and diversity were influenced by the physical processes of
638 water migration. They might also be explained by local competition between microorganisms
639 which could result in differentiation of the communities over time and a subsequent drift in
640 community composition. The physical transport induced by the recharge process would lead
641 to a higher diversity in the permanently saturated zone, even several meters below the zone of
642 water table variation. Our results suggest that the physical processes of water fluxes in the
643 aquifer play an important role in bacterial community structure.

644 The investigated site presents rather specific characteristics such as a water table depth which
645 remains relatively shallow and fluctuates rapidly (Martin et al., 2004). Various kinds of
646 geological formations, vadose thickness and heterogeneity would lead to various influences of
647 physical processes and water transfer kinetics. However, the recharge mechanisms can be
648 considered as ubiquitous and the overall characteristics of the hydrological cycle in the upper
649 part of the aquifers may concern a large number of various kinds of aquifers. More precisely,
650 the contrast between the permanently- and the variably-saturated zone, the seasonal effects of
651 diffusion and advection in the variably-saturated zone, and the seasonal connection of the
652 variably-saturated zone with the soil water may constitute a major driver to microbial
653 community structuring in many aquifers. Further studies should be however carried out to
654 assess to which extent the observed microbial patterns extend to other aquifers.

655

656

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663

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862 **TABLES**

863

864 Table 1: Dissolved oxygen (O₂), pH and temperature measurements; Nitrates, sulphates and
 865 dissolved organic carbon (DOC) concentrations in groundwater samples collected from the
 866 different wells from 2005 to 2007. No values are indicated at 6 m for the fourth campaign
 867 (Ker4) because the water table was below this level. No DOC measurements were done
 868 during the third and the fifth sampling campaigns for technical reasons. The quantification
 869 limit for carbon analysis is 0.85 mg L⁻¹.

870

<i>Physico - chemical parameters</i>												
<i>Campaign</i>	<i>Dissolved oxygen (mg.L⁻¹)</i>				<i>pH (Units)</i>				<i>Temperature (°C)</i>			
	<i>H6</i>	<i>H8</i>	<i>H10</i>	<i>H15</i>	<i>H6</i>	<i>H8</i>	<i>H10</i>	<i>H15</i>	<i>H6</i>	<i>H8</i>	<i>H10</i>	<i>H15</i>
<i>Samples</i>												
Ker1	4.4	4.5	4	5	4.8	5.5	5.5	5.4	17.9	16	17.1	18.1
Ker2	6.8	7.6	5.7	4.8	6.4	5.9	6.2	6	12.6	12.4	13	12.1
Ker3	7.9	7.6	6.2	6.5	5.9	5.1	5.2	5.3	12.5	12.5	12.6	12.9
Ker4	-	5.7	5.8	5.5	-	5.7	5.6	5.5	-	14.1	13.4	14.4
Ker5	9.4	8.2	8.7	6.5	6	5.2	5.4	5.2	11.3	12.1	10.2	12.2

<i>Chemical parameters</i>												
<i>Campaign</i>	<i>Nitrates (mg.L⁻¹)</i>				<i>Sulphates (mg.L⁻¹)</i>				<i>Dissolved Organic Carbon (mg.L⁻¹)</i>			
	<i>H6</i>	<i>H8</i>	<i>H10</i>	<i>H15</i>	<i>H6</i>	<i>H8</i>	<i>H10</i>	<i>H15</i>	<i>H6</i>	<i>H8</i>	<i>H10</i>	<i>H15</i>
<i>Samples</i>												
Ker1	56.1	71	61.8	62.6	65.4	22.8	20.2	17.6	3.73	0.98	1.42	1.45
Ker2	80.0	71.3	65.1	68	37.5	29.2	19.7	19.2	1.66	0.89	0.87	1.13
Ker3	87.3	73.5	69.8	72.4	35.9	21.6	20.1	18.5	-	-	-	-
Ker4	-	70.7	66	65.6	-	23	20.2	19.3	-	0.9	<QL	<QL
Ker5	57.2	67.7	69.2	64.8	22.2	21.9	19.7	19.5	-	-	-	-

871 Table 2: Similarity indexes calculated from the 16S rRNA gene T-RFLP analysis. Table A
 872 represents the similarity indexes of the different campaigns for each depth and table B the
 873 similarity indexes of the different depths for each campaign.

874 A. Time influence

<i>Depth</i>	<i>Samples</i>	Similarity indexes - Campaign				
		<i>Ker1</i>	<i>Ker2</i>	<i>Ker3</i>	<i>Ker4</i>	<i>Ker5</i>
6 m	Ker1	1				
	Ker2	0.16	1			
	Ker3	0.32	0.18	1		
	Ker4	-	-	-	-	
	Ker5	0.26	0.26	0.32	-	1
8 m	Ker1	1				
	Ker2	0.11	1			
	Ker3	0.11	0.2	1		
	Ker4	0.29	0.21	0.26	1	
	Ker5	0.26	0.21	0.17	0.41	1
10 m	Ker1	1				
	Ker2	0.24	1			
	Ker3	0.33	0.26	1		
	Ker4	0.44	0.24	0.25	1	
	Ker5	0.36	0.26	0.38	0.37	1
15 m	Ker1	1				
	Ker2	0.14	1			
	Ker3	0.27	0.29	1		
	Ker4	0.35	0.39	0.41	1	
	Ker5	0.18	0.44	0.32	0.68	1

875

876 B. Depth influence

<i>Campaign</i>	<i>Samples</i>	Similarity indexes - Depth			
		<i>6</i>	<i>8</i>	<i>10</i>	<i>15</i>
Ker1	6	1			
	8	0.2	1		
	10	0.42	0.41	1	
	15	0.36	0.38	0.27	1
Ker2	6	1			
	8	0.42	1		
	10	0.46	0.64	1	
	15	0.47	0.63	0.61	1
Ker3	6	1			
	8	0.50	1		
	10	0.32	0.38	1	
	15	0.51	0.42	0.34	1
Ker4	6	-			
	8	-	1		
	10	-	0.66	1	
	15	-	0.62	0.62	1
Ker5	6	1			
	8	0.58	1		
	10	0.47	0.60	1	
	15	0.40	0.57	0.59	1

877

878 Table 3: Diversity index (Shannon index) calculated from the 16S rRNA gene T-RFLP

879 analysis.

<i>Depth</i> <i>Samples</i>	Diversity indexes - Campaign				
	<i>Ker1</i>	<i>Ker2</i>	<i>Ker3</i>	<i>Ker4</i>	<i>Ker5</i>
6 m	3.69	2.40	4.63	-	4.29
8 m	3.94	4.89	4.37	3.90	4.89
10 m	4.63	3.83	4.74	4.12	5.07
15 m	3.15	4.96	5.08	4.32	5.76

880

881

882 **FIGURE Captions**

883 **Figure 1: Chemical concentration variation with time and depth.**

884 Depth of the water table *vs* time and chloride concentration changes in the water-table
885 fluctuation zone (6 m depth) and the permanently saturated zone (15 m depth). The water
886 table depth is given with the different sampling dates. Ker1 was sampled on 05/25/05, Ker2
887 on 01/18/06, Ker3 on 03/13/06, Ker4 on 11/22/06 and Ker5 on 01/25/07. Ker1, Ker3 and
888 Ker5 were sampled during high-water periods and Ker2 and Ker4 during low-water periods.

889 **Figure 2: Phylogenetic affinities of the environmental 16S rRNA gene sequences isolated** 890 **from environmental ecosystems, in a consensus tree.**

891 Only the closest known related 16S rRNA bacterial sequences are represented (i.e. closest
892 BLASTn hits and phylogenetic neighbours). The tree presented was constructed from 105
893 sequences using the NJ algorithm and K2P distance model and was congruent with the MP
894 and ML trees. Bootstrap values > 50% are indicated at the nodes (respectively MP/ML/NJ
895 estimated from 250/100/1000 iterations). The symbol * indicates bootstrap values higher than
896 95%. The sampling period and depth are presented along the tree on the right side of the
897 figure. K1 was sampled 05/25/05; K2: 01/18/06 and K3: 03/13/06.

898 **Figure 3: 16S rRNA gene bacterial community structuring with time (A) and depth (B).**

899 The non-centred PCA from T-RFLPs data highlighted bacterial community differentiation
900 with time for the 6 and 15 m water-samples. At 6 m depth, Ker4 is missing because no water
901 was present at this depth on the sampling date. 1, 2, 3, 4, and 5 are the gravity centres for each
902 modality: Ker1, Ker2, Ker3, Ker4 and Ker5

903 **Figure 4: Similarity between *narG* genes displayed as non-centred PCA versus time (A)** 904 **and depth (B).**

905 The non-centred PCAs were derived from the same database but are represented in relation to
906 time and depth.

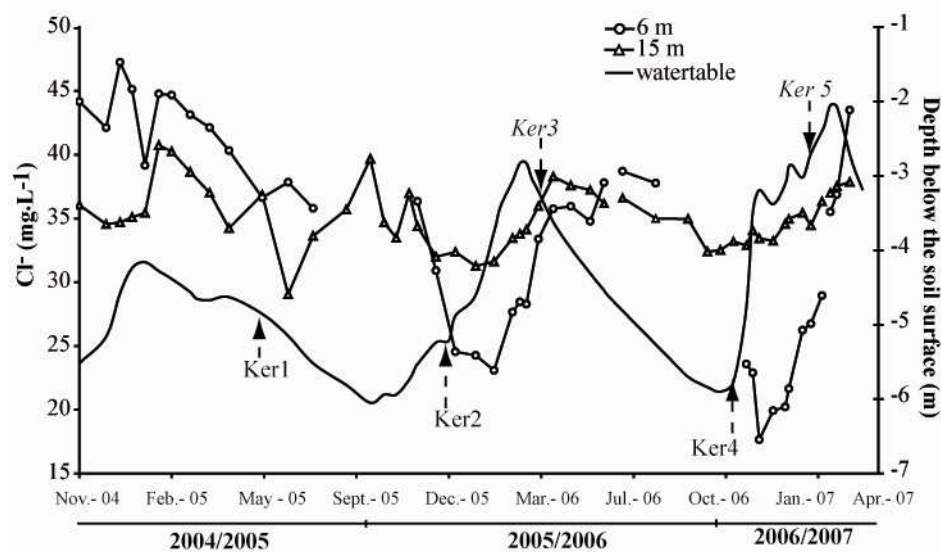
907 **Supporting Information**

908 **Fig. S1.** The Kerrien catchment is located in western France and the device used for sampling
909 water is a pan-flute piezometer (modified from Molénat et al. 2007).

910 **Fig. S2.** The number of bacterial cells in each sample was estimated by flow cytometry. The
911 symbols plotted are means of 3 independent measures. For each mean, the standard deviations
912 are plotted as bars. The dark symbols represent the samples before purging the well, while the
913 open symbols represent the samples collected after purging.

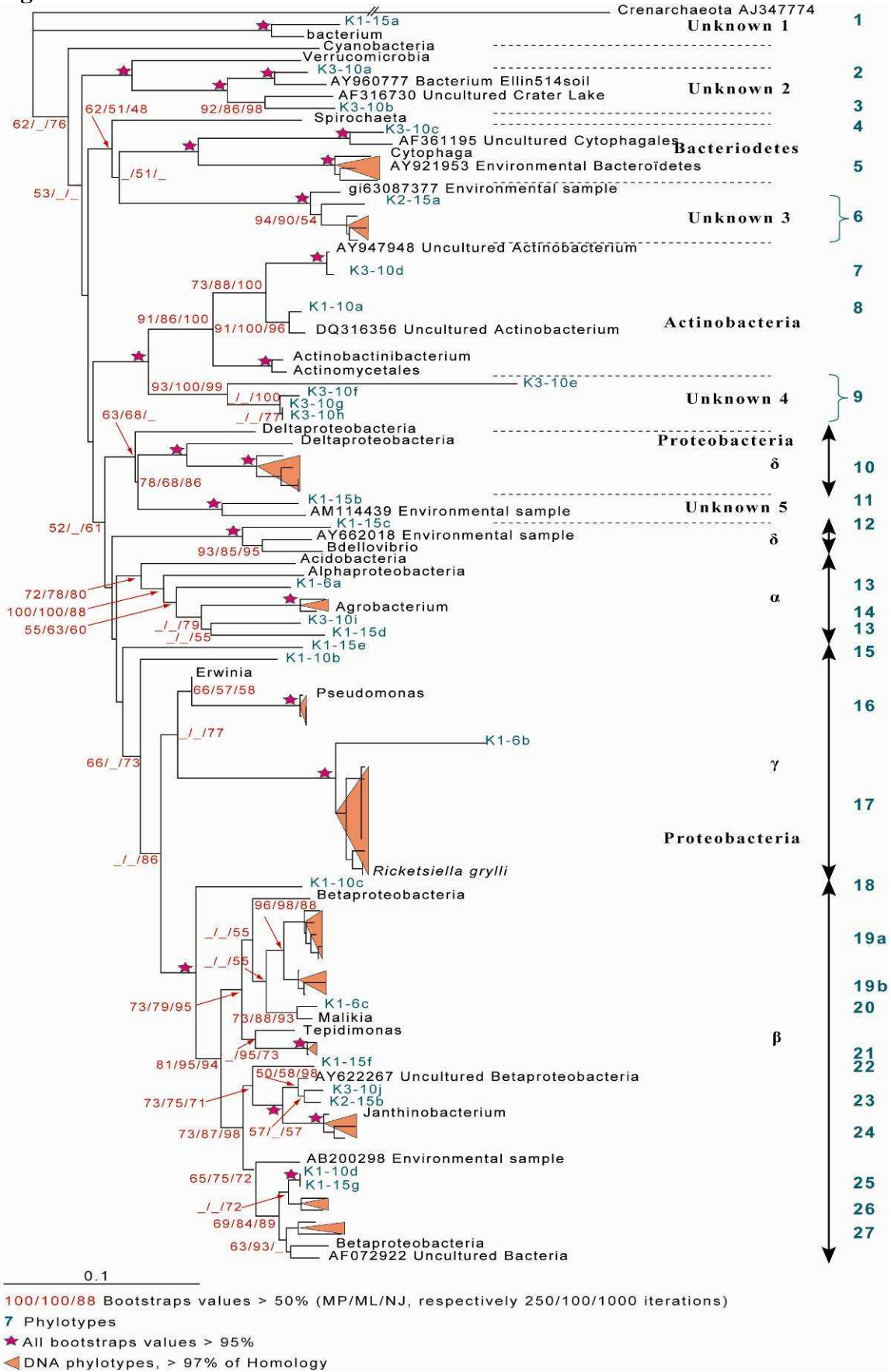
914 **Fig. S3.** The effect of the purge on the bacterial communities was tested by non-centred PCA.
915 The ellipses grouped 95% of the communities (dots) projected on the vectorial space for a
916 vine modality (i.e. before purge 'BP' vs after purge 'AP'). 'BP' and 'AP' represent the
917 gravity centres of the ellipses. For a given piezometer water samples before purge and after
918 purge were considered as true replicates. These 2 true replicates were expected more
919 heterogeneous in comparison to two samples before purge or two samples after purge.

920 **Figure 1.**



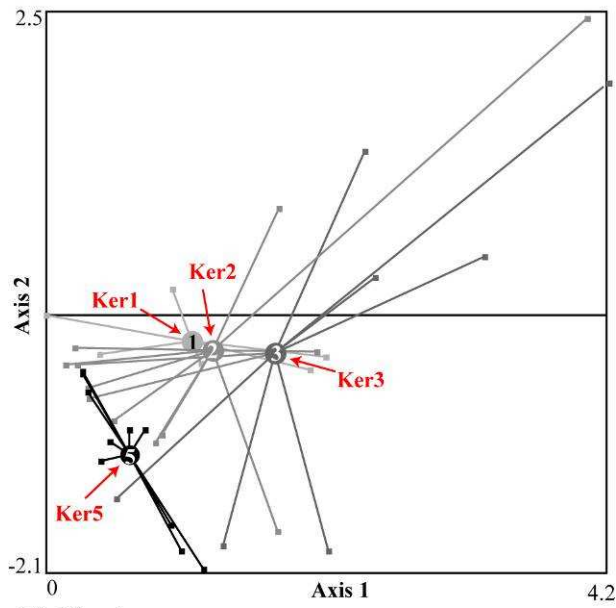
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923 **Figure 2.**

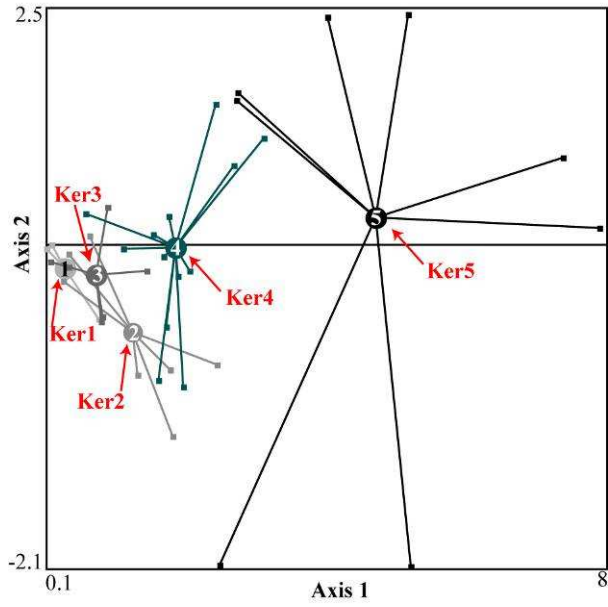


924

925 **Figure 3.**
(A) 6 meters



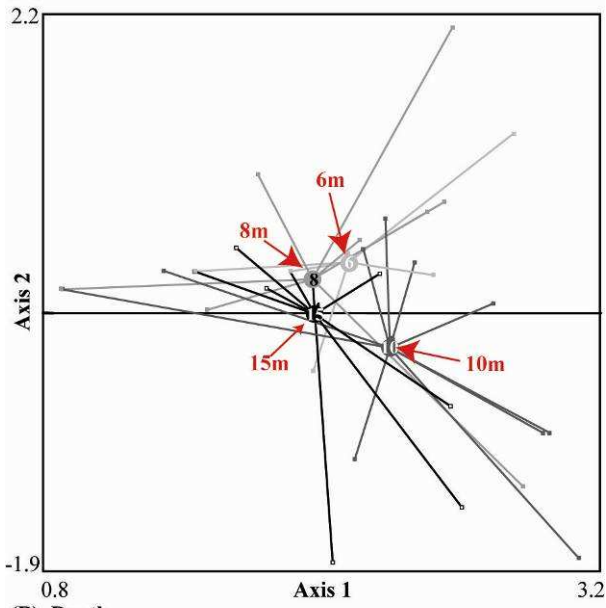
926 (B) 15 meters



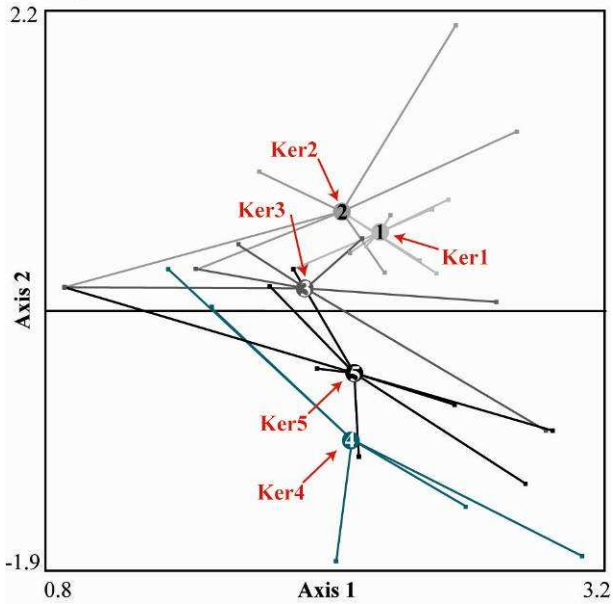
927

928 **Figure 4.**

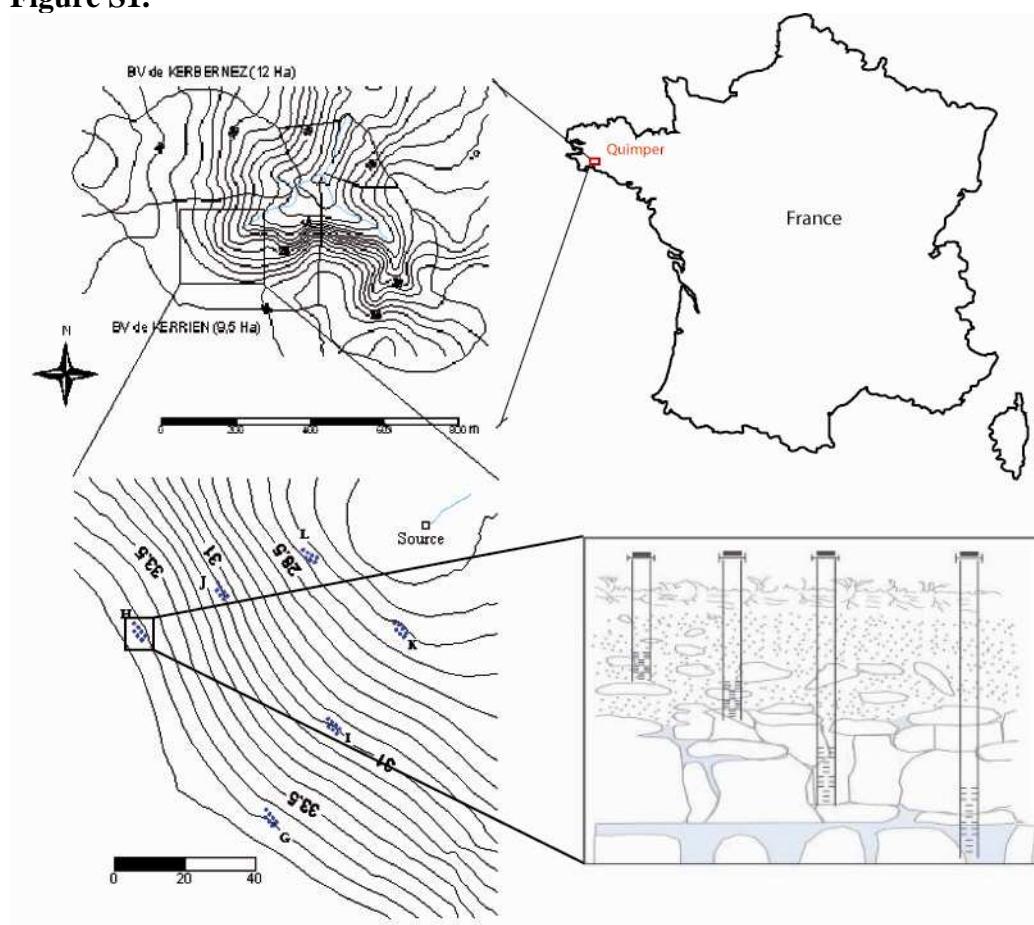
(A) Time



(B) Depth

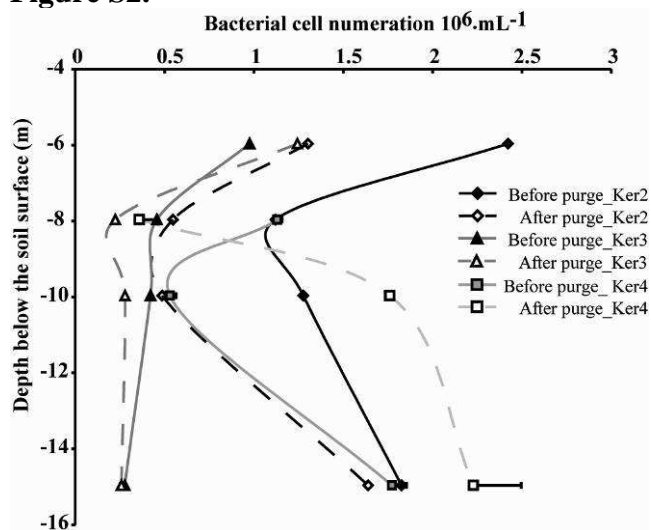


931 **Figure S1.**



932

933 **Figure S2.**



934

935 **Figure S3.**

