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Jérémy Voisin, B. Cournoyer, L. Marjolet, Antonin Vienney, Florian Mermillod-Blondin

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1 **Ecological assessment of groundwater ecosystems disturbed by recharge systems**
2 **using organic matter quality, biofilm characteristics and bacterial diversity**

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7 4 Jérémy VOISIN^{1,2}, Benoit COURNOYER², Laurence MARJOLET², Antonin VIENNEY¹,
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10 5 Florian MERMILLOD-BLONDIN^{1*}

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1- Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR5023, Écologie des
Hydrosystèmes Naturels et Anthropisés, Villeurbanne, F-69622, France

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20
21

2- Univ Lyon, Université Claude Bernard Lyon 1, VetAgro Sup, CNRS, UMR5557, INRA
UMR1418, Ecologie Microbienne, Research Team "Bacterial Opportunistic Pathogens and
Environment", Marcy L'Etoile, F-69280, France

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* Corresponding author:

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Florian Mermillod-Blondin

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Phone: 00 33 4 72 43 13 64

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E-mail: florian.mermillod-blondin@univ-lyon1.fr

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19 Abstract

20 Recharge of aquifers by urban stormwater may trigger significant ecological changes that can be
21 detrimental to the biodiversity and functioning of groundwater ecosystems. Here, the effects of
22 aquifer recharge (AR) on three levels of parameters were investigated: dissolved organic carbon
23 (DOC) quantity and quality, global biofilm characteristics, and diversity changes of bacterial
24 communities. As DOC enrichment by AR can be mitigated by vadose zone (VZ) thickness, 3 AR sites
25 with thin VZ (< 3 m) and 3 sites with thick VZ (> 10 m) were selected. For each AR site, clay beads
26 were incubated over a 10 days-long rainy period through wells in recharged and non-recharged
27 groundwaters. Total proteins, dehydrogenase and hydrolytic activities were monitored from clay
28 beads to assess biofilm development. Bacterial richness on beads was estimated by 16S rRNA-based
29 metabarcoding. AR was found to significantly increase DOC and biodegradable DOC (BDOC)
30 concentrations, biofilm development and bacterial richness especially in sites with thin VZ. VZ
31 thickness was inversely related to microbial growth indicators and bacterial richness in groundwater,
32 through a control of DOC availability. The proportion of *Bacteroidetes* 16S rRNA gene reads was
33 higher in recharged groundwater than in non-recharged groundwater, suggesting that this phylum
34 could be used as an indicator of DOC enrichment associated with AR. Quantitative PCR assays for
35 *Bacteroides* DNA confirmed these trends, and showed an enrichment of this bacterial group in DOC-
36 rich aquifer waters. The positive linear relationships between BDOC concentrations and biofilm
37 variables highlighted a strong C-limitation of groundwater impacting bacterial species sorting and
38 activity.

39 40 Keywords

41 Nutrient-driven diversity changes; vadose zone thickness; stormwater infiltration; clay bead biofilms
42 bacterial communities; aquifer 16S rRNA metabarcoding profiling

44 **Introduction**

45 In ecology, the species-energy theory (Wright 1983, Evans et al. 2005; Fuhrman 2009) is considered
46 as a universal law stating that species richness correlates positively with energy availability. Smith
47 (2007) reviewed 70 published studies in aquatic ecosystems and concluded that diversity-productivity
48 patterns are similar between micro- and macro-organisms. Nonetheless, the shape of the diversity-
49 productivity relationship was variable depending on the studied micro-organisms and environments
50 (Smith 2007; Prosser et al. 2007; Bernstein et al. 2017). Although these relationships have been
51 reported from many aquatic ecosystems including natural lakes, ponds, and oceans, few studies have
52 investigated the influence of energy availability on the diversity of microbial communities in
53 groundwater ecosystems (but see Hershey et al. 2018 for a deep karstic aquifer).

54 Groundwater ecosystems are among the most stable and energy-limited environments on Earth
55 because of the lack of photosynthesis and low inputs of organic matter infiltrating from surface
56 ecosystems (Gibert 1994; Griebler and Lueders, 2009). Nonetheless, connectivity and dissolved
57 organic matter (DOM) transfers between surfaces and subterranean systems can be enhanced by
58 aquifer recharge (AR) practices (e.g., Kolehmainen et al. 2007). In urban environments, these
59 practices were developed to collect stormwater runoff to recharge aquifers by infiltration through
60 porous media (Marsalek and Chocat, 2002; Hunt et al. 2010). AR practices have been shown to
61 increase DOM in groundwaters over the levels of those that were not influenced by recharge (Datry
62 et al. 2004; Foulquier et al. 2010; Mermillod-Blondin et al. 2013). DOM is the main source of food
63 for micro-organisms in groundwater ecosystems (Goldscheider et al. 2006), and its increase can lead
64 to denser and more active microbial communities (Foulquier et al. 2011, Landa et al. 2013). For
65 example, Mermillod-Blondin et al. (2013) showed that the growth, and the esterase and
66 dehydrogenase activities of biofilms were stimulated by DOM enrichment associated with aquifer
67 recharge in an urban context. Nevertheless, the influence of DOM supplied by AR systems on
68 microbial diversity is still poorly defined.

69 The aim of the present work was to evaluate the relative efficiencies of three levels of descriptors to

70 detect the effect of stormwater inputs on groundwater: DOM quality, biofilm characteristics and
71 bacterial diversity. A field experiment was conducted during a rainy period in 6 AR sites that were
72 divided into two groups: (1) those with a thin vadose zone (< 3 m) and (2) those with a thick vadose
73 zone (> 10 m). The vadose zone plays a key role on DOM retention during its transfer from surface
74 to groundwater (Pabich et al. 2001; Shen et al. 2015). Selection of sites with different vadose zone
75 thicknesses should lead to significant differences in groundwater disturbance. Dissolved organic
76 carbon (DOC) and biodegradable (BDOC) concentrations were measured together with the
77 incubation of artificial substrates (i.e., clay beads) in recharged and non-recharged groundwaters.
78 Clay beads were previously shown to be efficient artificial substrates that can allow reliable bacterial
79 diversity analyses of groundwaters (Voisin et al. 2016; Mermillod-Blondin et al. 2019). Microbial
80 productivity and activities of the biofilms on the clay beads could be monitored from biomass
81 measurements (total proteins), and from esterase and dehydrogenase activities. Bacterial diversity
82 was investigated through a next-generation sequencing (NGS) dataset of PCR amplified 16S rRNA
83 gene segments generated from biofilm DNA extracts. According to Datry et al. (2005), we expected
84 that DOM concentrations would be higher in AR sites with a thin vadose zone in comparison with
85 those with a thick vadose zone. Consequently, the hypothesis was that the influence of AR practices
86 on microbial biomass, activity and diversity should be highest in AR sites with the thinnest vadose
87 zone (< 3 m). In addition, positive correlations between DOM concentrations over the 6 sites and
88 bacterial richness of the clay bead biofilms developing in groundwaters should be observed.

90 **Material and methods**

91 **Sites**

92 Aquifer recharge (AR) sites were located on the eastern aquifer of the Lyon metropolitan area, France
93 (Fig. 1A). This aquifer (catchment area: 314 km²) is characterized by three corridors of highly
94 permeable glacio-fluvial sediments (hydraulic conductivity 10⁻³-10⁻² m/s) (Foulquier et al. 2009). The
95 aquifer is artificially recharged with stormwater runoff at multiple AR sites to compensate for the

96 reduced recharge caused by the sealing of urban surfaces. The AR sites of this study corresponded to
97 retention/infiltration basins that collected stormwater from residential, commercial, and industrial
98 areas.

100 **Experimental design**

101 Six AR sites (Figure 1AB) were selected: 3 with vadose zone thicknesses < 3 meters (IUT, Grange
102 Blanche (GB) and Minerve (MIN) basins) and 3 with vadose zone thicknesses > 10 meters (Django
103 Reinhardt (DJR), Raquin (RAQ) and Feyzin (FEY) basins). Each AR site is equipped with two wells:
104 a well located outside the area of influence of the stormwater plume induced by infiltration which
105 was used as a control well, and a well located in the immediate downstream vicinity of the infiltration
106 basin used as a recharge well because it intersected the stormwater plume induced by the infiltration..
107 Clay beads (expanded clay aggregates, Botanic®, Saint-Julien en Genevois, France) of a diameter of
108 8 mm were incubated in these wells to trap micro-organisms. These clay beads favor biofilm devel-
109 opments from the groundwater microbiota (Voisin et al. 2016; Mermillod-Blondin et al. 2019), and
110 enable a monitoring of parameters such as microbial productivity (total proteins), and esterase and
111 dehydrogenase activities. Before incubation, clay beads were heated to 550°C to eliminate organic
112 matter traces and then autoclaved at 121°C for 20 min. They were incubated in wells using small
113 polypropylene bags (110 × 40 mm) with a mesh size of 3.4 mm, previously treated with ethanol
114 (70%). A total of 30 clay beads were inserted per bag, and 3 bags were incubated per well after well
115 purging with a submerged pump (see below). From each bag, clay beads were used to measure all
116 microbial variables (total proteins, esterase and dehydrogenase activities, bacterial community struc-
117 ture, and qPCR assays on specific genes) in order to obtain mean biofilm parameters per well calcu-
118 lated from 3 values (corresponding to batches of clay beads from the three bags incubated in the same
119 well). According to previous laboratory and field experiments (Voisin et al. 2016; Mermillod-Blondin
120 et al. 2019), clay bead incubation lasted 10 days during a rainy period to obtain (1) a significant
121 microbial colonization of clay beads submerged in groundwaters and (2) a time-integrated monitoring

122 of micro-organisms in recharged groundwater during a rainy event. The experiment was performed
123 between September 12 and September 22 in 2015. During this period, precipitations were of 26.6 mm
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of micro-organisms in recharged groundwater during a rainy event. The experiment was performed between September 12 and September 22 in 2015. During this period, precipitations were of 26.6 mm on the 12, 34.6 mm on the 13, 1.6 mm on the 14, 49.6 mm on the 17, 2.8 mm on the 18, 3.2 mm on the 19, and 1 mm on the 20. For each AR site, groundwaters (recharged and non-recharged) were also collected from the same wells but at three dates over the clay bead incubation period (Day 0, Day 5 and Day 10) to obtain mean values of DOC and BDOC per well during the incubation period of clay beads. Groundwaters were sampled in wells using a submerged pump (PP36 inox, SDEC, Reignac-sur-Indre, France). The first 50 pumped liters were used to rinse the sampling equipment. The following liter was collected for analyses of DOC and BDOC analyses. Samples were stored in pre-combusted glass bottles and brought back to the laboratory in an isotherm box at 4°C.

Water physico-chemistry

DOC (dissolved organic carbon) was determined using a carbon analyzer (multi N/C® 3100, Analytik Jena, Jena, Germany) based on thermocatalytic oxidation (850°C) of organic carbon and infrared detection of CO₂, after removal of dissolved inorganic C with HCl. Biodegradable DOC (BDOC) was determined by the method of Servais et al. (1987, 1989) following Mermillod-Blondin et al. (2015). Briefly, water samples (100 mL) were filtered through a pre-washed 0.2 µm polycarbonate membrane and incubated with a bacterial inoculum at 20°C for 30 days in the dark. DOC concentrations were measured from filtered (0.2 µm) water samples at the start and the end of the incubation period to determine the initial DOC concentration and the remaining DOC concentration after 30 days of incubation (representing the refractory dissolved organic carbon, RDOC), respectively. BDOC concentration was then calculated as the difference between initial DOC concentration and RDOC concentration.

Microbial analysis

Microbial biomass

148 Microbial biomasses on clay beads were assessed from Lowry's total protein assay using the Sigma
149 Protein Assay Kit P-5656 (Sigma Diagnostics, St Louis, MO, USA) according to Peterson (1977).
150 For each incubated bag, 5 randomly-sampled clay beads were treated with 4 mL of a 50 % solution
151 of Lowry's reagent for 20 min. At the start of the reaction, the contact between proteins on beads and
152 the reagent was improved by vortexing at 1000 rpm for 1 minute. After the 20-min long reaction, 0.5
153 mL of Folin reagent was added, followed by a 30 min color reaction. The absorbance of the solution
154 was then measured at 750 nm with a spectrophotometer (Aquamate, ThermoSpectronic, Cambridge,
155 UK) and total protein content on beads was determined using a calibration curve performed with
156 bovine serum albumin. Total proteins were expressed in μg of proteins per cm^2 of clay bead, after
157 subtracting values measured from 3 batches of 5 sterile clay beads.

159 Microbial activity

160 Hydrolytic activity of biofilms was estimated using the fluorescein diacetate (FDA) hydrolysis
161 method (Fontvieille et al. 1992) following Voisin et al. (2016). For each incubated bag, 5 randomly-
162 sampled clay beads were incubated into 3 ml of a pH 7.6 phosphate buffer solution with 0.15 ml of
163 4.8 mM FDA solution. Incubation was performed at 15°C for 3 h. The reaction was stopped by
164 addition of 3 ml of acetone in the sample and storage at -20°C (Battin 1997). Fluorescein
165 concentration was estimated by measuring the absorbance at 490 nm of the filtered supernatant (0.45
166 μm , HAWP, Millipore, Billerica, MA, U.S.A.). Then, the hydrolytic activity of biofilms was
167 expressed in μmol of FDA hydrolyzed per hour and per cm^2 of clay bead, after subtracting values
168 measured from 3 batches of 5 sterile clay beads.

169 The 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) was used to measure
170 dehydrogenase activity (respiratory activity) as modified from Hourri-Davignon et al. (1989)
171 following Mermillod-Blondin et al. (2019). For each incubated bag, 5 randomly-sampled clay beads
172 were incubated in a 0.02% INT solution for 3 h at 15°C and the supernatant was then filtered on a
173 nylon membrane (0.22 μm , Millipore, Billerica, MA, U.S.A.) to retrieve floating microbial cells.

174 Extraction of INT-formazan from clay beads and nylon membrane containing microbial cells was
175 performed with 5 ml of methanol during 12 h. The INT-formazan concentration was determined by
176 absorbance measurement at 480 nm. Then, the dehydrogenase activity of biofilms was expressed in
177 μmol of INT-formazan produced per hour and per cm^2 of clay bead, after subtracting values measured
178 from 3 batches of 5 sterile clay beads.

130 16S rRNA gene bacterial community profiling

181 DNA extraction and qPCR assays

182 DNA extraction from clay beads biofilms was performed following Voisin et al. (2016). For each
183 incubated bag, microbial cells were detached from 15 randomly-selected clay beads by shaking at
184 2500 rpm for 2 minutes in 10 mL of 0.8 % NaCl. Cells and aggregates were retrieved from the NaCl
185 solution by a 0.22 μm filtration. DNA was extracted from filters using the FastDNA spin kit for soil
186 (MP biomedical). Then, the amount of extracted DNA was quantified spectrophotometrically using
187 a Nanodrop ND1000 (Thermo Fisher Scientific, USA).

188 qPCR assays were performed on a Bio-Rad CFX96 real-time PCR instrument with Bio-Rad CFX
189 Manager software, version 3.0 (Marnes-la-Coquette, France). Total *Bacteroides* DNA targets were
190 PCR amplified according to Layton et al. (2006) using the Brilliant II SYBR Green low ROX qPCR
191 master mix for TaqMan qPCR (Agilent, Vénissieux, France). The human-specific HF183 *Bacteroides*
192 qPCR assay was performed according to Seurinck et al. (2005), and the assay for the 16S rRNA gene
193 segment for total bacteria was performed according to Park and Crowley (2006) using primers 338F
194 and 518R, and using the Brilliant II SYBR green low ROX qPCR master mix for SYBR Green qPCR.
195 Melting T° was 60°C for all assays. Linearized plasmid DNA containing 16S rRNA genes from the
196 targeted DNAs were run as standards using 10-fold dilutions of the plasmids. These plasmids were
197 obtained from Marti et al. (2017). Presence of inhibitors was checked by spiking known amount of
198 plasmid harboring *int2* with 10 times dilution of sample DNA extracts (10^7 copies of plasmid per μl
199 of DNA diluted solution). Number of cycles needed to have a significant signal was compared with

200 wells where only plasmid harboring *int2* was added to the qPCR mix. When a higher number of cycles
201 was needed to observe a signal, a dilution by 5 or 10 fold was done and another run of tests was
202 performed to confirm the absence of PCR inhibitions. Negative controls without template DNA were
203 run in triplicate. Each assay was triplicated on distinct DNA extracts, and technical triplicates were
204 performed.

206 V5-V6 analyses of 16S rRNA gene PCR products

207 Sequencing of the V5-V6 16S rRNA gene segments were performed at MrDNA (Shallowater, TX,
208 USA) with the Illumina MiSeq technology. Primers 799F (5'-ACCMGGATTAGATACCKG-3')
209 and 1193R (5'-CRTCCMCACCTTCCTC-3') were used, and 20,000 reads (i.e. 2 x 300-bp paired-
210 end reads) were generated per sample. Data processing was realized with the MOTHUR software
211 version 1.35.1 (Schloss et al. (2009) following Voisin et al. (2018)). Briefly, raw sequences were
212 filtered by discarding those having (1) read sizes < 200 bp and > 400 pb, (2) reads containing
213 homopolymers > 8 bp, (3) more than one sequence mismatch to barcode DNA tags and more than
214 two mismatches with the forward sequencing primer, (4) reads containing ambiguous bases, and (5)
215 average sequencing quality score < 35 using a 50-bp sliding window (Schloss et al. 2011). After this
216 filtering step, reads were aligned against the SILVA database release 123 (Quast et al. 2013) and reads
217 that could not be reliably assigned to the V5-V6 region were discarded. A pre-cluster step was applied
218 following Huse et al. (2010). Potential chimeric sequences were detected and removed using
219 CHIMERA UCHIME (Edgar et al. 2011). Then, reads were clustered into operational taxonomic units
220 (OTUs) by a clustering at 97% similarity (Schloss and Westcott 2011). OTUs were affiliated to
221 taxonomic groups according to the SILVA database using an 80 % minimum bootstrap cutoff (Wang
222 et al. 2007). DNA sequences classified as *Archaea*, *Eukaryota*, chloroplasts or mitochondria were
223 discarded.

225 Data deposition

226 Sequence data are available at the European Nucleotide Archive (<https://www.ebi.ac.uk/ena>) under
227 the project accession #PRJEB29925. Sample accession #ERS2912777 (SAMEA5128392) = run2.
228 See Table S1 for barcode information.

230 **Data analysis**

231 The effects of artificial recharge (i.e., infiltration) and vadose zone thickness (VZT) on DOC and
232 BDOC concentrations, biofilm characteristics (i.e., proteins, FDA and INT assays) and diversity
233 (number of OTUs, Shannon diversity index, and Pielou's evenness index) were tested using repeated-
234 measure analyses of variance (2-way RM-ANOVA) with VZT as fixed factor and recharge as repeated
235 factor to take into account the non-independence of recharged and non-recharged wells in each site.
236 Pearson's correlations were computed to test the significance of the relations between DOM (i.e.,
237 DOC and BDOC) and clay bead biofilm variables (i.e. biomass, activities, OTU richness, and
238 diversity indices). Most variables were logit transformed to satisfy the normality assumption verified
239 by Shapiro's test. Sphericity assumption for RM-ANOVA was verified using Mauchly's sphericity
240 test. As Log transformed qPCR datasets did not satisfy the assumption of normality, Kruskal-Wallis
241 (KW) and Spearman correlation tests were applied on the qPCR datasets.

242 A non-metric multidimensional scaling (NMDS) was performed based on the Bray-Curtis
243 dissimilarity distances. Differences in bacterial community structures among biofilms from recharged
244 and non-recharged groundwaters in AR sites with thin and thick vadose zones (4 groups taking into
245 account 2 VZT conditions * 2 recharge conditions) were tested using permutational multivariate
246 analysis of variance (PERMANOVA; Anderson 2001). Statistical tests were based on 999
247 permutations of the Bray-Curtis matrix and significance was accepted at p-value < 0.05. All statistical
248 tests were performed using the R software (R Development Core Team 2008). The ade4 and Vegan
249 packages were used for nMDS and PERMANOVA analyses (Oksanen et al. 2007).

251 **Results**

252 **DOM concentrations in groundwater of AR sites**

253 DOC and BDOC concentrations in groundwater averaged $1.19 \pm 0.55 \text{ mg L}^{-1}$ and $0.56 \pm 0.27 \text{ mg L}^{-1}$, respectively. Both DOC and BDOC concentrations were significantly higher in recharged groundwaters than in non-recharged groundwaters (Fig. 2AB, 2-way RM-ANOVA, recharge effect, $p < 0.05$ for DOC and BDOC concentrations). DOC concentrations in recharged groundwater averaged $1.52 \pm 0.54 \text{ mg L}^{-1}$ whereas measured concentrations in non-recharged groundwater were $0.87 \pm 0.33 \text{ mg L}^{-1}$. The same pattern was observed for BDOC concentrations with higher values measured in recharged groundwater ($0.69 \pm 0.26 \text{ mg L}^{-1}$) in comparison with concentrations from non-recharged groundwater ($0.42 \pm 0.22 \text{ mg L}^{-1}$). The mean enrichment in DOM concentrations induced by groundwater recharge (recharged versus non-recharged groundwater) was more important in AR sites with thin vadose zones (mean enrichment of 0.89 mg L^{-1} and 0.37 mg L^{-1} for DOC and BDOC, respectively) than in AR sites with thick vadose zones (mean enrichment of 0.40 mg L^{-1} and 0.17 mg L^{-1} for DOC and BDOC, respectively). Consequently, DOC and BDOC concentrations in groundwaters were significantly higher in AR site with a thin vadose zone than in AR sites with a thick vadose zone (2-way RM-ANOVA, VZT effect, $p < 0.03$).

268 **Influence of groundwater recharge on biofilm biomass and activities**

269 Biofilm growth parameters were significantly influenced by recharge of groundwater with stormwater runoffs (2-way RM-ANOVA, recharge effect, $p < 0.05$). Total proteins measured on clay beads were significantly higher in biofilms developed in recharged groundwaters (mean total proteins: $7.34 \pm 5.09 \text{ } \mu\text{g per cm}^2$ of clay beads) than in biofilms recovered from non-recharged groundwaters (mean total proteins: $4.23 \pm 1.79 \text{ } \mu\text{g per cm}^2$ of clay beads) (Fig. 2C). The same trends were observed for hydrolytic and dehydrogenase activities measured on biofilms (Fig. 2DE) although no significant statistical results were obtained due to the high variability among sites (2-way RM-ANOVA, recharge effect, $0.2 > p > 0.15$ for hydrolytic activity and $0.1 > p > 0.05$ for dehydrogenase activity). On average, hydrolytic activity was more than 4-fold higher for biofilms developed in recharged

278 groundwaters compared to those developed in non-recharged groundwaters (Fig. 2D). Groundwater
279 recharge increased by more than 1.5-fold the dehydrogenase activity of biofilms over the activities
280 obtained from non-recharged groundwaters (Fig. 2E). Quantitative PCR assays used to determine
281 bacterial concentrations over the bead surfaces followed similar trends. High bacterial contents
282 among the clay bead biofilms going from 2×10^6 to 1.88×10^7 16S rRNA gene copies per cm^2 of clay
283 beads incubated in non-recharged groundwater, and 2.35×10^6 to 6×10^8 copies per cm^2 of clay beads
284 incubated in recharged groundwater (Table S2). 16S rRNA qPCR DNA counts were significantly
285 higher in clay bead biofilms from recharged wells with a thin vadose zone rather than those with a
286 thick vadose zone (Kruskal-Wallis tests, $p < 0.01$). This VZT effect was confirmed with the other
287 biofilm biomass and activities parameters also giving higher values in AR sites with thin vadose zones
288 rather than thick ones (2-way RM-ANOVA, VZT effect, $p < 0.05$ for the three variables). Although
289 statistical analyses were close to significant level (2-way RM-ANOVA, “recharge x VZT” interaction,
290 $0.1 > p > 0.05$ for dehydrogenase activity), biomass and activities tended to be more stimulated by
291 recharge in AR sites with vadose zone thicknesses < 3 m in AR sites with vadose zone thicknesses $>$
292 10 m.

293 **Influence of groundwater recharge on biofilm 16S rRNA gene diversity indices and bacterial** 294 **community structure**

295 The OTU numbers were estimated from 16S rRNA V5-V6 DNA segments generated from the clay
296 bead biofilm DNA extracts (after a re-sampling step set at 32,000 sequences per sample, after the
297 trimming-cleaning processes). These DNA reads were grouped into OTU when showing 3 % or less
298 divergence. Accordingly, the DNA extracts from recharged groundwaters were more diversified than
299 biofilms from non-recharged ones (2-way RM-ANOVA, recharge effect, $p < 0.05$). Numbers of
300 detected OTUs were 1224 ± 472 in recharged groundwater and 870 ± 202 in non-recharged
301 groundwaters (Fig. 2F). OTU abundance also depended on VZT (2-way RM-ANOVA, VZT effect, p
302 < 0.02). OTU increases were mainly observed in AR sites with vadose zone thicknesses < 3 meters

304 (mean increase of 607 OTUs). The other three sites with thicker vadose zones showed no clear
305 changes in OTU numbers associated with AR (mean decrease of 102 OTUs). Shannon diversity
306 indices computed from OTU numbers were higher in biofilms from recharged groundwaters than
307 non-recharged ones (Fig. 2G, 2-way RM-ANOVA, recharge effect, $p < 0.05$). Pielou's evenness index
308 indicated that these differences in OTU were associated with an increase in evenness among OTUs
309 from biofilms of the recharged sites (Fig. 2H, 2-way RM-ANOVA, recharge effect, $p < 0.05$). In
310 addition, the NMDS ordination of OTUs from clay beads showed significant differences in bacterial
311 community structure between biofilms developing in groundwater for the four tested conditions (2
312 recharge conditions * 2 VZT condition, Fig. 3, PERMANOVA, $p < 0.001$).

314 **Core bacterial taxa inferred from aquifer biofilm 16S rRNA gene sequences**

315 Only 130 OTUs which corresponded to 1.38 % of the total number of OTUs (9435) were shared
316 between the 36 clay bead biofilm samples. Nonetheless, these 130 OTUs represented 71.66 % of all
317 sequences. The largest groups of core OTUs were allocated to the following phyla: *Proteobacteria*
318 (109 OTUs with 87 % of sequences), followed by *Bacteroidetes* (13 OTUs with 11 % of sequences),
319 *Actinobacteria* (5 OTUs with 1.6 % of sequences) and *Nitrospirae* (3 OTUs with only 0.05 % of
320 sequences). *Proteobacteria* were mainly represented by *Gamma-Proteobacteria* (28 OTUs with 44 %
321 of sequences), *Beta-Proteobacteria* (50 OTUs with 32 % of sequences) and *Alpha-Proteobacteria*
322 (27 OTUs with 10% of sequences).

323 Core clay bead biofilm communities of non-recharged groundwaters and recharged groundwaters
324 were similar with *Proteobacteria* being predominant according to number of OTUs and read numbers
325 (Fig. 4). *Proteobacteria* was followed by *Bacteroidetes*, and *Actinobacteria*. Other phyla such as
326 *Nitrospirae* and *Elusimicrobia* were found but in smaller proportions in both core communities.
327 *Chlorobi* and *Chloroflexi* were only detected in core communities of recharged groundwaters, while
328 *Firmicutes* were only found in core communities of non-recharged groundwaters. Except these rare
329 phyla, the main difference between the core communities of recharged and non-recharged

330 groundwaters was observed on the relative proportions of *Bacteroidetes* (Fig. 4): *Bacteroidetes* were
331 represented more than 3-fold in the core community of recharged groundwaters (17.1 % of sequences)
332 than in core community of non-recharged (4.9 % of sequences). Quantitative PCR assays confirmed
333 these trends (Table S2). Concentrations of total *Bacteroides* were found significantly higher by qPCR
334 in clay bead biofilm DNA extracts from recharged wells than those recovered from non-recharged
335 wells (Table S2) (for each VZT category, Kruskal-Wallis, p-value <0.01). Furthermore, biofilms from
336 shallow aquifers were always found to have significantly higher total *Bacteroides* counts than those
337 from aquifers with higher VZT (KW p-value <0.01). Total *Bacteroides* DNA could not be detected
338 among most clay bead biofilms recovered from non-recharged wells from sites with a thick VZT
339 except for two samples in the RAQ site (Table S2).

341 **Relations between DOM and biofilm biomass, activities and diversity**

342 Biofilm characteristics such as qPCR datasets, microbial biomass estimated from Lowry's total
343 proteins estimations or microbial activities estimated from FDA and INT were positively and
344 significantly correlated with the concentrations of DOC and BDOC associated with stormwater
345 infiltration (e. g. Table 1), with the exception of the relation between FDA activity and DOC that was
346 not significant. Nevertheless, stronger correlations between these three microbial parameters with
347 BDOC were observed rather than with DOC. Number of bacterial cells among aquifer biofilms as
348 estimated by qPCR were positively correlated with DOC and BDOC (Spearman's rho factor > 0.6, p
349 < 0.01). A positive and significant correlation was also detected between OTU number and BDOC (r
350 = 0.79; p < 0.05) and between OTU number and DOC (r = 0.74, p < 0.05). When considering the
351 main bacterial phyla, a positive correlation was observed between the number of OTUs belonging to
352 *Bacteroidetes* and BDOC (r = 0.71; p < 0.01) and between *Bacteroidetes* and DOC (r = 0.67; p < 0.05)
353 (Fig. 5). This correlation held true using total *Bacteroides* qPCR counts (Spearman's rho factor > 0.7,
354 p < 0.01) (according to Table S2). Furthermore, the total *Bacteroides* qPCR counts were found
355 correlated with qPCR counts for the HF183 *Bacteroides* target which is specific for species associated

356 with the human intestinal tract (Table S2) (Spearman's rho factor > 0.7, $p < 0.01$). HF183 *Bacteroides*
357 qPCR counts were also correlated with the DOC and BDOC values (Spearman's rho factor > 0.56, p
2 < 0.01). HF183 were only detected in biofilms from AR sites with a thin vadose zone (all biofilm
358 4 samples from recharged groundwater of GB site and one biofilm sample from recharged groundwater
5 of IUT site, Table S2). The number of OTUs belonging to *Alpha-proteobacteria* and *Beta-*
360 9 *proteobacteria* also significantly increased with DOC ($r = 0.81$; $p < 0.01$ for *Alpha-proteobacteria*
10 and $r = 0.71$; $p < 0.05$ for *Beta-proteobacteria*) and BDOC concentrations ($r = 0.79$; $p < 0.01$ for
1362 14 *Alpha-proteobacteria* and $r = 0.65$; $p < 0.05$ for *Beta-proteobacteria*) (Fig. 5). In the dataset, one
15 other phylum, *Chlorobi*, showed a significant and positive correlation between OTU numbers and
17 BDOC concentrations ($r = 0.66$ and $p < 0.05$) (Fig. 5).
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367 Discussion

368 The influence of AR practices was very well illustrated by the DOC contents of groundwaters. The
29 relationships between DOC and vadose zone thicknesses confirmed the hypothesis that DOM enrich-
369 31 ment of groundwaters decreases with VZT (Pabich et al. 2001; Datry et al. 2006). Concentrations of
32 total dissolved organic carbon (DOC) and biodegradable dissolved organic carbon (BDOC) were sig-
371 36 nificantly higher in recharged than non-recharged groundwaters but mainly for AR sites with thin
37 vadose zones (< 3 m). This effect was not significant for AR sites with a thick vadose zone (> 10 m).
372 39 The influence of VZT on DOM reaching groundwater was thus linked to the DOM soil retention
40 capacity. Biotic (microbial consumption of BDOC, Marschner and Kalbitz 2003; Li et al. 2013; Shen
41 et al. 2015) and abiotic (sorption of DOM on clay particles, soil organic matter, or/and Fe and Al
42 43 oxides/hydroxides, McKnight et al. 1992; Kalbitz et al. 2000; Saidy et al. 2013) soil components are
44 likely involved in this VZT-related effect. Consequently, and according to several studies (e.g.,
45 46 Pabich et al. 2001; Shen et al. 2015), the thicker the VZ, the more efficient is its ability to limit
47 48 groundwater contamination with DOM.
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381 Based on groundwater samples from Cape Cod (USA), Pabich et al. (2001) determined a statistical
382 relationship between VZT (in m) and DOC concentration (in mg L⁻¹) in groundwater: DOC concen-
383 tration = DOC concentration in surface water * e^{-0.7*VZT}. By applying the same relationship using a
384 mean DOC concentration of 5 mg L⁻¹ in surface water (according to Voisin et al. 2018), we calculated
385 DOC concentrations ranging between 0.9 and 3.05 mg L⁻¹ for groundwaters influenced by recharge
386 in sites with vadose zones < 3 meters. Interestingly, the measured DOC concentrations at these sites
387 were in the same range (i.e., 1.6 – 2.3 mg L⁻¹). One would have expected that AR would have pro-
388 duced higher DOC concentrations than those expected from the model of Pabich et al. (2001) because
389 of the stronger hydrological connectivity between the surface and the aquifer in these AR sites (Foul-
390 quier et al. 2011). Two mechanisms could explain the concordance between our measurements and
391 simulations by the model of Pabich and colleagues: (1) soil and VZ of AR systems were very efficient
392 in DOC retention (Zhang et al. 2012; Mermillod-Blondin et al. 2015) and (2) DOC from infiltrating
393 waters was significantly diluted by groundwater (poor in DOC) in recharge zones of the aquifer (Foul-
394 quier et al. 2010). In some of our AR sites, two studies (Mermillod-Blondin et al. 2015; Voisin et al.
395 2018) tried to determine the respective influences of dilution and retention in organic matter dynamics
396 by using chloride as conservative tracer to evaluate the role of dilution. They found that the dilution
397 of surface water with groundwater could not explain the decrease of DOC concentrations from surface
398 waters to the aquifer. Then, it is expected that abiotic and biotic retention of DOC during water infil-
399 tration through the soil and VZ was the main mechanism involved in DOC dynamics in AR sites.
400 Nevertheless, this retention process was not efficient enough to limit significant DOC and BDOC
401 enrichments of groundwater in AR sites with thin VZ. The concentration of organic carbon was thus
402 a good indicator of the connectivity between surface and groundwater ecosystems.

403 As most groundwater ecosystems experience strong carbon limitation that severely limit growth of
404 microbial communities (Bengtsson 1989; Kazumi and Capone 1994; Baker et al. 2000; Goldscheider
405 2006), the biomass (total proteins), 16S rRNA gene counts, and activities of biofilm that grew on

406 artificial substrates (clay beads) introduced in the aquifer were positively linked with DOC enrich-
407 ment. The vadose zone thickness was also significantly related to the observed changes in microbial
2 activities that were measured: artificial recharge significantly stimulated micro-organisms for AR
308 4 sites with a thin vadose zone whereas it was not the case in AR sites with a thick vadose. To our
5 409 knowledge, these results are the first to demonstrate that VZT influences microbial growth in ground-
7 waters through its control over DOM availability in AR systems. Indeed, Datry et al. (2005) demon-
8 410 strated the influence of VZT on DOC concentrations and groundwater invertebrates but they did not
10 411 evaluate the response of the micro-organisms which are known to mediate energy fluxes from DOM
11 412 towards invertebrates (Cooney and Simon 2009). Foulquier et al. (2011) estimated bacterial cell num-
12 413 bers (i.e., DAPI staining counts and Lowry's total proteins assays) and microbial activities (i.e., es-
13 414 terase and dehydrogenase activities) on sediments collected from three MAR systems with a thin VZ
14 415 but they did not consider systems with thick VZ.
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27 418 The scarcity of studies on microbial biofilms from deep alluvial aquifers (i.e., when the vadose zone
28 419 thickness exceeds 10 m) results from technical difficulties and costs associated with pumping or cor-
29 420 ing for sediment collection (Chapelle 2001). Thus, the use of artificial substrates (clay beads) ap-
30 421 peared an efficient strategy to 422 compare biofilm growth development and activities among sites of
31 423 contrasted VZT including deep aquifers. The positive and significant correlations obtained between
32 424 microbial datasets from biofilms developing on clay beads and DOC and/or BDOC concentrations
33 425 (Table 1) confirmed the efficacy of this approach for monitoring trophic conditions in groundwater
34 426 ecosystems (Williamson et al. 2012; Mermillod-Blondin et al. 2013; Voisin et al. 2016).

47 426 The influence of AR practices on the OTU richness of biofilms collected from artificial substrates
48 427 (clay beads) was also related to the VZT. Indeed, OTU number was significantly higher in recharged
49 428 groundwaters than in non-recharged ones for AR sites with thin VZ. In contrast, no significant effect
50 429 of recharge was measured on OTU numbers in AR sites with thick VZ. These increasing OTU
51 430 numbers were found to match the increase of 16S rRNA Bacterial qPCR counts observed in recharged
52 431 wells of AR sites with a thin vadose zone in comparison with recharged wells from AR sites with a
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432 thick vadose zone. In addition, Shannon diversity and Pielou's evenness indices indicated that the
433 higher OTU richness of biofilms from recharged wells in comparison with biofilms from non-
2 recharged wells was not due to the addition of relatively rare or relatively abundant taxa in the
434 community. It rather seemed to be associated with an increasing in numbers of less frequent OTU at
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435 the non-recharged sites, leading to a greater evenness in the datasets between the OTUs of the
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436 recharged sites. This confirms that bacterial community structures were significantly influenced by
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437 recharge (more OTUs and higher evenness in the bacterial community). NMDS ordination of OTUs
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438 and PERMANOVA test also highlighted that bacterial community structures of biofilms incubated in
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439 groundwaters were significantly different among the four tested conditions (2 VZT conditions * 2
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440 recharge conditions). According to the observations of Hug et al. (2015) on groundwater
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441 microorganisms, the spatial distribution of the six AR sites (Fig. 1) would have led to high differences
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442 in bacterial community structures among these sites, reducing the ability to detect a significant
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443 influence of VZT and recharge conditions on bacterial communities using AR sites as replicates. As
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444 it was not the case, VZT and recharge could be considered as main factors structuring the bacterial
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445 communities of groundwater biofilms in the present study.
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347 The V5-V6 16S rRNA gene-inferred richness indices were found coherent with changes in DOC
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448 availability in groundwaters associated with recharge, and positive significant relations were obtained
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449 between OTU numbers and BDOC concentrations. These trends were further confirmed by qPCR
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450 assays targeting *Bacteroides* species. Biofilms from shallow aquifers were found to have significantly
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451 higher total *Bacteroides* counts than those from aquifers with higher VZT. Furthermore, the
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452 application of a more specific PCR screening for *Bacteroides* species specialized for the human
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453 intestinal tract confirmed a strong relation between their counts in an aquifer biofilm and DOC/BDOC.
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458 those coming from human wastes.

459 We also showed that biomass (concentrations of total proteins), activities (fluorescein diacetate
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460 (FDA) hydrolysis, INT-formazan reduction) and 16S rRNA genetic diversity of biofilms growing on
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461 clay beads (in groundwaters) were all correlated positively with DOC and BDOC concentrations.
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462 These results are in line with the “more individuals” hypothesis (Srivastava and Lawton 1998) of the
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463 species–energy theory (Wright 1983; Cardinale et al. 2009) which indicates that resource availability
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464 positively influenced species richness by increasing population sizes and reducing the probability of
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465 extinction. This positive response of bacterial diversity with resource availability is more likely
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466 expected in ecosystems experiencing strong nutrient limitations (see Bienhold et al. 2012 for an
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467 example in Arctic deep-sea sediments). Given the oligotrophic conditions encountered in
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468 groundwater environments, it is thus probable that increased DOC and BDOC concentrations
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469 associated with AR practices would enable the maintenance of copiotrophic bacteria that were not
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470 adapted to live in the more oligotrophic areas of aquifers (e.g., Fierer et al. 2007; Lever et al. 2015).
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471 These diversity changes were related to changes in the proportions of bacterial phyla among the core
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472 communities. The relative proportion of *Bacteroidetes* increased in the recharged groundwaters as
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473 observed from the OTU profiling and qPCR datasets. These observations are consistent with an
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474 increase of copiotrophic bacteria with increasing energy availability because members of the phylum
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475 *Bacteroidetes* are often abundant in nutrient-rich aquatic environments (e.g., Gomez-Pereira et al.
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476 2010; Landa et al. 2013; Marti et al. 2017). Indeed, this phylum has been detected in most aquatic
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477 habitats (e.g., Alonso et al. 2007; Diez-vives et al. 2014; Schauer et al. 2010) but bacteria of this group
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478 which are known to degrade polymeric organic matter (Cottrell and Kirchman 2000) are often
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479 associated with blooms of phytoplankton (e.g., Pinhassi et al. 2004; Teeling et al. 2012; Tada et al.
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480 2011) and fecal contaminations (e. g. Marti et al. 2017; Bernardin-Souibgui et al. 2018). The latter
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481 origin was confirmed for some species of this phylum which are specific of the human intestinal tract
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482 (HF183 DNA target). The HF183 DNA target was found in the aquifer of AR sites with a shallow
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483 aquifer (but not for those with a thick vadose zone), suggesting a potential transfer from stormwater
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484 runoff to the aquifer. It is also to be noted that qPCR screenings for *Bacteroides* species appeared
485 good indicators of the ability of a vadose zone at preventing bacterial contamination of the aquifer
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486 with surface bacteria. The total *Bacteroides* PCR screening could only detect this bacterial group
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487 among biofilm samples recovered from recharged wells of AR sites. In addition to the effects
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488 observed on *Bacteroidetes*, the diversities of most phyla in terms of OTU numbers showed positive
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489 linear relations with BDOC concentrations. The fact that dominant bacterial groups increased in
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490 diversity with nutrient (BDOC) availability in groundwater environment might have resulted from
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491 the harsh trophic conditions encountered by micro-organisms in subterranean ecosystems (Chapelle
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492 and Lovley 1990). Indeed, we measured relatively low BDOC concentrations (below 0.9 mg L⁻¹) in
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493 groundwater samples. However, Li et al. (2013) showed that an increase in BDOC concentration from
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494 0.5 mg L⁻¹ to 1.1 mg L⁻¹ was high enough to reduce 16S rRNA gene bacterial diversity of biofilms
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495 developing in sediment columns (under laboratory conditions). We can thus not exclude that a
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496 reduction of the diversity of some bacterial groups could occur due to BDOC enrichments of
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497 groundwater associated with AR practices if these enrichments lead to BDOC concentrations
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498 exceeding 1.1 mg L⁻¹. Here, coupling measurements of DOC availability, biofilm variables, bacterial
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499 richness and proportion of particular taxa among bacterial communities (e. g. all *Bacteroides* or
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500 particular species) appeared sufficient to infer the relative incidence of AR practices on aquifers. This
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501 approach based on functional indicators appears promising for water manager to evaluate
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502 groundwater ecosystem health. Moreover, the results of the present study clearly showed that the
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503 implementation of AR sites in porous aquifers would be recommended in areas with thick VZ (> 10
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504 m) to prevent groundwater contamination.
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516
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703 Figure captions

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705 Figure 1: Selected aquifer recharge (AR) sites in the eastern aquifer of the Lyon metropolitan area,
706 France. (a) Location of AR sites and (b) their main characteristics.

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708 Figure 2: (a) Concentrations of dissolved organic carbon (DOC) and (b) biodegradable dissolved
709 organic carbon (BDOC) in non-recharged (NR, white) and recharged (R, gray) groundwater (GW).

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710 (c) Total proteins (i.e., proxy of biofilm biomass), (d) hydrolytic (FDA), (e) dehydrogenase (INT)
711 activities, (f) OTU numbers, (g) Shannon diversity index, and (h) Pielou's evenness index of biofilms

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712 developed on artificial substrates (clay beads) incubated in non-recharged (NR, white) and recharged
713 (R, gray) groundwater (GW). The AR sites are used as replicates (n=3) for the two conditions of

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714 vadose zone (VZ) thickness: thick VZ (> 10 meters) on the left of the panel and thin VZ (< 3 meters)
715 on the right of the panel.

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716 Figure 3: NMDS ordination of V5-V6 16S rRNA gene OTU profiles from biofilms incubated in two
717 zones (recharged (gray centroid) and non-recharged (white centroid) groundwaters –GW-) over three
718 AR sites with a thin vadose zone (VZ < 3 m) and three AR sites with a thick vadose zone (> 10 m).

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719 Stress test = 0.103

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720 Figure 4: Relative numbers of core phyla among clay bead biofilms which developed over a 10 day
721 period among non-recharged (a and c) and recharged (b and d) groundwater. Panels a and b indicate

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722 the relative numbers of OTU per phylum, and, c and d, the relative numbers of reads per phylum.

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723 Figure 5: Linear regression analyses of BDOC values against number of OTU among selected phyla.
724 (a) total number of bacterial OTUs (b) *Bacteroidetes*, (c) *Chlorobi*, (d) *Alpha-proteobacteria* and (e)

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725 *Beta-proteobacteria*. Pearson's r factors are indicated with their respective p-value. For *Bacteroidetes*

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729 (panel b), the relationship between richness and BDOC values remains significant ($r = 0.7$, $p < 0.05$)

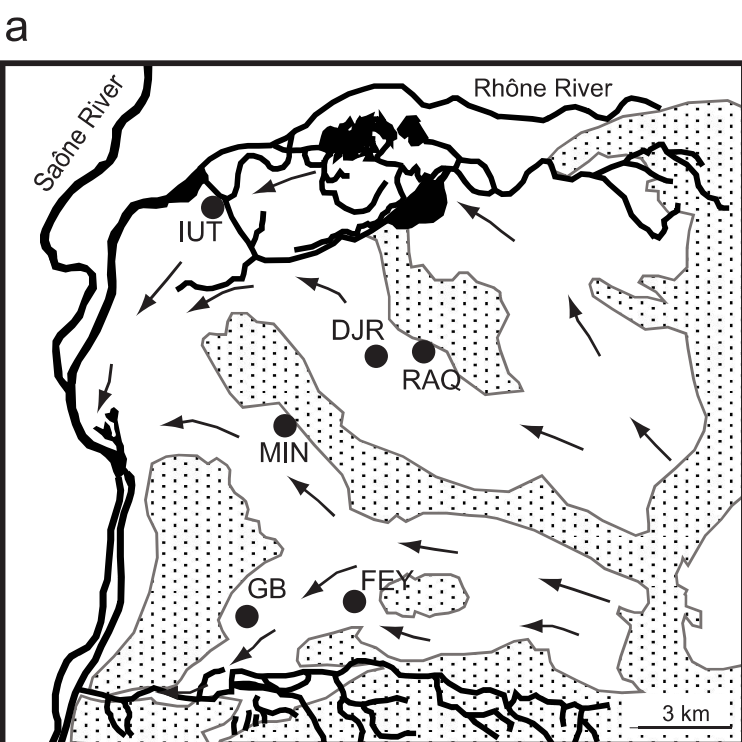
730 after suppression of the lowest and highest values of *Bacteroidetes* richness.




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Fig 1



-  Moraine hill (glacial till)
-  Groundwater flow direction
-  AR sites

b

Main characteristics of AR sites

AR sites	Catchment area (ha)	Infiltration basin surface (m ²)	Vadose zone thickness (m)
DJR	185	8750	11
FEY	315	2550	12
RAQ	26	285	17
MIN	165	4200	2
IUT	2.5	850	2.5
GB	300	3200	0.7

Fig 1

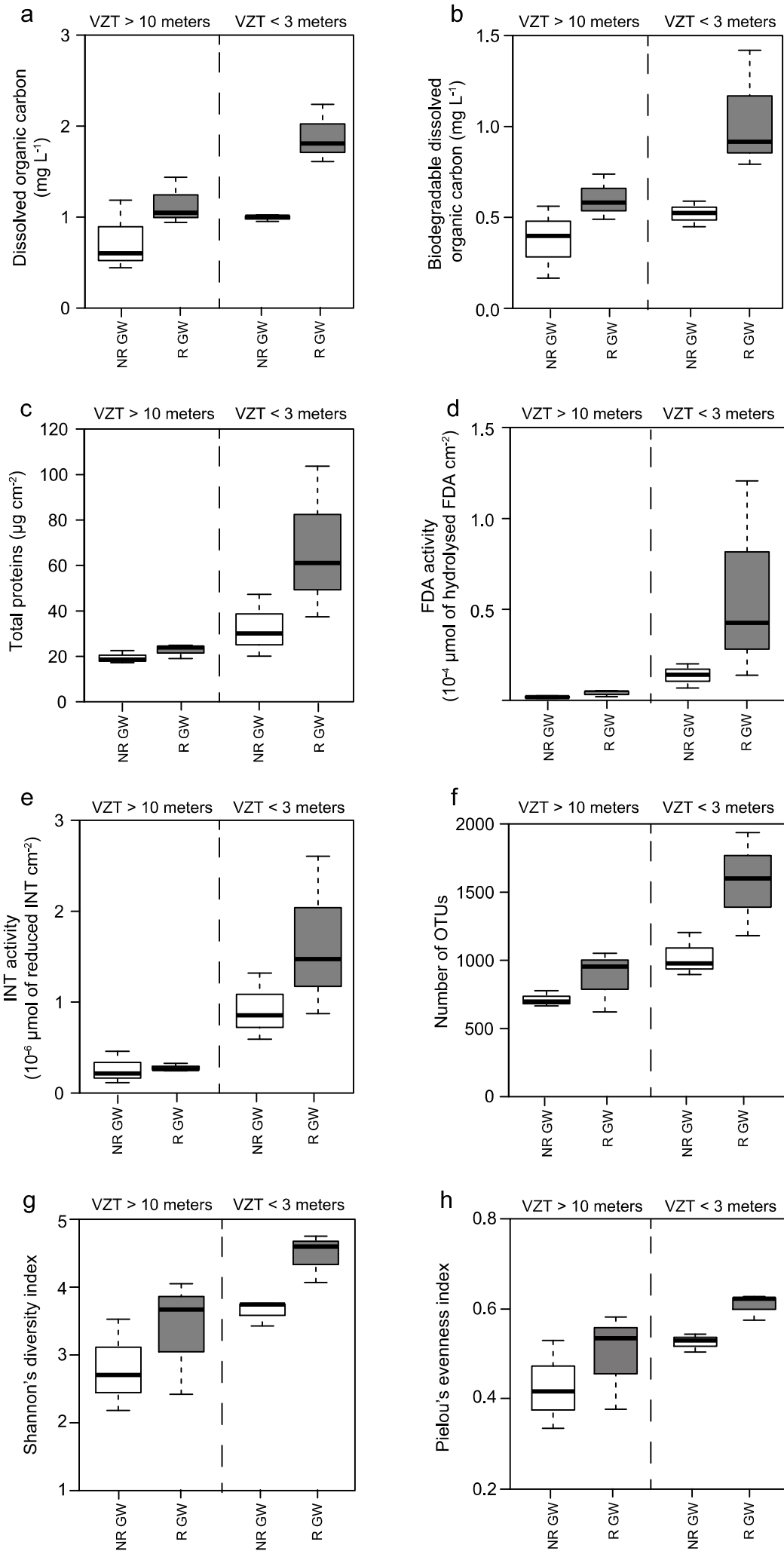


Fig 2

Fig 2

Fig 3

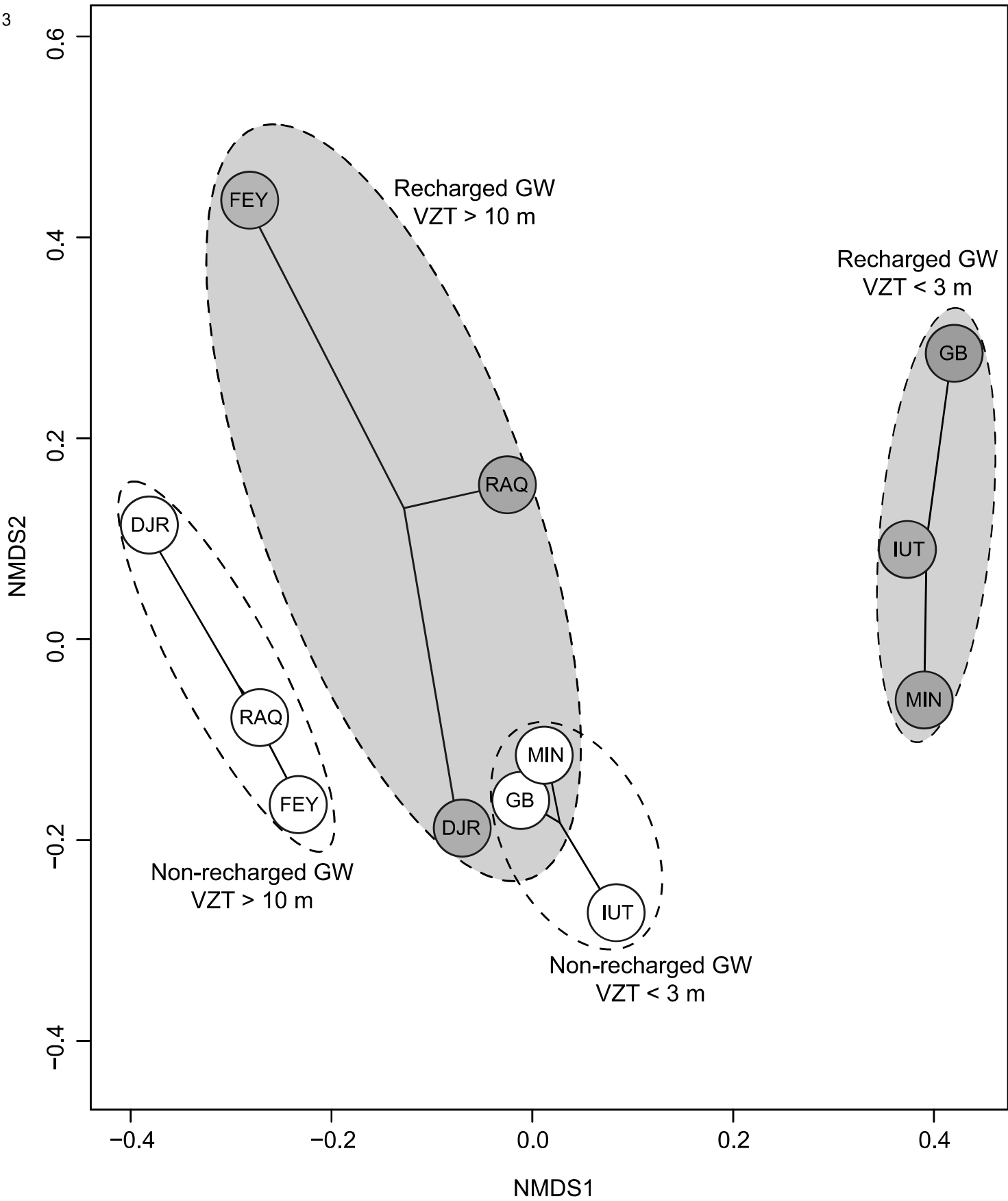


Fig 3

Fig 4

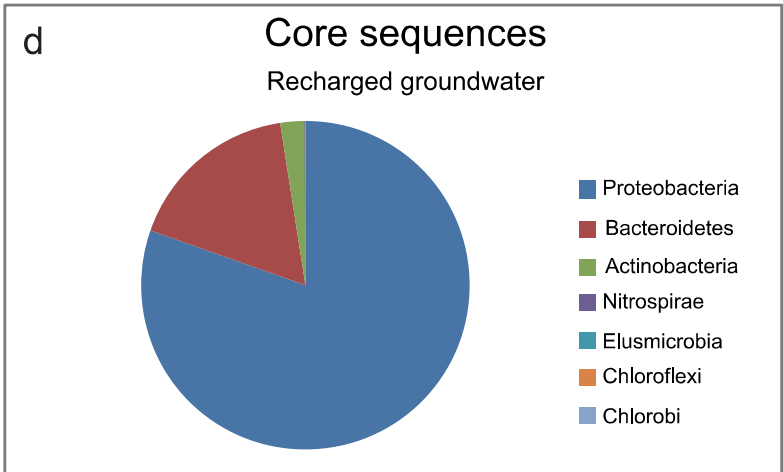
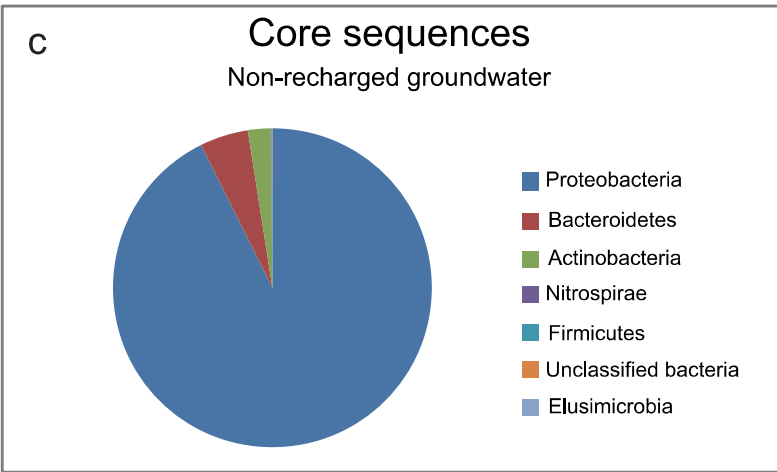
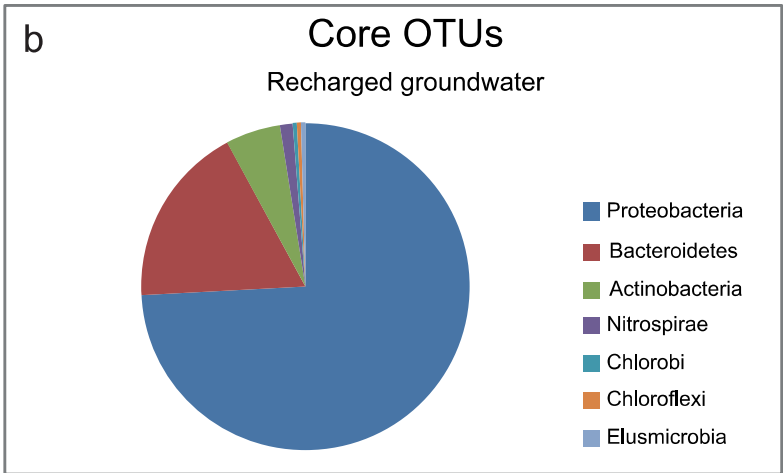
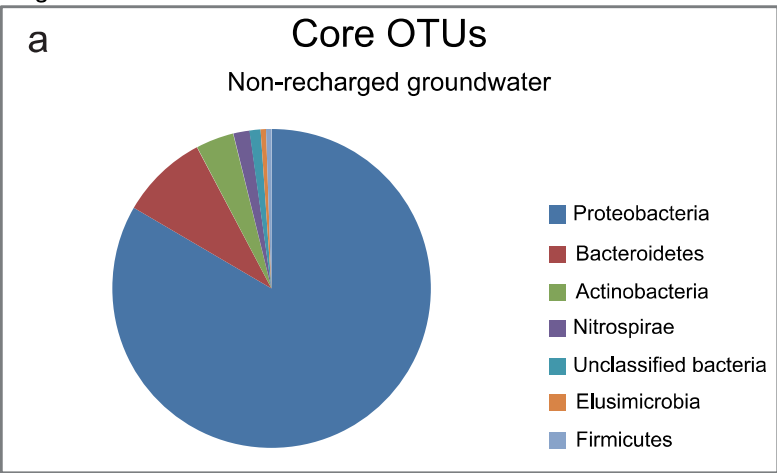


Fig 4

Fig 5

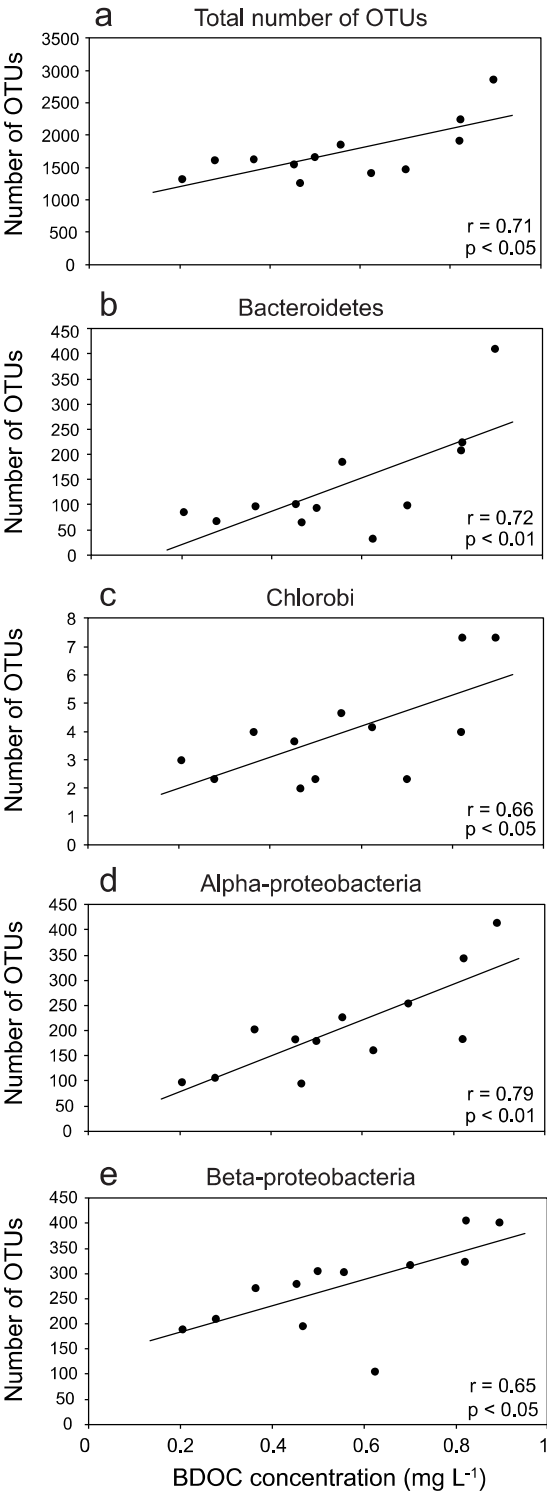


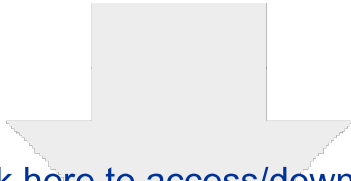
Fig 5

Table 1 Pearson's correlation coefficients between pairs of datasets produced in this study. DOC: dissolved organic carbon, BDOC: biodegradable organic carbon, FDA: Fluorescein diacetate (i.e., hydrolytic activity) and INT: 2-(p-iodophenyl)-3-(p-nitrophenyl)-5- phenyl tetrazolium chloride (i.e., dehydrogenase activity).

Variables	DOC	BDOC	Proteins	FDA	INT
BDOC	0.92***				
Proteins	0.65*	0.75**			
FDA	0.53	0.64*	0.97***		
INT	0.59*	0.70*	0.96***	0.95***	
OTU number	0.74**	0.79**	0.80**	0.78**	0.77**

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$





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Supplementary Material
Table S2 qPCR assays bc1.xlsx

