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1 Ultra-rare marine microbes contribute to key sulfur related ecosystem functions

2

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26 **Abstract**

27 The description of a rare biosphere within microbial communities has created great interest
28 because microbes play a fundamental role in the functioning of all ecosystems on earth.
29 Despite recent progress in understanding the ecology of the rare biosphere, the concept itself
30 is still discussed and fundamental questions remain. Here, we target the seed bank
31 compartment of the rare biosphere, assess the level of rarity at which microorganisms are still
32 able to colonize an ecosystem and investigate whether rare species are functionally redundant.
33 Using an original experimental design where wood in aquaria was inoculated with
34 increasingly diluted coastal seawater, we show that bacteria that represented as few as
35 0.00000002 % of the cells in the environment (or 1 cell in 10 L of seawater) were still able to
36 grow and play key roles within the ecosystem. Our experiment further showed that some
37 bacteria can be replaced by others that have the potential to fulfill the same metabolic tasks.
38 This finding suggests some functional redundancy within bacterial species. However, when
39 ultra-rare bacteria were progressively removed, productivity was reduced, and below a certain
40 threshold some processes were lost, and the function of the ecosystem was altered. Overall the
41 study shows that bacteria that are not detected by high-throughput sequencing approaches are
42 nevertheless viable and able to colonize new ecosystems, suggesting the need to consider
43 ultra-rare microbes in the marine environment.

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51 **Introduction**

52 Microbial communities play a fundamental role in the functioning of all ecosystems on earth
53 (Falkowski *et al.* 2008; McFall-Ngai *et al.* 2013). In the sea, they mediate key biogeochemical
54 cycles and are involved in nutrient cycling, primary production, organic matter mineralization
55 and ultimately climate regulation (Kirchman 2010). These processes are often conducted by a
56 few dominant and active species that are present in the environment at a given time (Pedrós-
57 Alió 2006). Such a feature is reflected by the representation of the distribution of
58 microorganisms along a skewed rank-abundance curve that shows the presence within a
59 community of a few dominant species and many less abundant organisms (Curtis *et al.* 2002;
60 Pedrós-Alió 2012). This long tail of rare microbes contains most of the tremendous diversity
61 of a microbial community (Crespo *et al.* 2016).

62 This large number of rare species was coined the “rare biosphere” following the first
63 application of high-throughput sequencing to describe marine microbial communities (Sogin
64 *et al.* 2006). The rare marine biosphere was first hypothesized to reflect the stochastic and
65 potentially unlimited dispersal capacity of microbes in the ocean. Traces of these dispersed
66 microorganisms would eventually be found everywhere. Furthermore, following Baas
67 Becking's statement that “everything is everywhere, but the environment selects”, these
68 microbes would represent a seed bank containing organisms that can grow when the
69 conditions become favorable (Jones & Lennon 2010; Lennon & Jones 2011; Pedrós-Alió
70 2006). However, it was shown that the rare biosphere represented a more complex picture. It
71 has patterns of biogeography that suggests the occurrence of selection processes (Galand *et al.*
72 2009). Furthermore, some rare microbes are active and at times are more active than the
73 abundant members of the community (Campbell *et al.* 2011; Jones & Lennon 2010), which
74 indicates that the rare biosphere is not only composed of a seed bank of dormant
75 microorganisms. It thus appears that there are large ecological differences between different

76 compartments of the rare biosphere (Alonso - Sáez *et al.* 2015; Hugoni *et al.* 2013; Pedrós-
77 Alió 2012). Despite recent progress in understanding the ecology of the rare biosphere, the
78 concept itself is still discussed, and fundamental questions remain. For instance, some data
79 suggest that some of the observed microbial diversity results from methodological artifacts
80 (Kunin *et al.* 2010; Lee *et al.* 2012; Patin *et al.* 2013) or that the observed diversity does not
81 represents viable microorganism but instead reflects free or dead DNA (Carini *et al.* 2016;
82 Lynch & Neufeld 2015). In addition, although sequencing now allows the in-depth probing of
83 the richness of the rare biosphere (Crespo *et al.* 2016), it was recently debated that there may
84 not be as many rare microbes as originally thought (Amann & Rossello-Mora 2016; Amann &
85 Rosselló-Móra 2016; Lennon & Locey 2016). However, there have been few if any
86 convincing experiments designed to explore the limits of rarity in natural samples.

87 The existence of the rare biosphere also begs the question of why there are so many
88 microbial species in the sea. High levels of diversity have been early explained by the
89 presence in the environment of different taxa that are able to conduct the same metabolic
90 processes. The paradigm of functional redundancy has long been assumed in microbial
91 ecology (Bell *et al.* 2005; Finlay *et al.* 1997), and the assumption is that a change in microbial
92 community composition will not have consequences for microbial-mediated processes
93 (Allison & Martiny 2008). The recent use of metagenomic profiles revealed high functional
94 redundancy for marine microbes at global scale (Sunagawa *et al.* 2015). Inversely, the fact
95 that some bacterial communities that are distinct in composition show different functions
96 (Leff *et al.* 2015) indicates that the paradigm of redundancy does not always hold in natural
97 microbial communities, as shown recently in soil ecosystems (Fierer *et al.* 2013). In aquatic
98 environments, recent findings showing intrapopulation complementary (Garcia *et al.* 2015)
99 support the theory of a high level of functional complementary in marine microorganisms

100 illustrated by reductive genomic evolution or genome streamlining (Giovannoni *et al.* 2014;
101 Morris *et al.* 2012).

102 Here, we focus on the seed bank compartment of the rare biosphere, which was
103 conceptualized by Pedrós-Alió (2006, 2012) as rare taxa that are not growing but that become
104 abundant when conditions become favorable, and investigate the extent of its diversity and
105 viability. More precisely, the goal of this study was to determine a threshold of abundance at
106 which rare organisms can still successfully colonize a complex substrate, to test whether the
107 rare species are functionally redundant and to verify whether they can be detected in the
108 environment. We earlier showed that wood incubated in seawater is a useful tool for testing
109 hypothesis about the functional ecology of communities (Kalenitchenko *et al.* 2016). Here,
110 wood incubated in aquaria were inoculated with different dilutions of coastal seawater, and
111 the growth of the bacterial communities was followed both within the wood and on the wood
112 surface, with a special focus on the bacteria involved in the sulfur cycle. These keystone
113 species are responsible for key ecosystem processes in the ocean. They are active during the
114 degradation of large organic falls on the sea floor (Bienhold *et al.* 2013; Kalenitchenko *et al.*
115 2015; Kalenitchenko *et al.* 2017), the mineralization of organic matter in marine sediments
116 (Jørgensen 1982) and biomass production at hydrothermal vents or cold seeps (Campbell *et al.*
117 2006; Martin *et al.* 2008). We chose to target these organisms because of their global
118 importance and because their activity can be conveniently monitored.

119

120 **Materials and methods**

121 **Experimental set up**

122 Wood logs originating from a same pine tree (*Pinus pinea*) in the Banyuls sur Mer
123 ‘ Biodiversarium’ botanical garden were cut the day before the experiment started. The
124 experimental design consisted of four separate 40-L aquaria that each contained four 15 cm in

125 diameter and 10 cm in length, fresh (non sterile), wood logs (Kalenitchenko *et al.* 2016). We
126 did not want to take the risk of changing the integrity of the chemical composition of the
127 wood by autoclave (possible impact on sugars). The aquaria were initially filled with
128 increasing dilutions of coastal seawater from the SOLA station in Banyuls Bay sampled at 3-
129 m depth on 27 January 2015. Seawater filtered through a UF100LL filtration module
130 (Polymem, Castanet-Tolosan, France) with a 0.01 μm pore size membrane was used for the
131 dilutions. The first aquaria was started with 100% (1/1 treatment) off coastal unfiltered
132 seawater, the second with 4 L of coastal water and 36 L of filtered water (1/10 treatment), the
133 third with 0.4 L of coastal water and 39.6 L of filtered water (1/100 treatment) and the last
134 with 100% filtered water (filtered treatment). Wood logs were immersed without water
135 renewal for the first 7 days of the experiment after which, the aquaria water was renewed with
136 piped in coastal seawater filtered through the 0.01 μm pore size filtration module (Polymem)
137 at a flow rate that allowed at least 2 renewals per day. The experiment lasted for 28 days.

138 One log was removed and sampled from each aquarium after 7 days (7d), 14 days
139 (14d), 21 days (21d) and 28 days (28d). At each sampling time, four replicate 1-cm long cores
140 were extracted from each log using a 4.35-mm-wide increment core borer. Mats growing on
141 the wood surface were sampled when present (21d and 28d) by scratching the entire wood
142 surface with a scalpel blade.

143

144 **DNA extraction and sequencing**

145 The microbial mat samples were lysed and wood core samples were powdered as described
146 earlier (Kalenitchenko *et al.* 2016; Kalenitchenko *et al.* 2015). The cell lysate was then
147 transferred to the Maxwell[®] 16 Blood DNA Purification kit cartridge and processed
148 automatically with the Maxwell[®] 16 automated extractor (Promega, Fitchburg, MA, USA).

149 DNA was also extracted from the original coastal seawater as described previously (Galand *et*
150 *al.* 2015). See the supplementary material and methods for a full description of the procedure.

151 A portion of the 16S rRNA gene was amplified using bacteria specific primers, 28F
152 (5'-TTTGATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3'), and then
153 sequenced by a commercial laboratory (Research and Testing Laboratory, Lubbock, TX) on
154 an Illumina Miseq sequencer to produce 2x300bp long, paired-end sequences. The raw
155 sequence data have been deposited in the NCBI Sequence Read Archive (accession no.
156 SRP099419 and BioProject accession no. PRJNA374511).

157

158 **Sequence analysis**

159 The 16S rRNA sequences were processed by following (Galand *et al.* 2015). In brief,
160 sequences were paired, producing ca. 500 bp fragments, quality trimmed, and chimeras were
161 removed. Sequences were grouped in operational taxonomic units (OTU) at 97% similarity
162 using the Uclust algorithm (Edgar 2010). The taxonomy of the most abundant sequence of
163 each OTU was assigned using the SILVA SSU 123 database (Pruesse *et al.* 2007). All
164 samples were resampled down to 5094 sequences per sample.

165 To identify the possible presence of rare species in seawater we used a dataset of sequences
166 from the Banyuls Bay microbial observatory time series. Samples have been gathered twice a
167 month from 2007 to 2014. DNA has been extracted and samples sequenced as described
168 earlier (Galand *et al.* 2015). A total of 7,418,629 sequences were used to search for rare
169 species in the time series. The occurrence of sequences that were not detected in the time
170 series was calculated to be $< 1 / 7,418,629$, which is < 0.00001 %.

171 Bacterial diversity was estimated by calculating the Shannon diversity index (H') and the
172 standardized effect size (SES) (Kembel *et al.* 2010), which is a standardized measure of
173 phylogenetic diversity. Phylogenetic diversity was computed using the Picante package
174 (Kembel *et al.* 2010) in R. To calculate SES, representative sequences from each OTU were

175 aligned using MUSCLE (Edgar 2004), and the alignment was then cleaned to remove non-
176 overlapping sequence regions. A phylogenetic tree was constructed using FASTTREE (Price
177 *et al.* 2010). The observed phylogenetic diversity was compared to the average phylogenetic
178 diversity in a randomly generated community (null model) and divided by the standard
179 deviation of the phylogenetic distances in the null model (Webb *et al.* 2008). The null model
180 randomizes community data matrixes with the independent swap algorithm to maintain the
181 species occurrence frequency and sample species richness (Kembel 2009). Positive SES
182 values indicate greater phylogenetic distance among co-occurring species than expected by
183 chance, whereas negative values indicate small phylogenetic distance.

184 The Bray Curtis similarity index was computed to compare the community composition
185 between samples with an MDS analysis. The effect of dilution and time factors on
186 composition differences was tested with PERMANOVA in the program PAST (Hammer *et al.*
187 2001). To identify OTUs or bacterial orders that had a significant difference in abundance
188 between groups, we used a nested ANOVA with the tools described in (McDonald 2014).
189 Differences in diversity (Shannon) were tested with ANOVA. Two outlier samples (1/1: 14d-
190 Replicate1, 1/10/ 21d-Replicate1) were removed from the analysis.

191

192 **Flow cytometry and most probable number**

193 Heterotrophic prokaryotic abundance in the aquaria water was measured by flow cytometry
194 (Supplementary methods). We used a method that is commonly used in microbiology, called
195 the most probable number method (MPN) (Oblinger & Koburger 1975), to estimate the
196 number of cells belonging to the OTUs that we monitored. This method relies on dilutions
197 and the observation of microbial growth to estimate the number of cells present in a starting
198 solution. To do so, we counted the number of replicate wood samples in which we detected
199 the OTUs of interest at the end of the experiment (28d). We then used an MPN table to

200 estimate the number of cells in the original seawater sampled from Banyuls Bay. Their
201 frequency was calculated by dividing the estimated number of cells by the total number of
202 cell counted by flow cytometry.

203

204 **Sulfide detection**

205 Continuous monitoring of sulfide was performed using autonomous potentiometric sensors
206 (NKE, Hennebont, France) equipped with lab made Ag/Ag₂S electrodes (Le Bris *et al.* 2012)
207 to detect when sulfide started to diffuse at the surface of the wood logs. To measure the
208 sulfide through the microbial mats, microsensor profiling was performed using amperometric
209 microsensors (H2S100) and a picoammeter (PA2000) (UNISENSE, Aarhus, Denmark).

210 Sulfide concentrations were profiled vertically, perpendicular to the surface of the wood, with
211 a resolution of 250 μm at each sampling time using a micromanipulator. Calibrations were
212 performed by standard additions of Na₂S stock solution in seawater as described in Laurent *et*
213 *al.* (2009) (Laurent *et al.* 2009) for the potentiometric electrodes. The amperometric electrode
214 was calibrated at pH of 6, 6.5, 7 and 7.5 using a Titrand pH controller (Metrohm, Herisau,
215 Switzerland). The slope of the electrode response to H₂S was thus confirmed to be pH
216 independent and was calculated combining the whole calibration series. The total free sulfide
217 concentration (i.e. both H₂S and HS) is determined from the pH variability at the wood-water
218 interface. To calculate the HS⁻ fraction, we used a pK_{a1}* of 6.7 at 13°C and salinity 37
219 (Rickard & Luther 2007).

220 The pH at the wood surface and wood water interface was measured with 1 mm
221 resolution with a mini electrode (1.5 mm diameter) (Diamond General Co., Ann Arbor, USA)
222 coupled with the sulfide microsensor. To prevent sulfide profiles from being disturbed by the
223 larger pH electrode, the tips of both electrodes were 1 cm apart. Triplicates of the combined

224 pH and H₂S profiles were acquired to determine mean concentration of total sulfide and the
225 corresponding standard deviation, which reflects the heterogeneity of the mat.

226

227 **Results**

228 **Sulfide concentrations**

229 We continuously measured sulfide concentrations inside the wood during the course of the
230 experiment. In the undiluted treatment, sulfide was first detected inside the wood after 13
231 days of incubation. In the 1/10 dilution, sulfide was detected after 21 days, and in the 1/100
232 dilution, the electrode potential only showed a small shift from baseline (< 7 mV), which
233 indicated that no significant amount of sulfide built up at the wood surface. In the aquaria
234 with filtered water only, the sulfide electrode potential in the wood did not change over the
235 experiment (Fig. 1a).

236 Surface mat sulfide profiles obtained with amperometric microsensors also reveal
237 marked differences between the treatments (Fig. 1b). The mats were first visible at 21d, and
238 the maximum sulfide concentration was higher in the undiluted experimental mats than in the
239 1/10 dilution mats. No mats were visible at the other dilutions. At 28d, the undiluted mat had
240 thicker sulfide layer and higher maximum sulfide concentrations at the wood surface
241 compared to those in the 1/10 dilution treatment (Fig. 1b). No sulfide was detected on the
242 1/100 and filtered treatments at 28d.

243

244 **Community diversity**

245 Community diversity measured as the Shannon index showed that at 7d, the communities
246 from the undiluted treatment had significantly higher diversity (ANOVA, $p < 0.05$), followed
247 by the 1/10 dilution, the 1/100 and the filtered treatments (Fig. S1). At 14d, the undiluted,
248 1/10 and 1/100 samples had similar diversity, whereas the diversity of the filtered treatment

249 community remained low. At 21d, the pattern of diversity did not change markedly, and at
250 28d, the diversity in the undiluted treatment decreased significantly.

251 For the phylogenetic diversity, the largest difference between samples was observed at
252 7d among diluted treatments. The 1/10 communities were the more phylogenetically clustered
253 (lowest SES values), followed by the 1/100 and filtered communities (ANOVA, $p < 0.05$). As
254 time passed, communities became less phylogenetically clustered (increasing SES values),
255 and there were no significant differences between treatments (Fig. S2).

256

257 **Bacterial community composition inside the wood**

258 Community composition between samples was compared by computing the Bray Curtis
259 dissimilarity index. For undiluted treatment (1/1) at 7d, the bacterial communities were very
260 different from those of the 7d communities in the diluted treatments. Dilution had a stronger
261 effect on community composition than incubation time had (Table S1 and S2). The
262 community composition changed among 7d, 14d and 21d. However, the undiluted 21d and
263 28d communities were very similar to each other (Fig. 2).

264 For the 1/10, 1/100 and filtered treatments, the 7d communities were more similar to
265 each other than to the communities from the undiluted treatment, as shown by their close
266 proximity on the MDS plot (Fig. 2). After 7d, the 1/10 communities became more similar to
267 the 1/1 communities, whereas the 1/100 and filtered communities became more similar to
268 each other with time (Fig. 2). In all dilution treatments, the communities changed with time
269 and continued changing after 21d. The diluted communities changed more than the undiluted
270 ones did, as shown by larger distances observed between samples on the plot.

271

272 **Composition at order level**

273 At the order level, in the diluted treatments, after 7 days (7d), there were significantly fewer
274 *Campylobacterales* and *Flavobacteriales* compared to the undiluted bacterial communities
275 (1/1). In the 1/10 dilution, the proportion of *Oceanospirillales* and *Alteromonadales* was
276 higher than that in the undiluted treatment. In the 1/100 and filtered treatments, the proportion
277 of *Vibrionales* was higher (Fig. 3).

278 After 14 days (14d), the samples from the undiluted treatment (1/1) maintained the
279 same groups as those present at 7 days but the proportion of *Aeromonadales* and
280 *Alteromonadales* decreased. The proportion of *Campylobacterales* increased significantly in
281 the 1/10 dilution. *Rhodospirillales* and *Flavobacteriales* sequences strongly increased in the
282 1/100 and filtered treatments (Fig. 3).

283 After 21 days (21d), the proportion of *Aeromonadales* and *Alteromonadales* continued
284 to decrease in the undiluted treatment (1/1), and 2 new groups increased (*Desulfovibrionales*
285 and *Bacteroidales*). *Desulfovibrionales* and *Bacteroidales* were also detected in the 1/10
286 dilution but at lower abundance. There were no significant changes otherwise for the 1/100
287 and filtered treatments (Fig. 3).

288 After 28 days (28d), the proportion of *Aeromonadales* and *Alteromonadales* kept
289 decreasing in the undiluted treatment. In the 1/10 dilution, *Desulfovibrionales* and
290 *Bacteroidales* sequences increased. In the 1/100 dilution, *Bacteroidales* became more
291 abundant (Fig. 3) but more variable between replicates in the samples from the filtered
292 treatment (Fig. S3).

293

294 **Dynamics at the OTU level**

295 We identified OTUs representing organisms known to be involved in sulfur cycling in
296 chemosynthetic ecosystems. For sulfate reducers, we identified two OTUs that grew well in
297 the undiluted treatment but not, or poorly, in the diluted treatments (Fig. 4). These two OTUs

298 were affiliated with *Desulfovibrio piezophilus* (OTU10 and OTU58, with 100% and 96 %
299 identity, respectively, over 442 bases). OTU10 was first detected at 21d in the undiluted
300 treatment and at 28d in the 1/10 dilution. It was only present at trace amounts in the 1/100 and
301 filtered water treatments (Fig. 4a). OTU58 was also detected in the samples at 21d in the
302 undiluted treatment but was not present in the 1/10 diluted treatment nor in the 1/100 or
303 filtered treatments (Fig. 4b).

304 We also identified potential sulfide oxidizers OTUs that grew better in the undiluted
305 treatment than in the diluted waters (Fig. S4). These OTUs were similar to *Arcobacter*
306 *bivalviorum* (OTU6, 100% identity) and *Arcobacter* sp. (OTU15, 94% identity). OTU6
307 sequences were detected at 7d in the undiluted treatment. In the 1/10 dilution, sequences
308 appeared mostly at 28d and were less abundant (Fig. S4a). OTU15 was only present in the
309 undiluted treatment and was detected at 21d (Fig. S4b).

310 Some of the OTUs were more abundant in samples from the diluted compared to the
311 undiluted treatment. OTU43 (100% identity over 452 bases to *Marinomonas foliarum*) was
312 more abundant at 7d in the undiluted and 1/10 dilution treatments but was overall more
313 abundant in the 1/100 dilution, especially from 14d (Fig. S5a). OTU19 (100% identity over
314 452 bases to *Vibrio oceanisediminis*) was rare in the undiluted treatment, more abundant and
315 present at all times tested in the 1/10 dilution and most abundant in the 1/100 dilution,
316 especially at 7d (Fig. S5b).

317

318 **Proportion of sequences and cell counts**

319 OTU10, OTU58, OTU6, OTU15, OTU19 and OTU43 sequences were not detected in the
320 sequences obtained from the original coastal seawater sample from Banyuls Bay. Because we
321 analyzed 23,743 sequences of bacteria from this inoculum water, the frequency of these
322 OTUs represented <0.004 % of the sequences. We further verified whether the sequences

323 were present in a database of 7,418,629 sequences sampled at Banyuls Bay over a period of 9
324 years. OTU10, OTU58, OTU6, OTU15, OTU19 and OTU43 were never detected, and their
325 occurrence was thus calculated to be < 0.00001 %.

326 Our flow cytometry counts validated the serial dilution of the Banyuls Bay seawater
327 for the experiment (Fig. S6). We used the most probable number method (MPN) to estimate
328 the number of cells belonging to the OTUs that we monitored (Table S3). Our MPN estimates
329 showed that some OTUs had occurrences as low as 1 cell in 10 L of seawater (OTU15). That
330 small proportion represents 0.00000002 % of the cells present in the aquaria.

331

332 **OTUs in the microbial mats**

333 We identified OTUs affiliated with *Arcobacter* sp. in the mats growing on the wood logs (Fig.
334 S7). Mats were visible starting at 21d. In the undiluted treatment, among the sequences that
335 were identified as *Arcobacter* sp., the OTU Arcobacter1 dominated, followed by the OTU
336 Arcobacter6 and a few sequences belonging to the OTU Arcobacter3 (Fig. 5). In the 1/10
337 dilution, the *Arcobacter* sequences belonged mainly to Arcobacter3. In the 1/100 dilution,
338 very few *Arcobacter* sequences were present. They were represented by Arcobacter31 and
339 Arcobacter3. In the samples from the filtered treatment, there were also few *Arcobacter*
340 sequences at 21d and 28d; half of those identified belonged to OTU1, and half belonged to
341 OTU3. Arcobacter1 was similar to the OTU6 found inside the wood. The other 3 main
342 *Arcobacter* OTUs were not detected inside the wood (Fig. 5).

343

344 **Discussion**

345 Our experiment demonstrated that seawater contains bacteria that are extremely rare but still
346 have the potential to grow and multiply rapidly when they encounter a suitable niche. Our
347 work suggests the existence of ultra-rare bacteria, microbes that are never detected by high

348 throughput sequencing approaches but that are nevertheless viable and able to colonize new
349 ecosystems with the potential to fulfill an important function in the marine ecosystem.

350 The presence of these ultra-rare microbes can, to date, only be revealed through
351 culturing or enrichment approaches. The organisms that grew in our experimental wood were
352 not detected by sequencing the inoculum water originating from Banyuls Bay. Their
353 occurrence in the inoculum in terms of sequence abundance was thus below 0.004%. These
354 ultra-rare microbes were not found either among sequences from Banyuls Bay collected over
355 8 years. By taking into account the total number of sequences from this long time series, these
356 ultra-rare microbes would represent < 0.00001 % of the naturally occurring community.

357 In terms of cell numbers, the flow cytometry counts and the most probable number
358 approach showed that, theoretically, the frequency of ultra-rare microbes was as low as 1 cell
359 in 10 L of seawater or as little as 0.00000002 % of the cells in the aquaria. They were not able
360 to grow below that threshold. Together, our count and sequencing data show a level of rarity
361 that is several orders of magnitude lower than thresholds used earlier to define the rare
362 biosphere in sequencing data: 0.1% (Pedrós-Alió 2012) or 0.01% (Galand *et al.* 2009). For the
363 first time, we also provide an estimation of rarity in terms of cell numbers. Theoretically, if
364 PCR or sequencing bias were not considered, one would thus need at least 8,038,282,890
365 sequences (the number of bacteria present in average in 10 L of Banyuls Bay seawater) to be
366 able to detect the ultra-rare *Arcobacter* cell (Fig. 6). Thus, current sequencing techniques may
367 not provide exhaustive maps of the microbial diversity even with one million final reads, a
368 number suggested recently to be required to yield quasi-exhaustive mapping of marine
369 bacterial diversity (Crespo *et al.* 2016).

370 These ultra-rare microbes could have been missed by our sequencing approach if they
371 had been present in the water as resting spores. Spore forming microbes are common (Lennon
372 & Jones 2011) and their DNA sometimes difficult to extract. Among the sulfate-reducing

373 bacteria, the *Firmicutes* form endospores that can be dispersed through the global ocean
374 (Hubert *et al.* 2009). However, among the bacteria that we identified, *Arcobacter* and
375 *Desulfovibrio* are considered non-spore forming bacteria (Vandamme *et al.* 2015) and should
376 be detected by PCR based diversity surveys. The growth of these organisms in our
377 experiments suggests that the ultra-rare biosphere contains living non-dormant bacteria, and
378 such viability shows that they do not represent taphonomic DNA originating from decaying
379 organisms, or extracellular DNA (Carini *et al.* 2016; Lynch & Neufeld 2015). In the context
380 of the ongoing debate around the presence of a “inflated biosphere” originating from PCR or
381 sequencing artifacts (Kunin *et al.* 2010; Lee *et al.* 2012; Patin *et al.* 2013), our findings argue
382 for the true existence of the rare biosphere.

383 In our study, we focused on microorganisms involved in the sulfur cycle. For instance,
384 sulfate reducing bacteria use the sulfate present in seawater as an electron acceptor to obtain
385 energy and release hydrogen sulfide. By measuring the concentration of sulfide, the
386 accumulation rates could be used to indirectly assess microbial activity and thus monitor this
387 key ecosystem process occurring in the wood and wood surface. Our data demonstrate that
388 after diluting the inoculum 10 times, the ecosystem processes were maintained. At a 100
389 times dilution, however, sulfide was no longer detected. Thus, when ultra-rare microbes were
390 progressively removed, ecosystem processes were maintained but only until a certain
391 threshold of rarity. Below that threshold some processes were lost and ecosystem functioning
392 was impaired. Interestingly, even though sulfide was still detected after the first dilution, it
393 appeared later and the concentration plateau was not reached, with a slower buildup of sulfide
394 at the interface. It might be that at 1/10 dilution, the activity started later because the
395 community had to restore a critical biomass before ecosystem processes could be detected.
396 Our data suggest, despite the fact that ecosystem functioning was maintained, that activity
397 was reduced when the original community was diluted.

398 Dilution had the effect of reducing the community diversity and the phylogenetic
399 diversity at the beginning of the experiment. Dilution certainly removed some rare species
400 and probably decreased the abundance of individuals within more abundant species. It
401 affected the composition of the community used as inoculum, which in turn affected the
402 composition of the communities colonizing the wood. As a result, the composition of the
403 wood communities in the diluted treatments differed from those observed in the wood from
404 the undiluted treatment. For instance, one sulfate reducing bacteria (*Desulfovibrio*, OTU 58)
405 and a potential sulfide oxidizer, *Arcobacter* (OTU 15), were never detected in the diluted
406 treatments. Interestingly, community diversity later increased with time and the functioning of
407 the community was maintained, as revealed by the production of sulfide. It clearly shows that
408 some microorganisms from the original community were replaced by others with the potential
409 to fulfill the same metabolic tasks. Functional redundancy can be difficult to demonstrate
410 because it requires knowledge about the microbial populations that perform a specific process
411 (Allison & Martiny 2008). Here, a change in microbial community composition did not have
412 any consequences for the microbial-mediated production of sulfide. It is an indication of the
413 functional redundancy within the bacterial species that are involved in the degradation of
414 large organic falls in the sea. Metagenomic data from planktonic marine bacteria recently
415 suggested high functional redundancy at global scale (Sunagawa *et al.* 2015). However, the
416 recent report that microorganisms in soils did not show a high degree of functional
417 redundancy (Fierer *et al.* 2013) could suggest that redundancy may vary depending on
418 ecosystems or, more likely, that the scale of the study is important. It has been emphasized
419 that redundancy has to be tested under common environmental conditions (Bradford & Fierer
420 2012).

421 In our study, a change in community composition, concomitant with the maintenance
422 of a function, was also clearly visible in the mats growing on the wood. Our results show that

423 at each level of dilution different *Arcobacter* bacterium were found in the mat. Again, it
424 points toward a certain level of functional redundancy within the genera. In addition, these
425 differences at the species level indicate that when some rare bacteria are lost or reduced in
426 abundance by dilution, other rare microbes can colonize the substrate and use the available
427 niche. Interestingly, some microorganisms that grew in the wood in the diluted treatments
428 were not detected in the woods from the undiluted treatment. For example, *Marinomonas*
429 (OTU43) and *Vibrio oceanisediminis* (OTU19) were less abundant or not detected in the
430 undiluted treatment. Dilution probably liberated a niche for these microorganisms that
431 otherwise would have been outcompeted. It should also be noted that a few *Arcobacter* were
432 present in the lowest dilutions after 21d, even though sulfide was not detected, which possibly
433 reflects the complete consumption of the sulfide produced and that wood could have produced
434 sulfide if the experiment had been longer.

435 In conclusion, we demonstrate the presence in the sea of very rare but viable bacteria
436 that are key to important ecosystem processes, such as organic matter degradation. These
437 functioning bacteria occurred at concentrations as low as 1 cell per 10 L of water and thus
438 would not be detected by current amplicon based environmental diversity surveys. In the
439 debate on the extent of microbial diversity, we found that the bacterial community richness
440 remains under estimated (Crespo *et al.* 2016; Locey & Lennon 2016). The rare biosphere
441 described to date only represents the emerged part of an iceberg floating in a sea of ultra-rare
442 microbes, which represent an underappreciated source of hidden diversity.

443

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449

450 **Author contributions.** P.E.G. and D.K. designed research, D.K, E.P., N.L.B. and P.E.G.
451 performed research, P.E.G contributed new reagents/analytic tools, D.K, N.L.B. and P.E.G.
452 analyzed data, D.K, E.P., N.L.B. and P.E.G. wrote the paper.

453

454 **Data Accessibility.** The raw sequence data have been deposited in the NCBI Sequence Read
455 Archive (accession no. SRP099419 and BioProject accession no. PRJNA374511).

456

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590

591 **Figure legends**

592 Figure 1. (a) Electrode potential as function of time indicating the presence of sulfide on the
593 surface of wood inoculated with different dilutions of seawater (1/1, 1/10, 1/100 and filtered).
594 A decrease in electrode potential indicates an accumulation of sulfide. 7d, 14d, 21d and 28d
595 indicate sampling times. (b) Microprofiles of sulfide concentration through the mats
596 colonizing the wood surface in the undiluted (1/1) (black lines) and 1/10 dilution experiments
597 (grey lines) measured after 21 days (dashed lines) and 28 days (full lines). Error bars
598 represent the standard deviation obtained from triplicate measurements.

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600 Figure 2. Trajectories of the bacterial communities. The similarity between bacterial
601 communities growing inside the wood is represented by an MDS ordination based on the
602 Bray-Curtis index. Colors group communities sampled from a same dilution treatment. The
603 size of the points is proportional to the time elapsed after the incubation started. The percent
604 value for each axis represents the proportion of total variation explained.

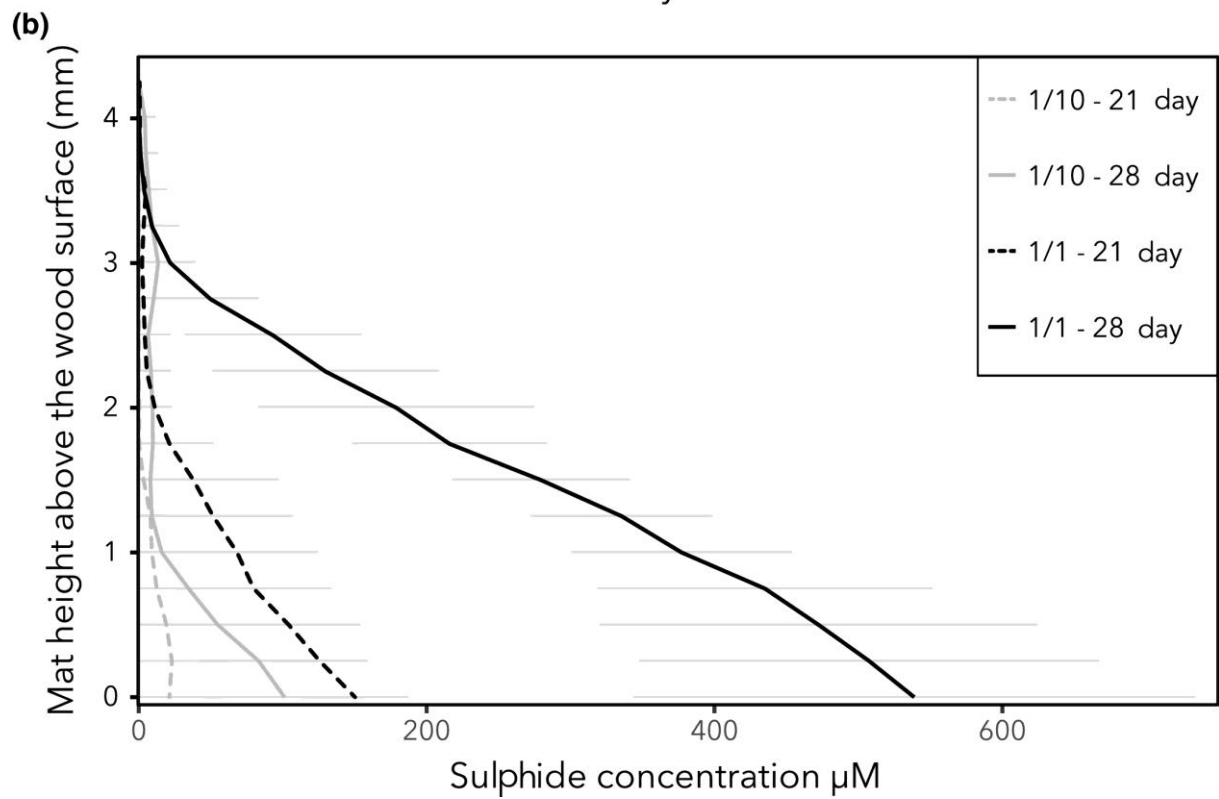
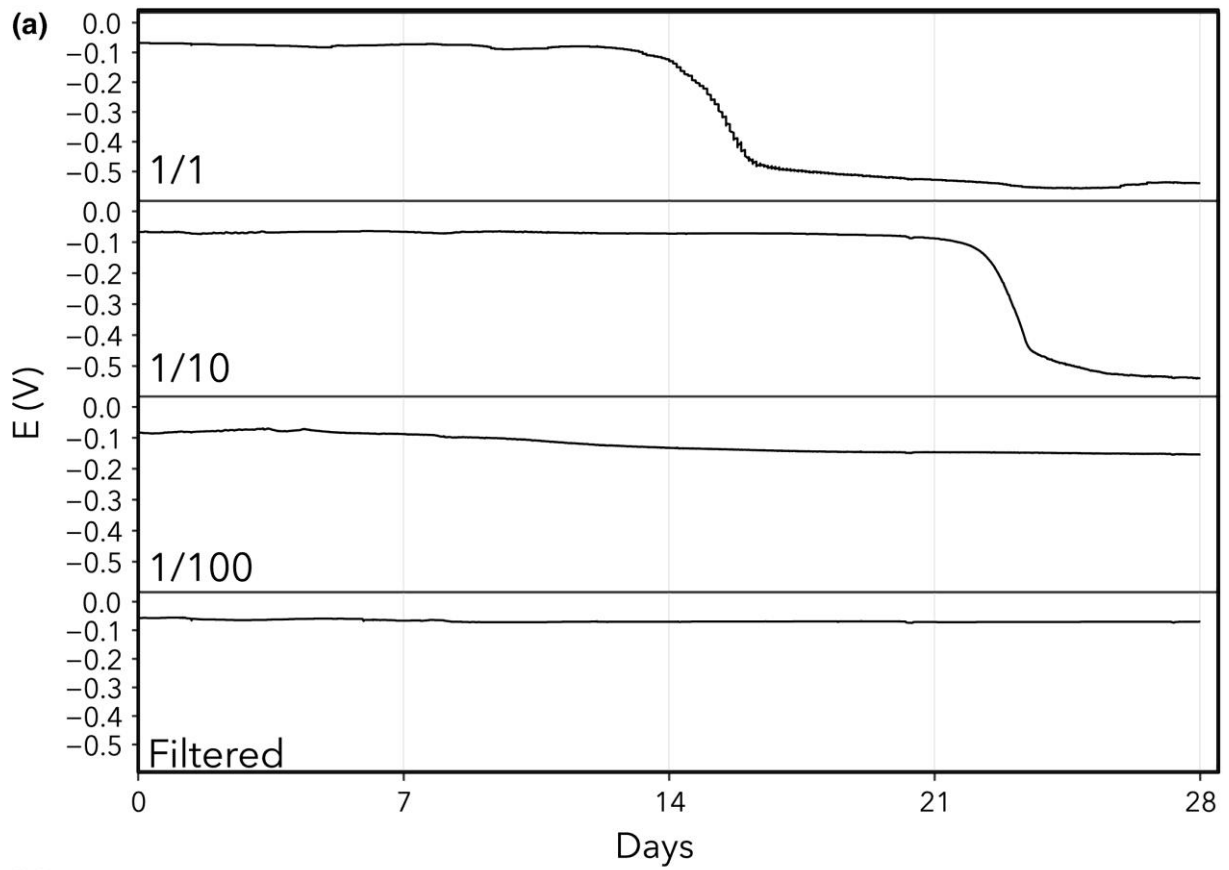
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606 Figure 3. Proportions of sequences at the order level detected inside woods incubated in
607 aquaria inoculated with seawater with a range of dilutions (1/1, 1/10, 1/100 and only filtered)
608 and sampled at four different time points (7 to 28 days). Each bar shows the average values of
609 4 replicate samples.

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Figure 4. Abundance of sequences belonging to two sulfate reducing bacteria identified as *Desulfovibrio piezophilus* OTU10 (a) and *Desulfovibrio* sp. OTU58 (b) during the experiment in the 4 different inoculation conditions. The number of sequences represents averages over four replicates.

Figure 5. Proportion of the 4 most abundant *Arcobacter* OTUs in the mats colonizing the wood surface in the 4 different treatment conditions (1/1, 1/10, 1/100 and filtered) at 21d and 28d. The proportion is relative to the total number of sequences found in each sample. The whole wood surface was sampled for sequencing.



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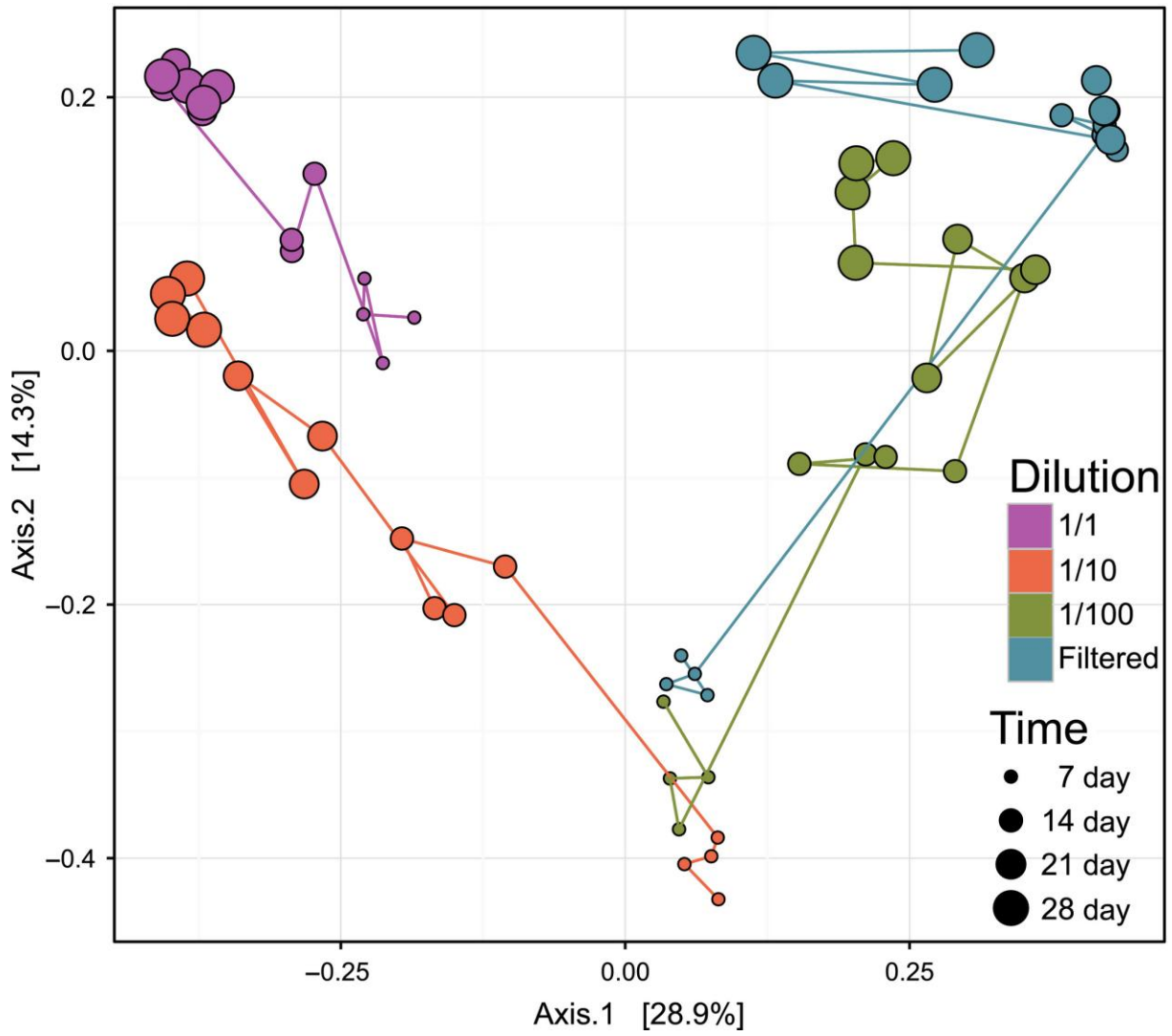
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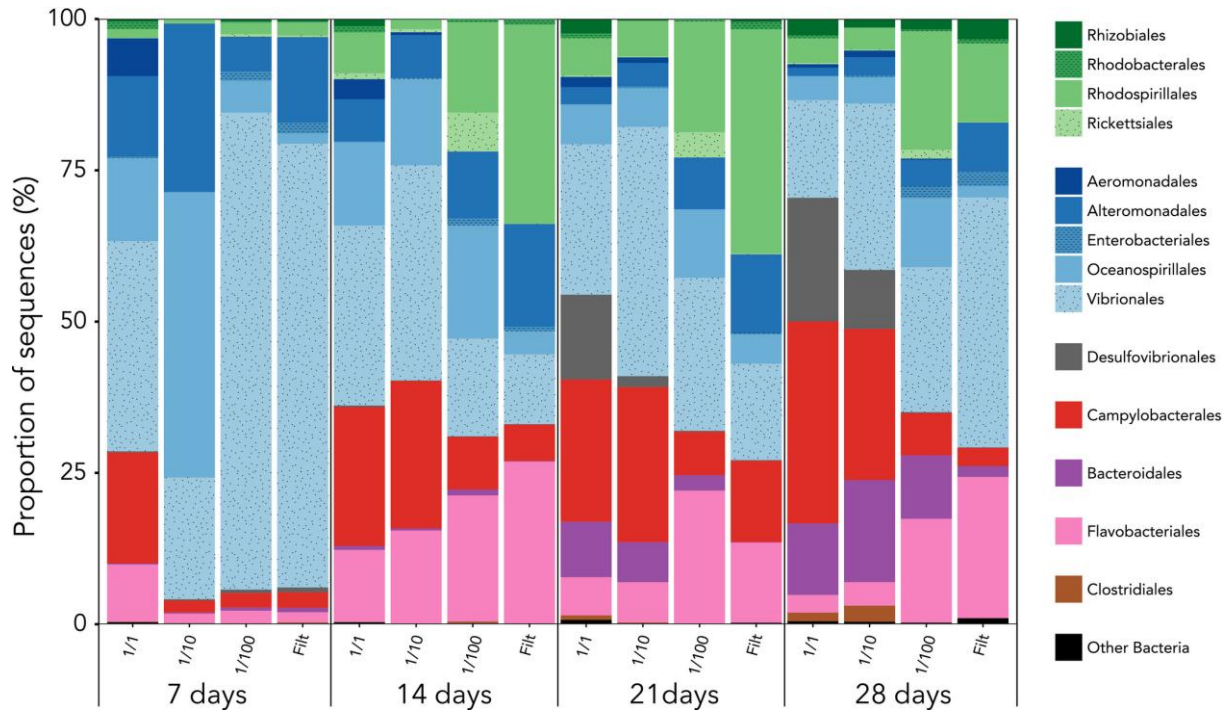
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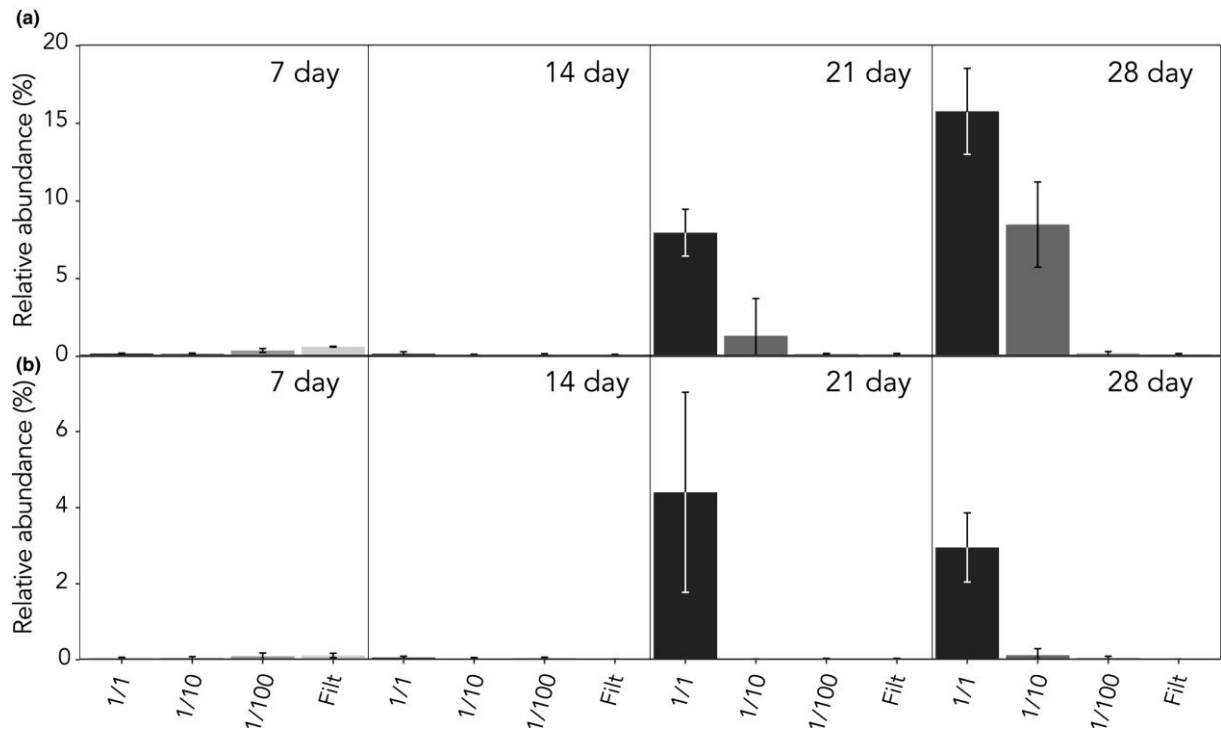
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645 Fig. 2



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647 Fig. 3

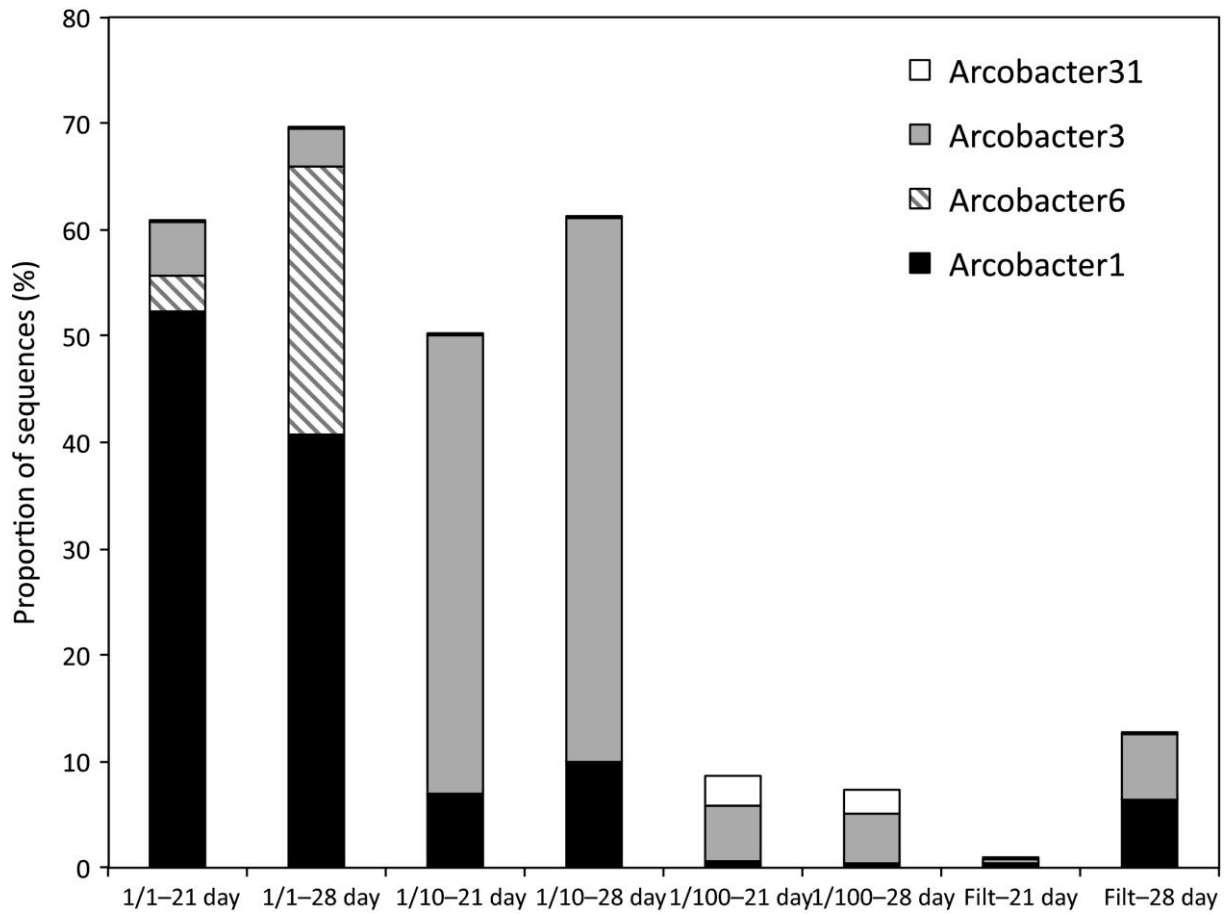
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651 Fig. 4

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653 Fig. 5
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