

# Ultrarare marine microbes contribute to key sulphur-related ecosystem functions

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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**

The microbial mat samples were lysed and wood core samples were powdered as described
earlier (Kalenitchenko *et al.* 2016; Kalenitchenko *et al.* 2015). The cell lysate was then
transferred to the Maxwell<sup>®</sup> 16 Blood DNA Purification kit cartridge and processed
automatically with the Maxwell<sup>®</sup> 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

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

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

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%.

Bacterial diversity was estimated by calculating the Shannon diversity index (H') and the standardized effect size (SES) (Kembel *et al.* 2010), which is a standardized measure of phylogenetic diversity. Phylogenetic diversity was computed using the Picante package (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.

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

191

#### 192 Flow cytometry and most probable number

Heterotrophic prokaryotic abundance in the aquaria water was measured by flow cytometry (Supplementary methods). We used a method that is commonly used in microbiology, called the most probable number method (MPN) (Oblinger & Koburger 1975), to estimate the number of cells belonging to the OTUs that we monitored. This method relies on dilutions and the observation of microbial growth to estimate the number of cells present in a starting solution. To do so, we counted the number of replicate wood samples in which we detected the OTUs of interest at the end of the experiment (28d). We then used an MPN table to estimate the number of cells in the original seawater sampled from Banyuls Bay. Their
frequency was calculated by dividing the estimated number of cells by the total number of
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/Ag<sub>2</sub>S 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 Na<sub>2</sub>S 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 Titrando pH controller (Metrohm, Herisau, 215 Switzerland). The slope of the electrode response to  $H_2S$  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 H<sub>2</sub>S and HS) is determined from the pH variability at the wood-water 218 interface. To calculate the HS<sup>-</sup> fraction, we used a pKa<sub>1</sub>\* of 6.7 at 13°C and salinity 37 219 (Rickard & Luther 2007).

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

pH and H<sub>2</sub>S profiles were acquired to determine mean concentration of total sulfide and the
 corresponding standard deviation, which reflects the heterogeneity of the mat.

226

#### 227 **Results**

#### 228 Sulfide concentrations

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

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

from the undiluted treatment had significantly higher diversity (ANOVA, p<0.05), followed

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** 

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

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

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

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

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

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

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

331

#### **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

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

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

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

These ultra-rare microbes could have been missed by our sequencing approach if they had been present in the water as resting spores. Spore forming microbes are common (Lennon & 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).

In our study, a change in community composition, concomitant with the maintenanceof 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 debate on the extent of microbial diversity, we found that the bacterial community richness 439 440 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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#### 591 Figure legends

- 592 Figure 1. (a) Electrode potential as function of time indicating the presence of sulfide on the
- surface of wood inoculated with different dilutions of seawater (1/1, 1/10, 1/100 and filtered).
- A decrease in electrode potential indicates an accumulation of sulfide. 7d, 14d, 21d and 28d
- indicate sampling times. (b) Microprofiles of sulfide concentration through the mats
- 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
- represent the standard deviation obtained from triplicate measurements.
- 599
- 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.

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

611	Figure 4. Abundance of sequences belonging to two sulfate reducing bacteria identified as
612	Desulfovibrio piezophilus OTU10 (a) and Desulfovibrio sp. OTU58 (b) during the experiment
613	in the 4 different inoculation conditions. The number of sequences represents averages over
614	four replicates.
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616	Figure 5. Proportion of the 4 most abundant Arcobacter OTUs in the mats colonizing the
617	wood surface in the 4 different treatment conditions (1/1, 1/10, 1/100 and filtered) at 21d and
618	28d. The proportion is relative to the total number of sequences found in each sample. The
619	whole wood surface was sampled for sequencing.
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651 Fig. 4

