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► To cite this version:

Maria-Cristina Ciobanu, Gaëtan Burgaud, Alexis Dufresne, Anja Breuker, Vanessa Rédou, et al.. Microorganisms persist at record depths in the subseafloor of the Canterbury Basin.. The International Society of Microbiological Ecology Journal, 2014, 8, pp.1370-80. 10.1038/ismej.2013.250 . hal-01056621

HAL Id: hal-01056621

<https://hal.univ-brest.fr/hal-01056621>

Submitted on 11 Mar 2016

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1 **Microorganisms persist at record depths in the seafloor of the**
2 **Canterbury Basin**

3

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18

19 Subject category: Microbial population and community ecology

20 Keywords: deep biosphere, subsurface life, eukaryote, record depth

21 Running title: Deep seafloor biosphere of the Canterbury basin

22

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27 The subsurface realm is colonized by microbial communities to depths of more
28 than 1000 meters below the seafloor (mbsf), but little is known about overall
29 diversity and microbial distribution patterns at the most profound depths. Here we
30 show that not only *Bacteria* and *Archaea*, but also *Eukarya* occur at record depths
31 in the subseafloor of the Canterbury Basin. Shifts in microbial community
32 composition along a core of nearly 2 km reflect vertical taxa zonation influenced
33 by sediment depth. Representatives of some microbial taxa were also cultivated
34 using methods mimicking *in situ* conditions. These results suggest that diverse
35 microorganisms persist down to 1922 mbsf in the seafloor of the Canterbury Basin
36 and extend the previously known depth limits of microbial evidence (i) from 159 to
37 1740 mbsf for *Eukarya* and (ii) from 518 to 1922 mbsf for *Bacteria*.

38

39 **Introduction**

40 In addition to terrestrial and marine near-surface habitats, the deep biosphere is
41 considered to be a third realm for microbial life. Subseafloor sediments provide a
42 habitat for large numbers of microbial cells, as revealed by cell counts (Parkes et al.,
43 2000) or CARD-FISH (Schippers et al., 2005). Although recent data have shown that
44 the global biomass in subseafloor sediments is smaller than given by earlier estimates,
45 the deep subseafloor biosphere still constitutes a large fraction (2.9×10^{29} cells) of
46 Earth's living biomass (Kallmeyer et al., 2012).

47 The subsurface microbiota is diverse and complex, hosting metabolically active
48 communities down to depths of more than one thousand meters below the seafloor
49 (mbsf), as revealed by molecular, metagenomic and metatranscriptomic studies (Lipp *et*
50 *al.*, 2008; Roussel *et al.*, 2008; Biddle *et al.*, 2011; Pawlowski *et al.*, 2011; Orsi *et al.*

51 2013a). It harbors representatives from the three domains of life, i.e., numerous endemic
52 and/or as yet uncultured *Archaea* and *Bacteria* (e.g., Orcutt *et al.*, 2011; Inagaki *et al.*
53 2006), in addition to bacterial endospores (Lomstein *et al.*, 2012), protists and fungi
54 belonging to *Eukarya* (Schippers *et al.*, 2006; Edgcomb *et al.*, 2011; Orsi *et al.*, 2013a,
55 2013b). Occurrence of capsid-encoding organisms has also been confirmed (Engelhardt
56 *et al.*, 2011). Although background molecular data on bacterial and archaeal lineages
57 inhabiting subsurface sediment above 1000 mbsf exists (e.g., Orcutt *et al.*, 2011;
58 Inagaki *et al.* 2006), most deep-subsurface microorganisms detected so far were
59 refractory to cultivation (Sass and Parkes, 2011). The diversity of deeply buried
60 microorganisms remains untapped, as subseafloor prokaryotic culturability in most
61 studies is less than 0.1% of all microscopically detected cells (D'Hondt *et al.*, 2004).
62 Remarkably, when wide enrichment collections targeting different physiological groups
63 such as fermenters, sulfate-reducers and methanogens were performed using different
64 subseafloor sediments, these often led to the isolation of the same few “generalist”
65 bacteria (e.g., Batzke *et al.*, 2007). In most cases, the retrieved bacterial genera were
66 adapted to a broader spectrum of environmental conditions (e.g., broad temperature
67 range for growth) compared to their surface counterparts (Sass and Parkes, 2011).
68 So far, within subseafloor sediments, active *Bacteria* have been identified down to 518
69 mbsf (Bale *et al.*, 1997), active *Archaea* down to 1626 mbsf (Roussel *et al.*, 2008), and
70 active microeukaryotes down to 159 mbsf (Orsi *et al.*, 2013a), but we are still eager to
71 know the depth limit of the deep subsurface biosphere. Limits to microbial habitability
72 in subseafloor sediments are set by a variety of physical and chemical parameters like
73 temperature, pH, pressure, salinity, porosity, availability of energy, nutrients and water,
74 and maybe also by age. The present study site is not characterized by particularly

75 extreme conditions but stands out from sites previously examined by its depth and low
76 porosity. The depth limit of the deep biosphere remains an important issue to place
77 bounds on the volume of the seafloor biosphere and to guide the search for deep life
78 capabilities/adaptation and the role of microorganisms in global nutrient cycles. We
79 hypothesized that life could exist in even deeper sediments if pore space was sufficient.
80 In this study, we investigated the subsurface microbial communities from a core of 1927
81 m length collected in the Canterbury Basin (344 m water depth), off the coast of New
82 Zealand at site U1352, which was drilled during the Integrated Ocean Drilling Program
83 (IODP) Expedition 317 with DS *Joides Resolution*. Our purpose was to investigate
84 vertical distribution of microbial communities, abundance and evenness of taxa above
85 and below 1000 mbsf depth. We developed a highly stringent massive parallel tagged-
86 amplicon sequencing of 16S-18S hypervariable regions of small-subunit (SSU) rRNA
87 gene (Fig. S1-S2, Tables S1-S3), coupled with cell counts, real-time PCR (phylogenetic
88 and functional genes) and cultivation approaches. This rigorous method was applied to
89 sediment/carbonate rocks spanning epochs from the Holocene to late Eocene.

90

91 **Materials and methods**

92 *Site description and sampling*

93 Three holes (A, B and C) were drilled at Site U1352 (44°56'26.62''S; 172°1'36.30''E),
94 reaching a total depth of 1927.5 m CSF-A, and thus spanning the Holocene to late Eocene
95 epochs. Fluorescent microspheres were used as tracers for contamination during drilling.
96 Sampling was processed under strict contamination controls onboard and offshore and only
97 samples with no detectable contamination were used for this study (Fulthorpe *et al.*, 2011).
98 Onboard, only the center parts of unconsolidated sediments and intact pieces of rocks that had

99 been exposed to UV radiation after washing were kept for microbiological analyses, as reported
100 elsewhere (Expedition 317 Scientists, 2011). Subsamples were immediately frozen at -80°C for
101 onshore molecular analyses, stored at 4°C under an anaerobic gas phase for later cultivation,
102 and stored at 4°C in a 3% NaCl/3% formalin solution for cell counting. Detailed information on
103 sampling/subsampling of sediment, contamination controls and on depth scale terminology are
104 provided in Supplementary Text.

105

106 *Lithological, physical and geochemical data*

107 Environmental data were acquired onboard during IODP Expedition 317, as reported elsewhere
108 (Fulthorpe *et al.*, 2011).

109

110 *DNA extraction, PCR amplification and contamination controls*

111 DNA extractions were made from 16 samples collected all along the core. In order to avoid
112 contamination, all handling was carried out in a PCR cabinet exclusively dedicated to low
113 biomass sediment samples (PCR cabinet; Captair[®]Bio, Erlab), using Biopur 1.5 mL Safe-Lock
114 micro test tubes (Eppendorf), ultrapure PCR water (Ozyme) and UV-treated (> 40 min)
115 plasticware and micropipettes. Negative controls (reaction mixture without DNA) were included
116 in each set of PCR reactions. In addition, a negative control (e.g., negative DNA extraction) was
117 prepared for each work stage, to ensure that no contamination with exogenous amplifiable DNA
118 occurred during the different stages of sample treatment. The FastDNA[™] Spin Kit for Soil
119 (#6560-200, MP Biomedicals[®]) was used to perform DNA extractions, with few modifications.
120 Detailed information on DNA extractions and PCR amplifications are provided in
121 Supplementary Information. Primer sequences used in this study are detailed in Table S2 and
122 primer sets for direct and nested PCR amplifications are detailed in Fig. S2.

123

124 *454-Pyrosequencing*

125 For each DNA extract, four independent 25 μ L PCR amplifications were run with fusion primer
126 pairs specific for *Bacteria*, *Archaea* and *Eukarya*, as detailed in Table S3. PCR products were
127 pooled two by two, so as to have two independent replicates for pyrosequencing. Potential
128 contaminants from lab reagents were excluded through the sequencing of negative-control
129 samples and the removal of OTUs containing sequences retrieved in negative controls. Detailed
130 information on 454-pyrosequencing, quality filtration, trimming, clustering and taxonomic
131 affiliation are provided in Supplementary Text.

132

133 *Cell counts*

134 Total prokaryotic cells were enumerated in triplicate from 13 uncontaminated sediment samples
135 collected all along the core, using the cell extraction protocol (protocol FCM-A) described by
136 Morono *et al.* (2011) until step 9. Then, all supernatants containing extracted cells were filtered
137 onto 0.2 μ m filters (Anodisc, Whatman) and stained with SYBR[®]Green I (Invitrogen), as
138 described elsewhere (Noble and Fuhrman, 1998). Filters were counted in epifluorescence mode,
139 with an Olympus BX60 microscope (objective 100 \times , pH3, WIB filter) (details in
140 Supplementary Text).

141

142 *Real-time PCR measurements*

143 Quantifications of different lineages and diverse functional genes were performed all along the
144 core by quantitative, real-time PCR (Q-PCR). Quantifications of *Bacteria*, *Archaea*, *Eukarya*,
145 JS1-*Chloroflexi*, and *Geobacteriaceae* were performed using previously described Q-PCR
146 assays based on the detection of 16S or 18S rRNA (Schippers *et al.*, 2012). These assays were
147 carried out using TaqMan[®] or SYBR[®]Green chemistries. DNA copy numbers were also
148 determined for the following functional genes: *mcrA* for alpha subunit of the methyl coenzyme
149 M reductase, *dsrA* for the alpha subunit of the sulfite (bi)reductase, *aprA* for the alpha subunit

150 of the adenosine-5'-phosphosulfate reductase and *cbbL* for the large subunit of the enzyme
151 ribulose-1.5-bisphosphate carboxylase/oxygenase (RubisCO, form I “red-like”), as described
152 elsewhere (Schippers *et al.*, 2012).

153

154 *Cultures and approaches used for their analysis*

155 A sediment slurry membrane system was used for cultivation (Ferrari *et al.*, 2008) (Fig. S8;
156 details in Supplementary Information). Different anaerobic metabolisms found in the subsurface
157 biosphere were targeted in culture: fermentation, sulfate-reduction and
158 methanogenesis/acetogenesis. Media, culture conditions, viability and identification procedures
159 of cells are described in Supplementary Text.

160

161 *Statistical analyses*

162 Principal Component Analysis (PCA) was used to help in visualization of high-dimensional
163 data. An order abundance matrix was combined with environmental parameters, using
164 XLSTAT, to assess relationships between microbial taxa and ecological variables (Addinsoft
165 USA, New York, USA). A second complementary approach was based on regularized canonical
166 correlation analyses (RCCA), which were performed to highlight correlations between the order
167 abundance matrices (X) and the environmental parameters (Y) using the R software CCA
168 package.

169

170 **Results and Discussion**

171 *Core description*

172 The core lithology was characterized by horizontal gradual layers, from unconsolidated
173 sediments (clay, marl) to carbonate rocks (Fig. 1). The core was composed of three
174 lithological units (UI, UII and UIII). Unit I (0–711 m CSF-A, meters of core depth
175 below seafloor computed by conventional method A, corresponding to mbsf; see “IODP
176 depth scale terminology” at www.iodp.org/program-policies/) was predominantly
177 characterized by a transition from mud-rich sediment to marl. Unit II (711–1853 m
178 CSF-A) consisted of hemipelagic/pelagic sediment from calcareous sandy mud to sandy
179 sandstone. Unit III (1853–1924 m CSF-A) was characterized by a sharp change
180 (Marshall unconformity: ~12 Ma are missing) that occurred at 1853 m CSF-A, and was
181 formed of hemipelagic to pelagic foraminifer-bearing nannofossil limestone of early
182 Oligocene to late Eocene age (Fig. 1). The temperature at the bottom of the hole was
183 estimated to be in the range 60°C–100°C on the basis of thermal conductivity
184 measurements and geochemical results (Fulthorpe *et al.*, 2011). Below 1000 m CSF-A,
185 sediments were replaced by consolidated sedimentary calcium carbonate rocks with
186 porous horizons of glauconite. Porosity decreased with depth and mean pore-size was
187 around 2-4 μm at the hole bottom. In carbonate rocks, numerous fractures and stylolites
188 were observed (Fig. S3). Organic carbon content was low (<0.6 wt %), with only a few
189 samples having >1 wt % TOC (Fig. 1). The organic matter quality changed from
190 relatively labile volatile material in the shallower sediments to more stable protokerogen
191 with increasing depth. Methane and ethane both occurred below 11.7 and 18.2 m CSF-
192 A and the relative ethane content increased with increasing burial depth and temperature
193 (Fig. 1). Low but increasing concentrations of C3-C5 and occasionally C6 alkanes were
194 also measured with depth (18). pH values were close to 7.5 and stable from the surface
195 to 1164 m CSF-A (18). Sulfate concentration decreased gradually in the first meters of

196 the core and reached the detection limit at ~16 m CSF-A (the SMTZ: sulfate-methane
197 transition zone, was placed between 15.2 and 16.6 m CSF-A), then it remained close to
198 the detection limit (~0.85 mM) down to 1433 m CSF-A (Fig. 1).

199

200 *Vertical distribution of cells*

201 We analyzed and compared cell abundances and cell concentrations reported for
202 different geographic sites using a standardized procedure based on cell extraction and
203 dissolution of silicates (Noble and Fuhrman, 1998; Kallmeyer *et al.*, 2008; Morono *et*
204 *al.*, 2011)(Fig. 2). Mean cell numbers decreased with depth from about $1.5 \times 10^6 \pm 4.7$
205 $\times 10^4$ cells·cm⁻³ (n = 8) at the surface (3.76 and 15.1 m CSF-A) to $2.5 \times 10^4 \pm 4.9 \times 10^3$
206 cells·cm⁻³ (n = 7) within the deepest samples (1911 and 1922 m CSF-A). The detection
207 limit, calculated in our conditions (Kallmeyer *et al.*, 2008), was 2.94×10^3 cells·cm⁻³.
208 The depth profile (down to 600 m CSF-A) was consistent with the general depth
209 distribution of prokaryotic cells from other subsurface sediments (Kallmeyer *et al.*,
210 2012).

211

212 *Vertical distribution of microbial taxa*

213 It is not clear what controls abundance of *Bacteria* and *Archaea* within deep marine
214 sediments (Schippers *et al.*, 2005; Lipp *et al.*, 2008; Schippers *et al.*, 2012). Here, a
215 real-time PCR approach was applied to quantify representatives of the three life
216 domains. Calculated detection limits for *Bacteria*, *Archaea* and *Eukarya* were
217 respectively 1.6×10^4 , 1.1×10^3 and 2.9×10^3 SSU rRNA gene copies per gram of

218 sediment (wet weight). *Archaea* were the most abundant within the first meters, while
219 *Bacteria* dominated the rest of the core (Fig. 2). Archaeal SSU rRNA gene copy
220 numbers strongly decreased with depth (from 1.8×10^6 to 1×10^3 gene copies·g⁻¹,
221 corresponding roughly to 1×10^6 to 6×10^2 cells·g⁻¹) and were no longer detectable
222 below 650 m CSF-A. A similar depth distribution was observed for eukaryotic SSU
223 rRNA gene copy numbers, but abundances were relatively constant with depth ($\sim 10^4$
224 copies·g⁻¹). Bacterial SSU rRNA gene copy numbers were low ($\sim 10^6$ copies·g⁻¹ $\approx 2.5 \times$
225 10^5 cells·g⁻¹) at the surface and decreased with depth up to 1600 m CSF-A (8×10^4
226 copies·g⁻¹ $\approx 2 \times 10^4$ cells·g⁻¹).

227 Along with these measures, deep sequencing allowed the detection limits to be lowered
228 and masked lineages to be revealed. We pyrosequenced bacterial (V4-V5), archaeal
229 (V1-V3) and eukaryotic (V1-V3) SSU rRNA gene amplicons from sixteen depth
230 horizons and one negative control, pooled together in one single dataset with two PCR-
231 replicates per sample to overcome PCR and sequencing errors (Fig. S1). Sequences
232 were grouped into OTUs (Operational Taxonomic Units) with a 97% identity threshold.
233 Sequence composition of the OTUs was then analyzed, and OTUs entirely composed of
234 sequences that had appeared in a single PCR only were excluded from the diversity
235 analyses. All the sequences kept appeared at least twice independently. Potential
236 contaminants from lab reagents were excluded through the sequencing of negative-
237 control samples and the removal of OTUs containing sequences retrieved in negative
238 controls. The remaining OTUs were used to calculate non-parametric diversity indices
239 (Fig. 3, Fig. S4-S6) and compared to the SILVA 111 database for taxonomic affiliation.
240 Pyrosequencing results were congruent with the data discussed above. Archaeal
241 sequences could not be amplified and sequenced for samples below 634 m CSF-A, as

242 observed with real-time PCR analyses. The non-detection of archaeal 16S rRNA genes
243 below 650 m CSF-A using two different amplification methods suggests that *Archaea*
244 are likely rare or absent at great depths in the Canterbury Basin. Eukaryotic sequences
245 were detected down to 1740 m CSF-A and bacterial sequences were found up to the
246 maximal depth of 1922 m CSF-A. The observed species richness (i.e., number of
247 OTUs) was extremely low in comparison with other microbial habitats investigated so
248 far, including extreme environments (e.g., Roalkvam *et al.*, 2012). Indeed, only 198, 16
249 and 40 unique bacterial, archaeal and eukaryotic OTUs, at 3% dissimilarity level, were
250 detected in the entire cored sequence (Fig. S4, Tables S4-S5). Chao1 estimator revealed
251 a vertical decrease in microbial richness with increasing depth (Fig. 3). Richness
252 estimates for *Archaea* and *Eukarya* dropped off gradually with depth and reached only 2
253 and 4 OTUs respectively at the deepest depth for which a PCR signal was obtained.
254 Beta diversity estimators (i.e., diversity among samples) revealed a strong
255 differentiation between communities with depth and a strong vertical structuration (Fig.
256 S5).

257 Archaeal diversity showed high abundances of MBG-B (Marine Benthic Group B) and
258 MCG (Miscellaneous Crenarchaeotal Group), two archaeal groups typically found in
259 subseafloor sediments (Lloyd *et al.*, 2013). Representatives of the as yet uncultured
260 lineages MBG-B, MBG-E (Marine Benthic Group E) and MCG were the predominating
261 taxa in surficial layers, while MCG was the most consistently detected archaeal lineage
262 down to 346 m CSF-A (Fig. 2). MBG-B and MCG members are heterotrophic *Archaea*
263 frequently found in surficial marine sediments (Biddle *et al.*, 2006; Lloyd *et al.*, 2013).
264 *Thermococcales* dominated archaeal diversity of the sediment horizon at 634 m CSF-A.
265 Methanogens and anaerobic methanotrophs (ANME) were not detected, in agreement

266 with the real-time PCR analysis for *mcrA*. Their absence from the dataset might be due
267 to the intervals sampled which do not correspond to the SMTZ.

268 In *Eukarya*, few protist OTUs (Stramenopiles and uncultured *Eukaryota*) were detected
269 down to 583 m CSF-A. Sequences affiliated with the bacterivorous protists *Bicosoecida*
270 were detected at 346 m CSF-A, raising the question of the existence of a subsurface
271 complex trophic web. In agreement with recently published papers (Edgcomb *et al.*,
272 2011; Orsi *et al.*, 2013a, 2013b), fungi appeared to be the most frequently detected
273 eukaryotes in the Canterbury Basin, with 56 to 100% of the SSU rRNA gene sequences.
274 Different shifts between *Ascomycota* and *Basidiomycota* were observed along the core
275 (Fig. 2). *Tremellomycetes* (order *Tremellales*), *Sordariomycetes* and *Eurotiomycetes*
276 dominated shallow depths while *Saccharomycetes* were detected at depths between 630
277 and 1365 m CSF-A. Deeper layers were dominated by *Wallemiomycetes*,
278 *Microbotryomycetes* and *Tremellomycetes* (order *Filobasidiales*, not found at shallow
279 depths). These heterotrophic fungi have been described in deep sediments of other
280 locations (e.g., Nagano and Nagahama, 2012; Richards *et al.*, 2012) and demonstrated
281 to be active members of microbial communities (Orsi *et al.*, 2013b). So, fungi represent
282 an important component of sediment ecosystems through their impact on nutrient
283 cycling and mineral weathering.

284 *Bacteria* were dominated by *Chloroflexi* and *Proteobacteria*, two heterotrophic
285 bacterial groups well-represented in subsurface sediments (Fig. 2). They comprised 67%
286 of the sequences and 69% of the OTUs in total. However, the abundances of the two
287 phyla were negatively correlated. *Chloroflexi* dominated microbial communities at
288 shallow depths (above 600 m CSF-A) and their abundances and richness decreased
289 rapidly. Reciprocally, *Proteobacteria* were found all along the core, but their relative

290 abundance showed a sharp increase below 343 m CSF-A. Among the other lineages
291 observed in this study, *Planctomycetes*, *Nitrospirae* and the candidate division OP9
292 were major contributors of the amplicon pool at shallow depths. Below 600 m CSF-A,
293 *Acidobacteria*, *Firmicutes* (a phylum containing spore-formers), and two loosely
294 defined groups of uncultured *Bacteria* (ML635J-21 and MLE1-12) were the most
295 consistently detected lineages. Real-time PCR quantification of the JS1-*Chloroflexi*
296 group confirmed these results as $\sim 10^3$ to 10^6 SSU rRNA gene copies·g⁻¹ were detected
297 between the sediment surface and 1532 m CSF-A. *Deltaproteobacteria* were detected
298 above the SMTZ and at great depths. Genes encoding a functional dissimilatory sulfite
299 (bi)reductase (*dsrA*), a key enzyme of dissimilatory sulfate reduction frequently
300 encountered among *Deltaproteobacteria*, was quantified above the SMTZ and in layers
301 up to 1000 m deep in the sediment. The gene became undetectable below this depth,
302 either because it may decrease below the detection limit or because the detected
303 *Deltaproteobacteria* cannot respire sulfate.

304

305 *Diversity and environmental factors*

306 Principal component analyses (PCA) coupled with regularized canonical correlation
307 analyses (RCCA) were performed to visualize relationships between environmental
308 factors and microbial taxa. We first evaluated the relationships between all
309 environmental parameters measured (i.e., depth, pH, salinity, porosity, alkalinity and
310 concentrations of calcium, calcium carbonate, ammonium, magnesium, sulfate,
311 inorganic carbon, organic carbon, methane and ethane) to design a network of
312 correlations. Only the six most explanatory variables were kept (Fig. S6). This

313 complementary analysis reinforced the conclusion about microbial distribution pattern
314 and vertical community composition, depth being defined as a main factor explaining
315 diversity changes (Fig. S7).

316

317

318 *Handling deeply buried microorganisms*

319 Cultivation approaches allowed prokaryotic and eukaryotic strains to be grown,
320 corresponding to a fraction of the microbial communities detected all along the core,
321 underlining that these microorganisms were viable. Fungal strains were obtained at 21
322 to 765 m CSF-A, using elevated hydrostatic pressure to mimic *in situ* conditions (Fig. 4
323 A-C, Table S6). Sequencing of the ITS1 rRNA regions allowed identification of a
324 *Cadophora* representative that had already been found in extreme environments, i.e.,
325 Antarctic environments (Tosi *et al.*, 2002) and deep-sea hydrothermal vents (Burgaud *et*
326 *al.*, 2009) (Table S6). Fifty-seven anaerobic fungi, currently under description, have
327 also been isolated from these sediments (Rédou and Burgaud, unpublished data). In
328 addition to the important finding that living fungi could be cultivated from the sediment
329 samples, microbial colonies were grown anaerobically at 60-70°C from calcareous
330 chalk/limestone samples collected at 1827 and 1922 m CSF-A (Fig. 4 D-E), using a
331 microcultivation method (Fig. S8). The microcolonies were successfully transferred to
332 liquid media and subcultured. From the different tests performed, it was impossible to
333 grow true methanogens and true sulfate-reducers. Only bacterial fermentative strains
334 degrading the organic compounds supplied (i.e., low quantity of yeast extract) have
335 grown. Within these subcultures, mean cell densities were low, around 4×10^5 cells·mL⁻¹
336 ¹ and growth rates were slow (in 2.5 years of culture, only 6 to 9 subcultures at 1/40 or

337 1/50 have been performed). Cells were able to grow at atmospheric pressure and at the
338 estimated *in situ* pressure (22 MPa). They were composed of viable very small rods,
339 coccobacilli and cocci of 300 to 800 nm in diameter, often forming aggregates (Fig. 4 F-
340 I). These small sizes and this cellular organization as consortia raises questions about
341 the living conditions of these cells and their (in)dependence with regard to other cells.
342 The smallest diameter of a cell that assures its viability was calculated as ~ 200 nm
343 (Velimorov, 2001). The major lineages identified in DNA and RNA libraries from these
344 subcultures belonged to *Alpha-*, *Beta-*, *Gamma-proteobacteria*, *Actinobacteria* and
345 *Armatimonadetes* (Fig. 5). With the exception of *Armatimonadetes*, all these taxa were
346 detected from pyrosequencing in crude samples from 1827 and 1922 m CSF-A. The
347 majority of the sequences had relatives recovered from environments with similar
348 physical-chemical characteristics (Lin *et al.*, 2006; Mason *et al.*, 2010) (i.e., hot and
349 reduced habitats) compared with the Canterbury subseafloor. Considering the 'ubiquity'
350 of these taxa, one can hypothesize that they are generalist bacteria which would have
351 been maintained during progressive burial of sediments or by transportation through
352 circulating fluids. They might have acquired metabolic capabilities enabling them to
353 resist the associated environmental changes. However, this hypothesis needs to be
354 analyzed in detail. Furthermore, similar SSU rRNA gene sequences do not
355 automatically correspond to identical physiologies, identical phenotypes or similar
356 functions.

357

358 *Impact of potential contaminants on native microbial populations*

359 Contamination is a crucial issue when working with subseafloor sediments. In general,
360 contamination during drilling is still difficult to predict. During IODP Expedition 317,

361 the level of contamination during drilling was evaluated by using fluorescent
362 microspheres and only samples with no detectable contamination were kept for
363 microbiological analyses. Nevertheless, samples without microspheres are not
364 necessarily uncontaminated (Smith *et al.*, 2000). Contamination generally decreases
365 from the exterior to the interior of both sediment and rocks cores (e.g., Lever *et al.*,
366 2006). In consequence, only the interior of sediment cores and intact pieces of rocks that
367 had been exposed to UV light after washing were used for the analyses. In addition, for
368 molecular experiments deeply frozen samples of more than 1 cm in diameter were
369 sterilized by flaming. Afterwards, all possible contaminations during the wet-lab steps
370 have been strictly controlled and minimized (see Supplementary Text). The cutting-
371 edge strategy applied for the pyrosequencing and bioinformatic analyses allowed
372 removing potential spurious sequences and OTUs likely to contain contaminants, by
373 sequencing of negative controls, a duplicate procedure and an associated bioinformatics
374 pipeline. In addition to these precautions, the level of potential contamination of our
375 samples was estimated by calculating the number of contaminating cells per gram of
376 sediment and per gram of sedimentary rock based on the mean contamination values
377 with drilling fluids and mean cell abundances in surface waters reported in the literature.
378 The mean potential contamination was estimated as (i) $0.011 \pm 0.018 \mu\text{L}$ of drilling
379 fluid per gram for unconsolidated sediments drilled using advanced piston coring (APC)
380 and (ii) $0.027 \pm 0.029 \mu\text{L}\cdot\text{g}^{-1}$ for rocks collected using rotary core barrel (RCB) (Lever
381 *et al.*, 2006). Considering these levels of contamination, mean cell counts of 5×10^5
382 $\text{cells}\cdot\text{mL}^{-1}$ in surface waters in the ocean (Whitman *et al.*, 1998) and average densities of
383 $1.85 \text{ g}\cdot\text{cm}^{-3}$ in sediments and $1.99 \text{ g}\cdot\text{cm}^{-3}$ in sedimentary rocks at site U1352, potential
384 contamination of the interior of the core sample should be expected very low with 5 to

385 11 cells·g⁻¹ of sediment only. A second reported estimate indicates that less than 50
386 cells per gram of sediment contaminated APC core centers drilled with *Joides*
387 *Resolution* and that XCB cores were generally more contaminated with contamination
388 levels 3 to 10 times higher in XCB cores than in APC core centers (House *et al.*, 2003).
389 Considering these different estimates of potential contamination, the observed cell
390 counts at site U1352 were 2 to 5 orders of magnitude higher in the studied samples. If
391 contamination cannot be excluded, in the worst case, non-indigenous cells represent
392 only up to 1% of total cells in the sample. Therefore, it is most likely that more than
393 99% of the counted cells are native to the sampled sediment/rocks. This implies that the
394 vast majority of the prokaryotic and eukaryotic DNA subjected to pyrosequencing was
395 therefore derived from the sediment native cells. By extension, assuming that most of
396 the prokaryotic DNA extracted from sediments samples is from native cells, the fact that
397 cultivated bacteria match OTUs abundant in the crude sediment samples supports the
398 idea that these cultivated strains are isolates of native bacteria. Consequently, the
399 potential impact of contaminants on each category of data (cell counts, molecular data
400 and cultures) is likely very low.

401

402 *Ecological implications and future prospects*

403 We have underlined that the seafloor of the Canterbury basin hosts microorganisms
404 which comprise *Bacteria*, *Archaea* and *Eukarya*. Some of these microorganisms are
405 alive and, at least to a certain extent, revivable. The communities exhibit a quite low
406 phylogenetic diversity, but this does not necessarily correspond to a low functional
407 diversity. This poor diversity could be explained if natural selection has produced (i)
408 taxa adapted to harsh subsurface conditions (i.e., specialists), which would be expected

409 in the case of a low connectivity among habitats; and/or (ii) taxa with a broad
410 physiological plasticity, allowing them to survive in a diversity of nutritional and
411 physical-chemical conditions (i.e., generalists). In fact, some taxa detected through their
412 16S/18S rRNA gene sequences are thought to be endemic to subsurface habitats, while
413 others seem ubiquitous and are consistently encountered in common and extreme
414 environments. The bacterial strains in cultures are related to opportunistic or generalist
415 taxa isolated from a broad array of redox environments, which raises the question of the
416 existence of microbial metabolic versatility and also questions the species concept, since
417 behind a given name or a given OTU can lay a variety of microorganisms with different
418 ecological lifestyles. Metabolic versatility has already been demonstrated in well-known
419 taxa. For example, some *Thermococcales* strains, which are usually fermenters that
420 reduce sulfur compounds, can grow in oligotrophic conditions or can oxidize carbon
421 monoxide (Sokolova *et al.*, 2004). Heterotrophy is likely to be the major mode of
422 carbon assimilation within microbial communities of subsurface marine sediments
423 (Batzke *et al.*, 2007). Our culture data support this hypothesis. Genome and
424 metagenome analyses would allow functions to be predicted on a finer scale, to assess
425 and hypothesize the individual ecological functions within the analyzed habitat or
426 ecosystem (Vandenkoornhuyse *et al.*, 2010). The detection of fungal sequences at great
427 depths and our success in the cultivation of fungal strains leads us to ask what role they
428 play in deep carbon cycling and what involvement they have in dynamics/regulation of
429 prokaryotic populations, if they are active *in situ*.

430 The broad polyphasic approach developed in this study provides direct evidence that
431 viable microorganisms can be present in rocks that are hardened but not totally
432 cemented, where stylolites and micro-fluid circulations exist. Our data demonstrate that

433 the combination of physical, chemical and energetic constraints encountered from 0 to
434 1922 m CSF-A in the subseafloor of the Canterbury Basin still allow microorganisms to
435 persist down to at least 650, 1740 and 1922 m CSF-A for *Archaea*, fungi and *Bacteria*,
436 respectively. It extends the subseafloor sedimentary depths at which subseafloor
437 organisms are known to be present to 1740 m for fungi and to 1922 m for *Bacteria*.
438 Nevertheless, one cannot exclude that some of the detected sequences belong to
439 microorganisms in dormancy. More extensive sequencing efforts will be required, i.e.,
440 direct metatranscriptomics, to describe more directly the microbial communities along
441 with functional signatures, and to compile data on biogeochemical processes and fluxes.
442

443 **Acknowledgements**

444 Samples, shipboard facilities and expedition support were provided by IODP. The authors thank
445 the co-chiefs, crew and shipboard scientific parties of IODP Expedition 317. The Joint Research
446 Unit UMR 6197 (CNRS-Ifremer-UBO), LUBEM, GDR Ecchis, EU program MaCuMBA,
447 DIVVIR project of the FRB and the BGR supported molecular and cultural post-cruise
448 analyses. The study was supported by grants from the French Ministry of Higher Education and
449 Research for MCC, VR and FG; from the Région Bretagne for FG; and from the DFG for AS
450 (grant SCH535/7-2) and for JSL (grant HI616/11-1). We thank reviewers for their constructive
451 comments. We thank also C Struckmeyer, M Guégan, H Leclerc, C Argouarch, S Coudouel, A
452 Dheilly and O Quenez for their contribution to this work.

453

454 Supplementary information is available at the ISMEJ's website.

455 The authors declare no conflict of interest.

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575 **Figure legends**

576

577 **Figure 1** Main physical-chemical characteristics of the studied core. Black arrows
578 indicate measures of *in situ* temperatures used to calculate a thermal gradient of
579 46°C/km. This thermal gradient together with the interpretation of the thermal maturity
580 gradient defined by T_{\max} measurements, allowed estimating a bottom-hole temperature
581 comprised between 60 and 100 °C (Source: IODP report 317) (Fulthorpe *et al.*, 2011).

582

583 **Figure 2** Lithological structure and age of lithological deposits at site U1352 in the
584 Canterbury Basin compared with depth distribution of cell counts, 16S/18S rRNA gene-
585 tag sequences and DNA copy numbers of genetic markers and functional genes. On the
586 left, cell concentrations counted at site U1352 (red dots) and neighboring site U1351
587 (blue squares), according to depth (m CSF-A), compared with the general depth
588 distribution of cells (grey dots) in subseafloor sediments (Kallmeyer *et al.*, 2012). In the
589 center, Phylum Class_Order distribution of archaeal, eukaryotic and bacterial 16S/18S
590 rRNA gene-tag sequences (based on SILVA111 classification) from OTU containing
591 100 or more sequences (the remaining sequences were grouped into “Other”). On the
592 right, copy numbers of the disulfite reductase genes A, of the 18S rRNA genes from
593 total *Eukarya* and of 16S rRNA genes from total *Bacteria*, *Archaea* and
594 *JS1/Chloroflexi*-related bacteria. *Geobacteriaceae* were detected only up to 15 m CSF-
595 A ($\sim 5 \times 10^3$ copies per g). No amplification from greater depth was shown. The
596 functional genes *mcrA*, *aprA* and *cbbL* were not detected at all. Legend: § In other
597 classifications, MCG affiliate with the *Thaumarchaeota*. # Lineages of plants and algae.
598 * Depth horizons where ≥ 100 reads of plants and Chrysophyceae were detected.

599

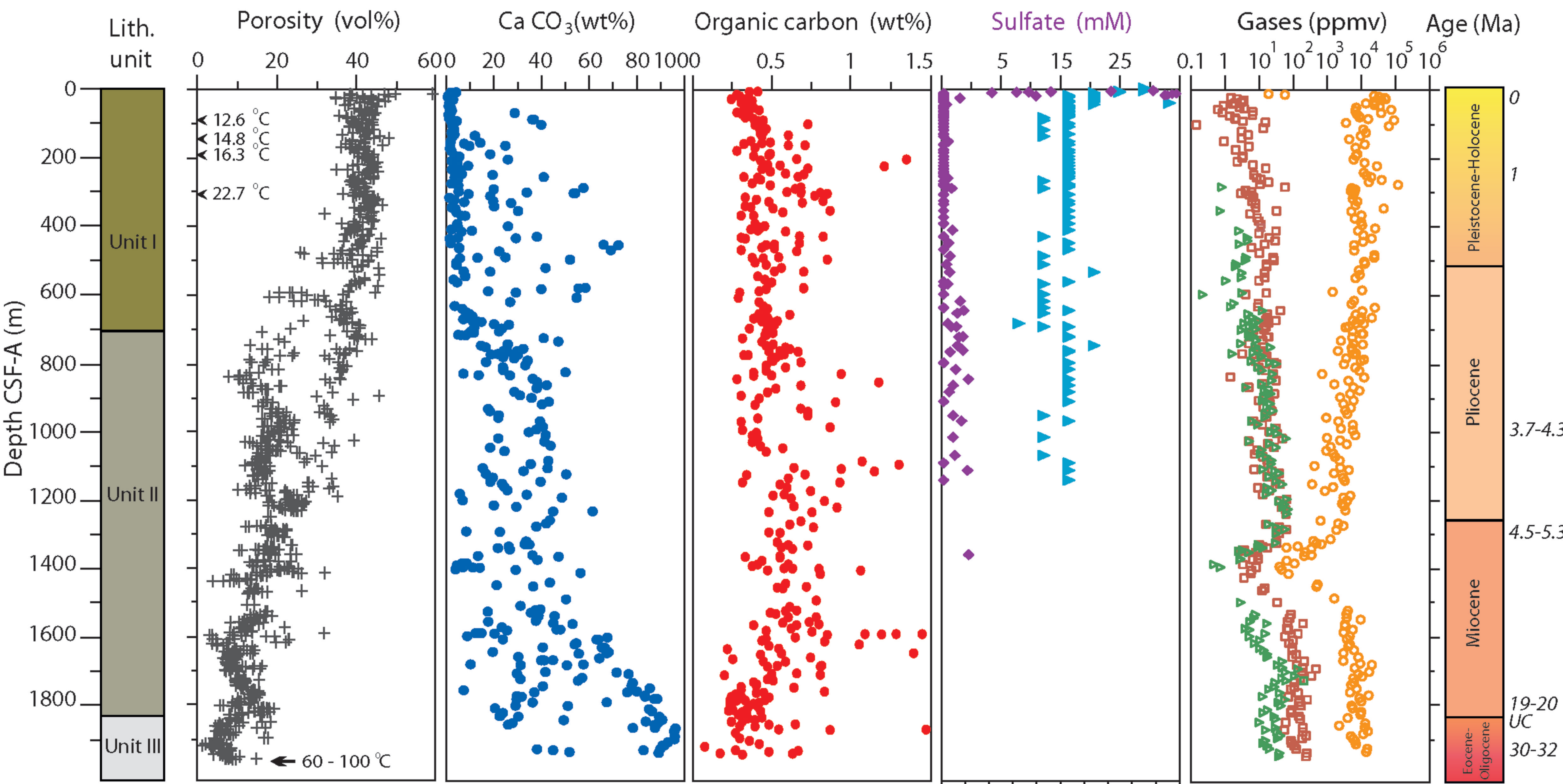
600 **Figure 3** Community richness (Chao1 non-parametric estimator) for archaeal,
601 eukaryotic and bacterial sequences making up the OTUs (calculated by MOTHUR at
602 3% difference between OTUs).

603

604 **Figure 4** Epifluorescence microphotographs of initial enrichment cultures and
605 subsequent liquid subcultures hybridized with the Cy3-labelled probe Euk516 (staining
606 of nuclei with DAPI) (top panel), or stained with SYBR[®]Green I (middle panel + G) or
607 with the dual staining LIVE/DEAD[®]Bacterial Viability Kit (H-I). (A-C) Identification
608 of fungus-like eukaryotic cells with fluorescently-labeled 18S rRNA oligonucleotide
609 probes in initial enrichment cultures with sediments from 21 m CSF-A on PDB 3%, at 4
610 MPa (A), with sediments from 37 m CSF-A on PDB 3%, at 11 MPa (B) and with
611 sediments from 765 m CSF-A on PDB 0%, at 11 MPa (C). (D-E) Microcolonies
612 observed on polycarbonate membranes (initial enrichment cultures) after 15 days of
613 incubation with sediments from 1922 m CSF-A on H₂/CO₂ + YE. (F-G) Cellular
614 aggregates observed in the 7th liquid subcultures performed after the initial enrichment
615 with sediments from 1827 m CSF-A on YE + peptone + casamino acids. (H-I) Cell
616 structural integrity was observed in the 7th liquid subcultures performed after the initial
617 enrichment with sediments from 1827 m CSF-A on acetate + YE (H) and with
618 sediments from 1922 m CSF-A on YE + peptone + casamino acids (I). Legend: YE,
619 Yeast Extract; PDB, Potato Dextrose Broth.

620

621 **Figure 5** Phylogenetic position of the bacterial 16S rRNA gene sequences detected in
622 RNA extracts from the fifth to eighth liquid subculture following the initial enrichment
623 step on a sediment substrate membrane system. The phylogenetic reconstruction was
624 performed using the Neighbor-Joining algorithm. Bootstrap values based on 1000
625 replications are indicated at the branch nodes. Representative bacterial 16S rRNA gene
626 sequences of H₂/CO₂ + yeast extract (YE)-based cultures and acetate + YE-based
627 cultures from the 137R section (1827 m CSF-A), as well as YE + peptone (pept.) +
628 casamino acids (CA)-based culture from the 148R section (1922 m CSF-A) are shown
629 in bold blue letters. Sequences from basalt or an underground aquifer where the
630 temperature is high (52<T°<80°C) are shown in brown; Sequences from environments
631 rich in hydrocarbons or radioactive metals are shown in orange. Accession numbers are
632 given in brackets.



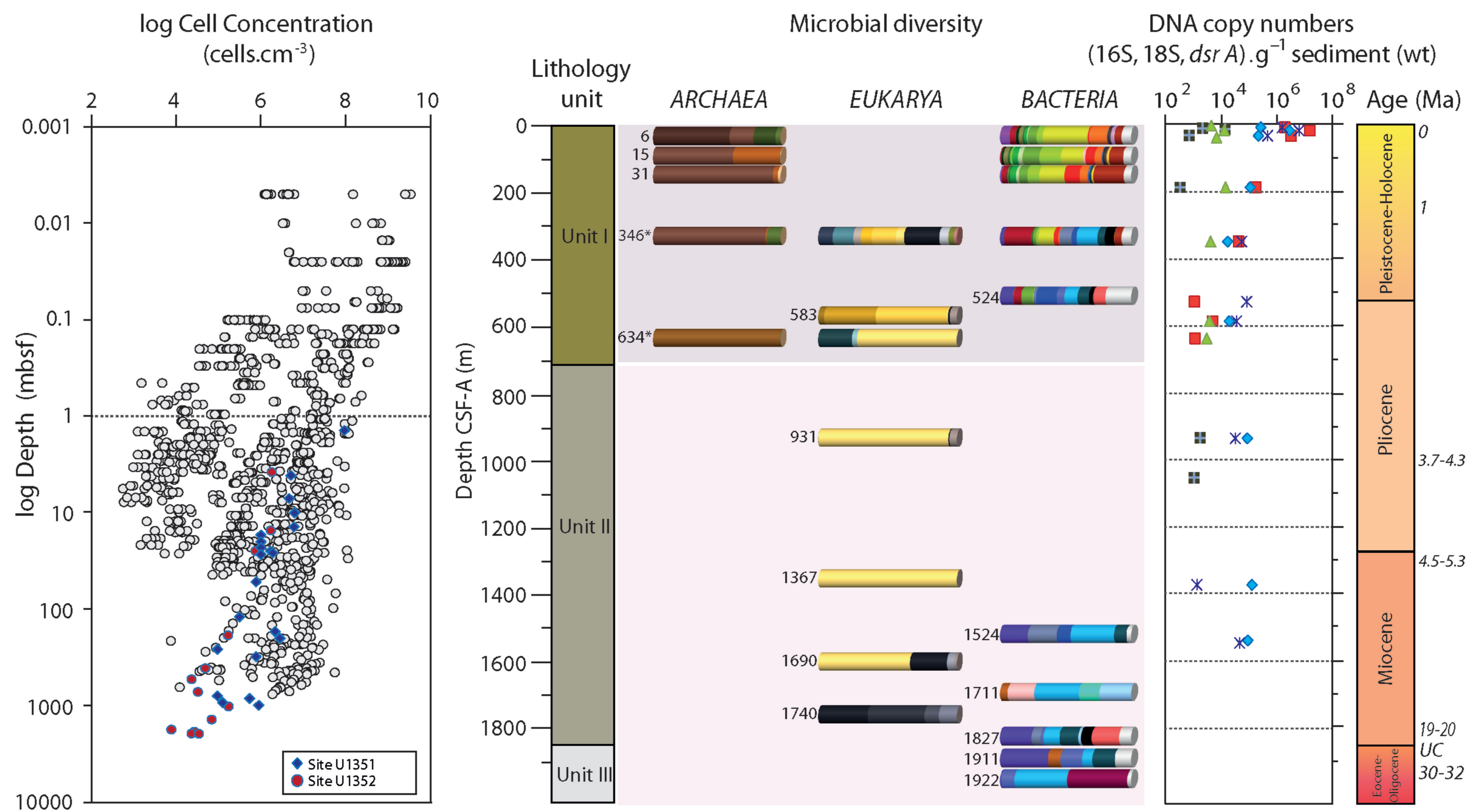
Lithology

- Mud, sand, shells/ sandy mud/ calcareous sandy mud
- Sandy marlstone/ lithified marlstone/ limestone
- Nannofossil limestone

UC : Unconformity

CSF-A (m) : Core depth below seafloor computed by conventional method A

- Methane
- Ethane
- Propane



Archaea

Crenarchaeota

- MBG - B
- MCG s
- Unclassified Crenarchaeota

Euryarchaeota

- Thermococcales
- MBG - E
- SAGMEG
- Other

Eukarya

Streptophyta #

- Liliopsida_Poales
- Eudicotyledons_Malpighiales
- Eudicotyledons_Rosales
- Eudicotyledons_Solanales

Stramenopiles

- Bicosoecida_Bicosoecida
- Chrysophyceae
- Ochromonadales #

Ascomycota

- Dothideomycetes_Capnodiales
- Eurotiomycetes_Chaetothiriales
- Eurotiomycetes_Mycocaliciales
- Unclassified_Sordariomycetes
- Saccharomycetes_Saccharomycetales
- Uncultured_Eukaryota
- Other

Basidiomycota

- Tremellomycetes_Tremellales
- Tremellomycetes_Filobasidiales
- Wallemiomycetes_Wallemiales
- Microbotryomycetes
- Microbotryomycetes incertae sedis
- Exobasidiomycetes_Malasseziales

Bacteria

- Acidobacteria_Acidobacteria_DA052
- Acidobacteria_Acidobacteria_S035
- Actinobacteria_Actinobacteria_Corynebacteriales
- Actinobacteria_OPB41
- Firmicutes_Bacilli_Bacillales
- Nitrospirae_Nitrospira_Nitrospirales
- Planctomycetes_Phycisphaerae_MSBL9
- Spirochaetes_Spirochaetes_Kazan-3B-09
- BH180-139
- Candidate_division_OP9
- Uncultured_Bacteria_ML635J-21
- Uncultured_Bacteria_MLE1-12
- Other

Chloroflexi

- Caldilineae_Caldilineales
- GIF3
- GIF9
- JG30-KF-CM66
- MSB-5B2
- MSBL5
- Napoli4B-65
- Sh765B-AG-111
- vadinBA26

Proteobacteria

- Alphaproteobacteria_Caulobacterales
- Alphaproteobacteria_Rhizobiales
- Alphaproteobacteria_Sphingomonadales
- Betaproteobacteria_Burkholderiales
- Betaproteobacteria_Neisseriales
- Deltaproteobacteria_Desulfarculales
- Deltaproteobacteria_Desulfobacteriales
- Deltaproteobacteria_Myxococcales
- Gammaproteobacteria_Pseudomonadales
- Gammaproteobacteria_Xanthomonadales

Real time-PCR

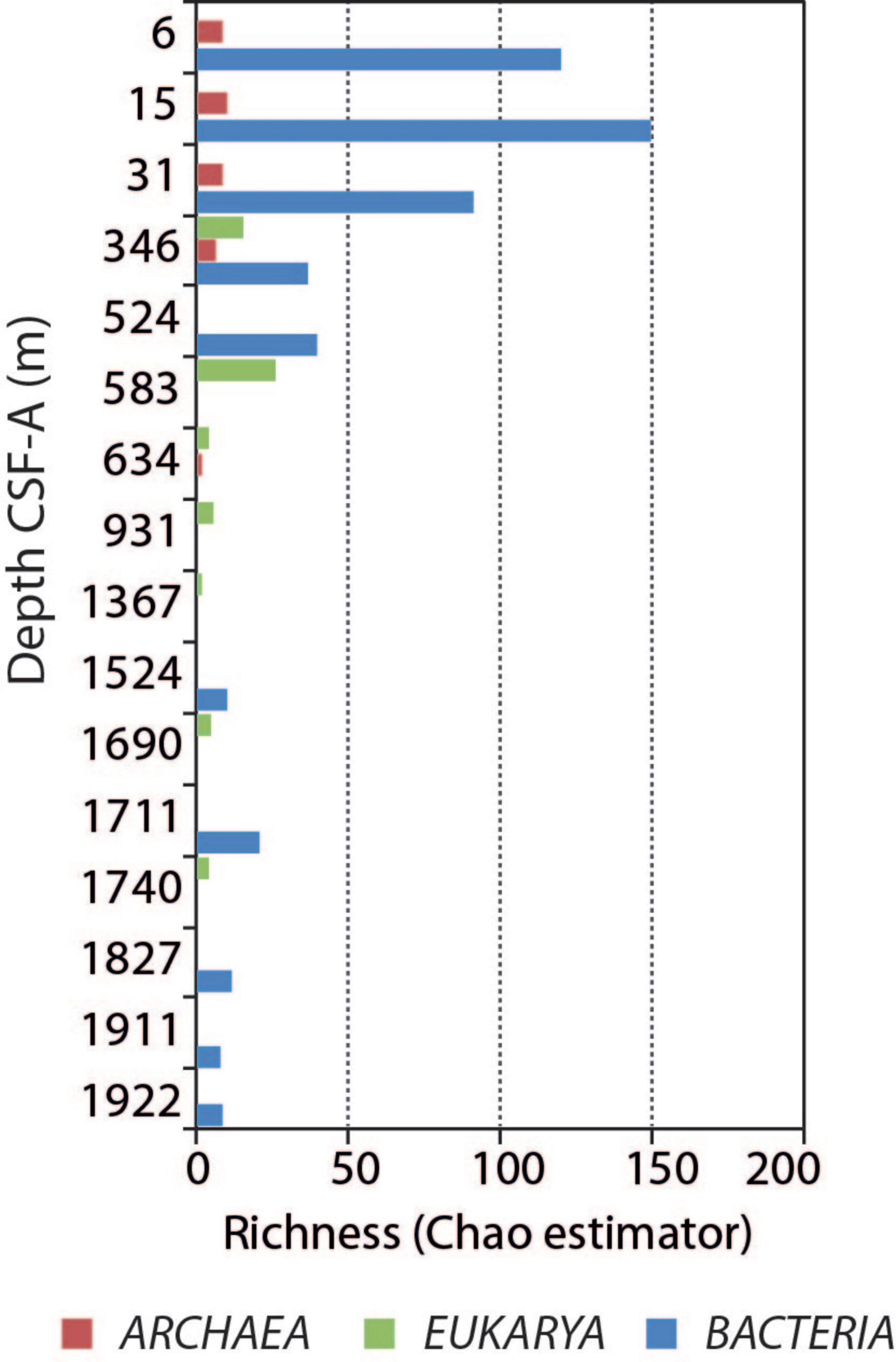
- Bacteria (blue diamond)
- Archaea (red square)
- Eukarya (green triangle)
- JS1/Chloroflexi (purple asterisk)
- dsrA* (grey square)

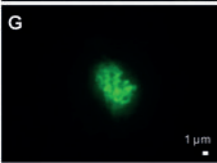
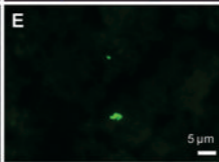
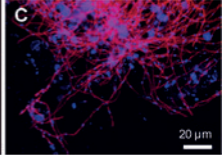
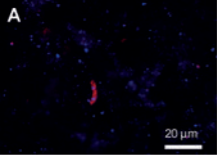
Lithology

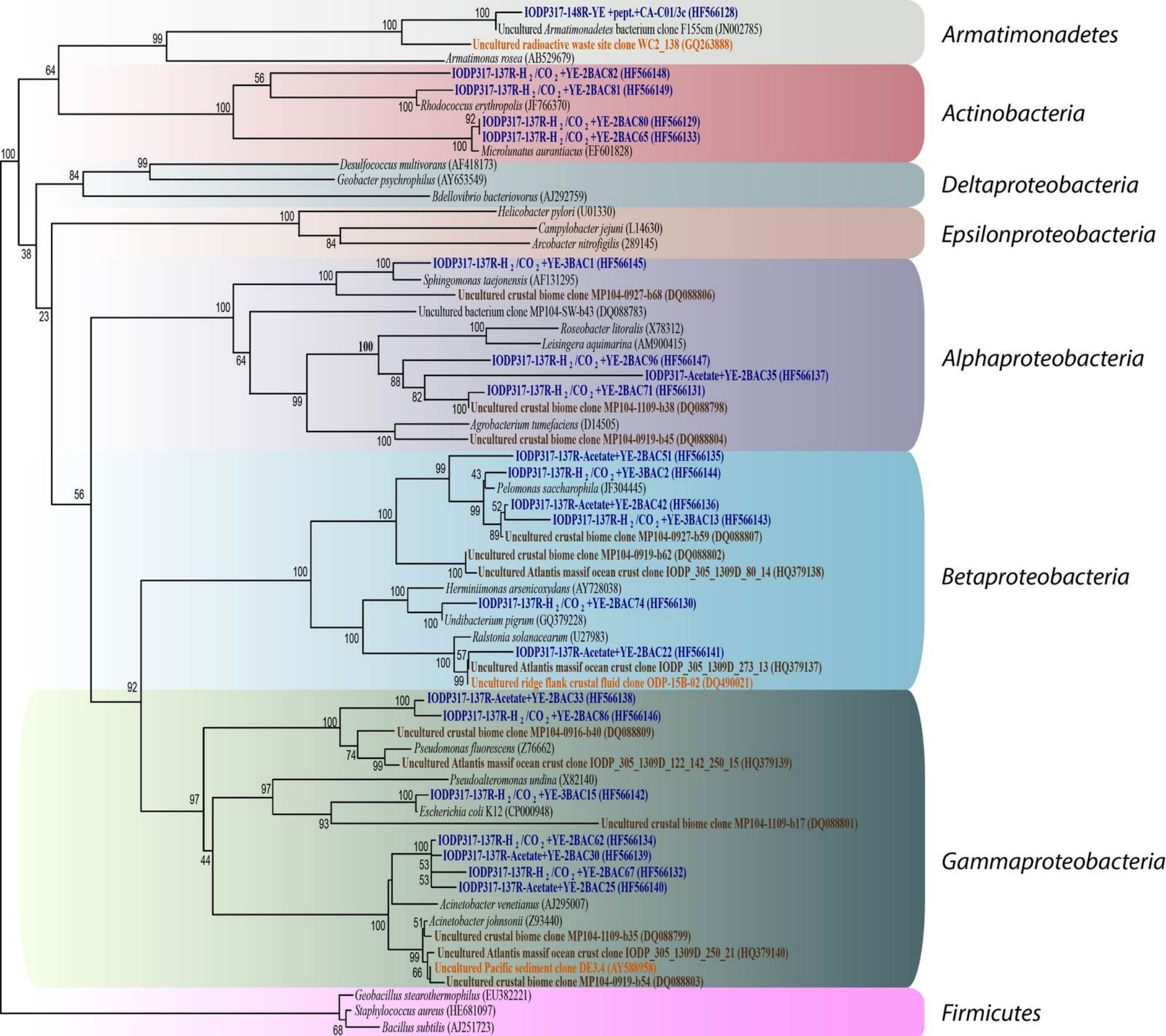
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0.02