Microorganisms persist at record depths in the subseafloor of the Canterbury Basin.

Maria-Cristina Ciobanu, Gaëtan Burgaud, Alexis Dufresne, Anja Breuker, Vanessa Rédou, Sarah Ben Maamar, Frédéric Gaboyer, Odile Vandenabeele-Trambouze, Julius Sebastian Lipp, Axel Schippers, et al.

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2 **Canterbury Basin**

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4 MC Ciobanu[1,2,3], G Burgaud[4,8], A Dufresne[5,8], A Breuker[6], V Rédou[4], S Ben Maamar[1], F

5 Gaboyer[1,2,3], O Vandenabeele-Trambouze[2,1,3], JS Lipp[7], A Schippers[6], P Vandenkoornhuyse[5], G

6 Barbier[4], M Jebbar[1,2,3], A Godfroy[3,1,2] & K Alain[2,1,3].

7

8 [1] Université de Bretagne Occidentale (UBO, UEB), IUEM – UMR 6197, Laboratoire de

9 Microbiologie des Environnements Extrêmes (LMEE), Plouzané, France

10 [2] CNRS, IUEM – UMR 6197, LMEE, Plouzané, France

11 [3] Ifremer, UMR6197, LMEE, Plouzané, France

12 [4] Université de Brest, UEB, Laboratoire Universitaire de Biodiversité et d'Ecologie Microbienne

13 EA 3882, IFR148 SFR ScInBioS, ESIAB, Plouzané, France.

14 [5] Université de Rennes I, CNRS, UMR 6553 ECOBIO, Rennes, France.

15 [6] Bundesanstalt für Geowissenschaften und Rohstoffe (BGR), Hannover, Germany.

16 [7] Organic Geochemistry Group, Department of Geosciences and MARUM Center for Marine

17 Environmental Sciences, University of Bremen, Bremen, Germany.

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23 Correspondence: K Alain, Laboratoire de Microbiologie des Environnements Extrêmes

24 (LMEE), UMR 6197 CNRS-UBO-Ifremer, Plouzané, France. E-mail: Karine.Alain@univ-

25 brest.fr

26 [8]These authors contributed equally to this work

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

Introduction

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

The subsurface microbiota is diverse and complex, hosting metabolically active communities down to depths of more than one thousand meters below the seafloor (mbsf), as revealed by molecular, metagenomic and metatranscriptomic studies (Lipp _et 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 subseafloor 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

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 μL 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 μm 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×, 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 *Taq*Man® 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 ~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

13

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$^-$

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 ± 0.018 μL of drilling

379 fluid per gram for unconsolidated sediments drilled using advanced piston coring (APC)

380 and (ii) 0.027 ± 0.029 μL$\cdot$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 cells$\cdot$mL^{-1} in surface waters in the ocean (Whitman *et al.,* 1998) and average densities of

383 1.85 g$\cdot$cm^{-3} in sediments and 1.99 g$\cdot$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 subseafloor 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

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 $\geq$ 100 reads of plants and Chrysophyceae were detected.

599

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

603

Figure 4 Epifluorescence microphotographs of initial enrichment cultures and subsequent liquid subcultures hybridized with the Cy3-labelled probe Euk516 (staining of nuclei with DAPI) (top panel), or stained with SYBR®Green I (middle panel + G) or with the dual staining LIVE/DEAD®Bacterial Viability Kit (H-I). (A-C) Identification of fungus-like eukaryotic cells with fluorescently-labeled 18S rRNA oligonucleotide probes in initial enrichment cultures with sediments from 21 m CSF-A on PDB 3%, at 4 MPa (A), with sediments from 37 m CSF-A on PDB 3%, at 11 MPa (B) and with sediments from 765 m CSF-A on PDB 0%, at 11 MPa (C). (D-E) Microcolonies observed on polycarbonate membranes (initial enrichment cultures) after 15 days of incubation with sediments from 1922 m CSF-A on H_2/CO_2 + YE. (F-G) Cellular aggregates observed in the 7th liquid subcultures performed after the initial enrichment with sediments from 1827 m CSF-A on YE + peptone + casamino acids. (H-I) Cell structural integrity was observed in the 7th liquid subcultures performed after the initial enrichment with sediments from 1827 m CSF-A on acetate + YE (H) and with sediments from 1922 m CSF-A on YE + peptone + casamino acids (I). Legend: YE, 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 H_2/CO_2 + 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.
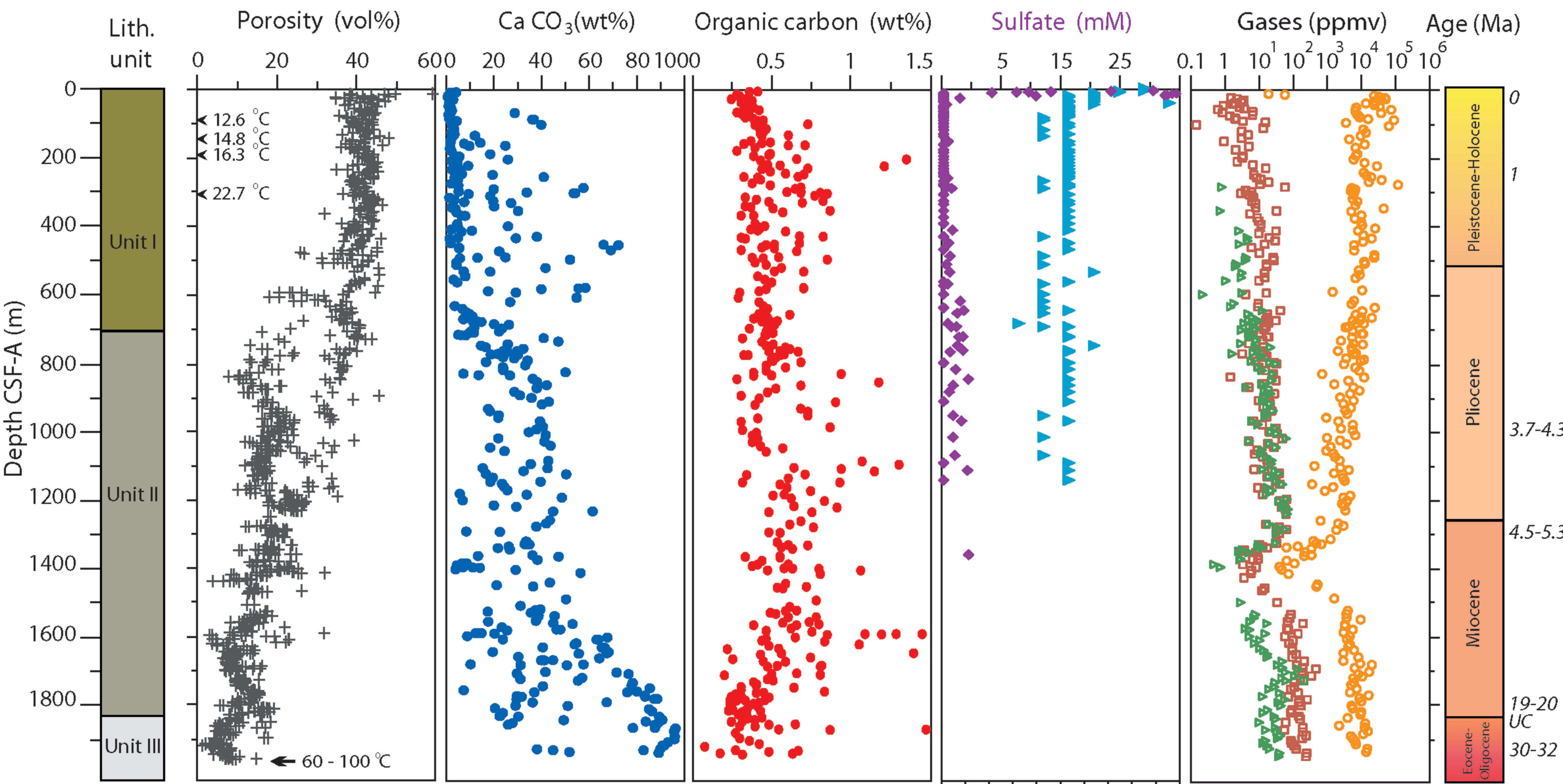
Lithology

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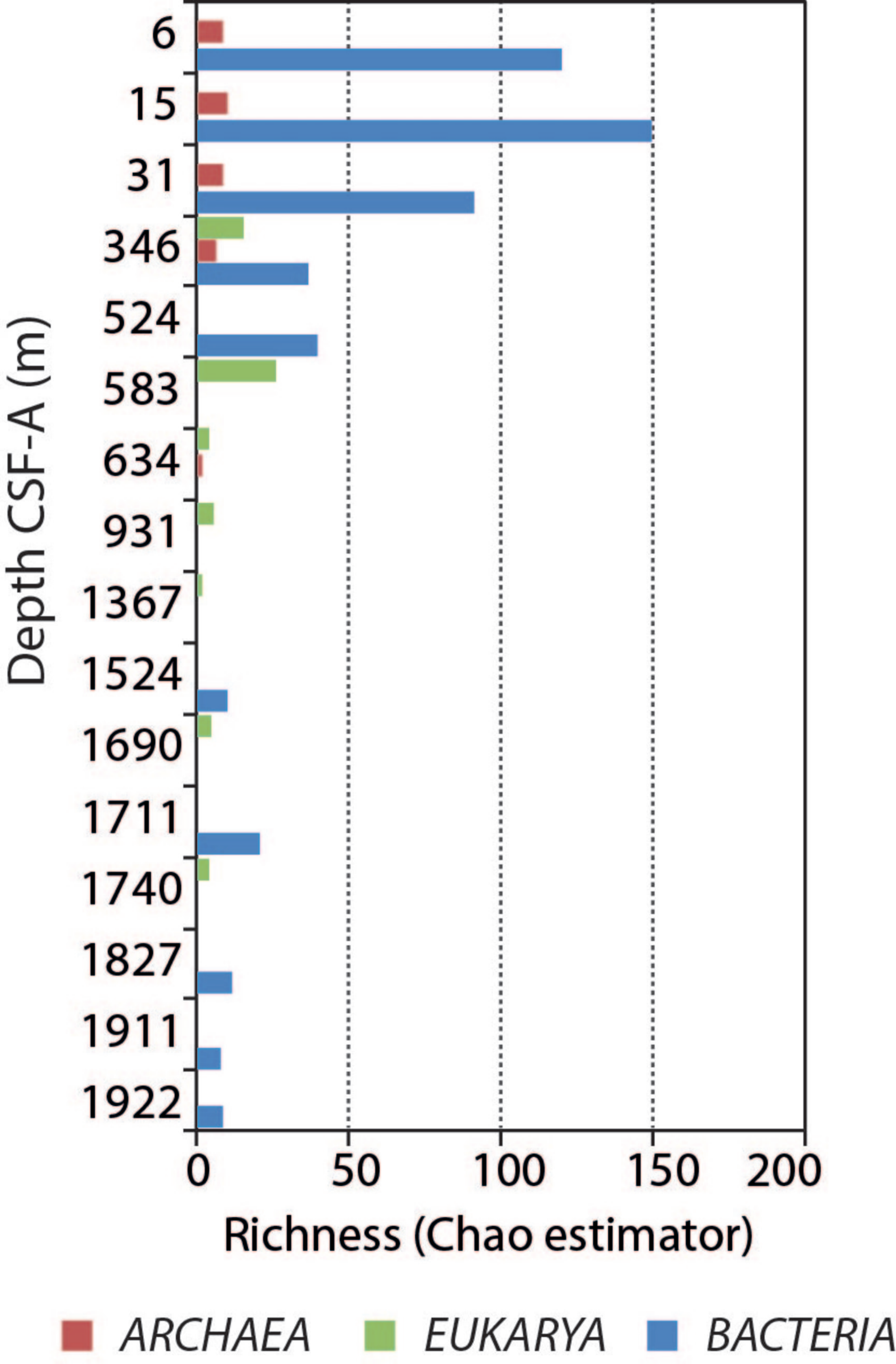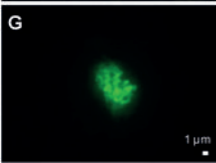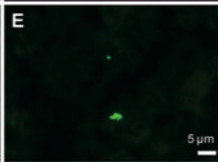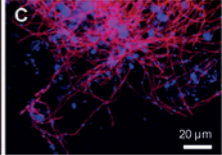- Nannofossil limestone

UC : Unconformity

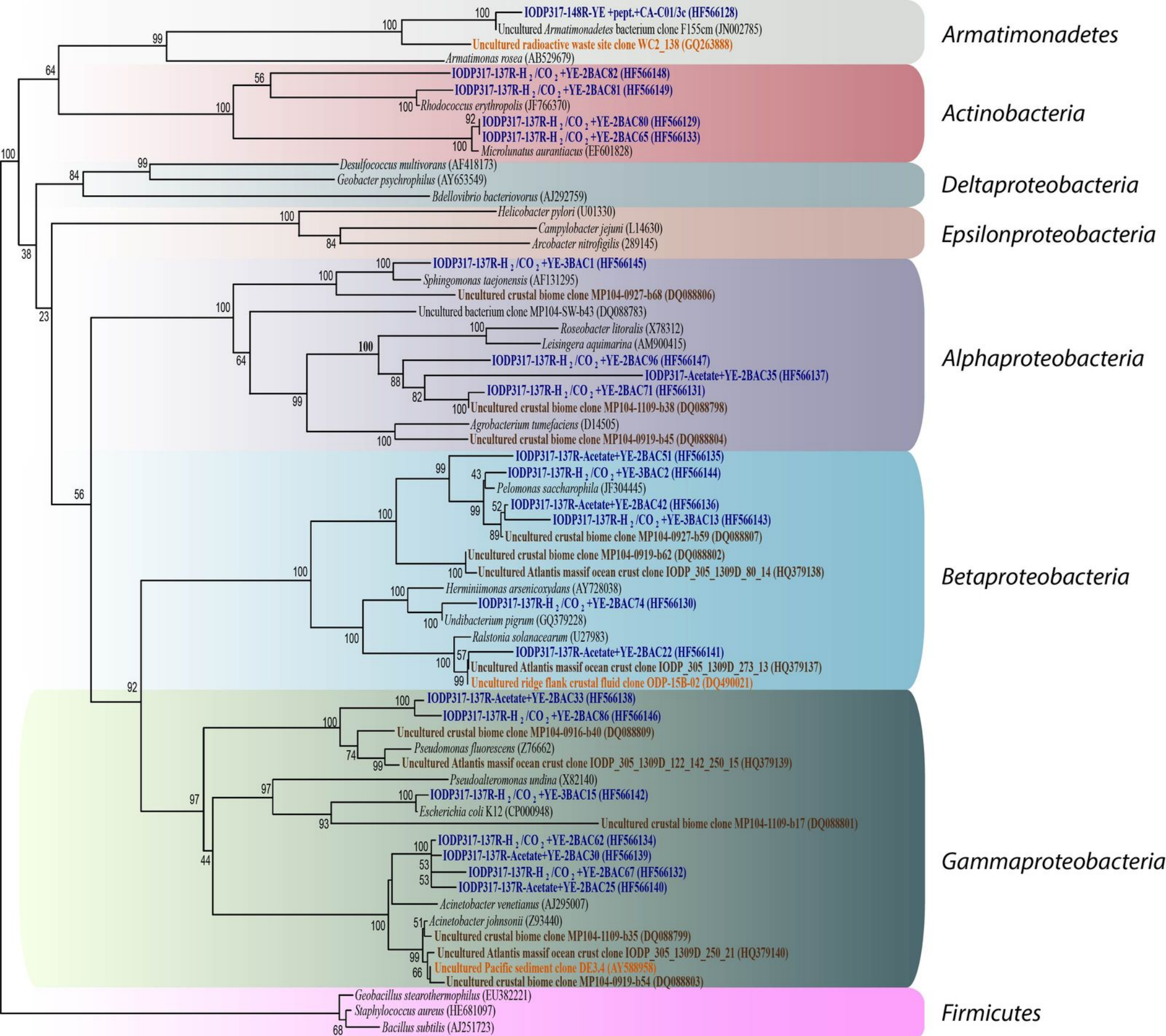CSF-A (m) : Core depth below
seafloor computed by
conventional method A

- ○ Methane
- □ Ethane
- △ Propane

log Cell Concentration
(cells.cm⁻³)

log Cell Concentration (cells.cm^{-3})

Lithology unit

Microbial diversity

ARCHAEA EUKARYA BACTERIA

DNA copy numbers
(16S, 18S, *dsr A*).g^{-1} sediment (wt)

Age (Ma)

log Depth (mbsf)

Depth CSF-A (m)

Unit I

Unit II

Unit III

Pleistocene-Holocene

Pliocene

Miocene

Eocene-Oligocene

0

1

3.7-4.3

4.5-5.3

19-20

UC

30-32

Site U1351
Site U1352

Archaea

Crenarchaeota
MBG - B
MCG §
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_Chaetothyriales
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
BHI80-139
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_Desulfobacterales
Deltaproteobacteria_Myxococcales
Gammaproteobacteria_Pseudomonadales
Gammaproteobacteria_Xanthomonadales

Real time-PCR
Bacteria
Archaea
Eukarya
JS1/*Chloroflexi*
dsr A

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

Scale bar: 0.02