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**Abstract:** Oceanic deep hypersaline anoxic basins (DHABs) are characterized by drastic changes in physico-chemical conditions in the transition from overlaying seawater to brine body. Brine-seawater interfaces (BSIs) of several DHABs across the Mediterranean Sea have been shown to possess methanogenic and sulfate-reducing activities, yet no systematic studies have been conducted to address the potential functional diversity of methanogenic and sulfate-reducing communities in the Red Sea DHABs. Here, we evaluated the relative abundance of Bacteria and Archaea using quantitative PCR and conducted phylogenetic analyses of nearly full-length 16S rRNA genes as well as functional marker genes encoding the alpha subunits of methyl-coenzyme M reductase (*mcrA*) and dissimilatory sulfite reductase (*dsrA*). Bacteria predominated over Archaea in most locations, the majority of which being affiliated with Deltaproteobacteria, while Thaumarchaeota were the most prevalent Archaea in all sampled locations. The upper convective layers of Atlantis II Deep, which bear increasingly harsh environmental conditions, were dominated by members of the class Thermoplasmata (Marine Benthic Group E and Mediterranean Sea Brine Lakes Group 1). Our study revealed unique microbial compositions, the presence of niche-specific groups, and collectively a higher diversity of the sulfate-reducing communities compared to the methanogenic communities in all five studied locations.

## \*Detailed Response to Reviewers

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Dear Dr. Jebbar,

Please find attached our revised version of RESMIC-D-15-00081 for publication in Research in Microbiology. The only comment from the referee was on the measurements of CO<sub>2</sub> in our samples. As these were done by a commercial service provide by GEOMAR in Germany, we now refer to their website.

We hope that you will find the manuscript now suitable for publication in Research in Microbiology.

Best regards,

Uli Stingl

1 **Diversity of Methanogens and Sulfate-Reducing Bacteria**  
2 **in the interfaces of five deep-sea anoxic brines of the Red Sea**

3

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24 **Abstract**

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26 physico-chemical conditions in the transition from overlaying seawater to brine body. Brine-  
27 seawater interfaces (BSIs) of several DHABs across the Mediterranean Sea have been shown to  
28 possess methanogenic and sulfate-reducing activities, yet no systematic studies have been  
29 conducted to address the potential functional diversity of methanogenic and sulfate-reducing  
30 communities in the Red Sea DHABs. Here, we evaluated the relative abundance of Bacteria and  
31 Archaea using quantitative PCR and conducted phylogenetic analyses of nearly full-length 16S  
32 rRNA genes as well as functional marker genes encoding the alpha subunits of methyl-coenzyme  
33 M reductase (*mcrA*) and dissimilatory sulfite reductase (*dsrA*). Bacteria predominated over  
34 Archaea in most locations, the majority of which being affiliated with *Deltaproteobacteria*,  
35 while *Thaumarchaeota* were the most prevalent Archaea in all sampled locations. The upper  
36 convective layers of Atlantis II Deep, which bear increasingly harsh environmental conditions,  
37 were dominated by members of the class *Thermoplasmata* (Marine Benthic Group E and  
38 Mediterranean Sea Brine Lakes Group 1). Our study revealed unique microbial compositions, the  
39 presence of niche-specific groups, and collectively a higher diversity of the sulfate-reducing  
40 communities compared to the methanogenic communities in all five studied locations.

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42

## 43 1. Introduction

44 Hypersaline water bodies at the bottom of the ocean (brine pools) are present in the  
45 Mediterranean Sea, the Gulf of Mexico, and the Red Sea [1]. In the Red Sea, a total of 25 such  
46 deep-sea hypersaline brine pools have been discovered at depths ranging from 1,193 to 2,850  
47 meters below sea level [1, 2]. These environments are extremely saline (up to 26% salinity),  
48 anoxic, rich in heavy metals, and characterized by drastic changes in physicochemical conditions  
49 when compared to the overlaying seawater [3].

50 The interface between the brine pools and the seawater (BSI) represents a highly peculiar  
51 environment that harbors a high microbial diversity and biomass [4-6]. The increase in microbial  
52 biomass can be explained by the drastic changes in density, which result in an *in situ* particle trap  
53 for debris sinking through the water column, thus increasing the concentrations of available  
54 nutrients [6, 7]. In addition, the BSI is also characterized by sharp changes in physicochemical  
55 parameters including salinity, oxygen concentration, temperature, and redox potential, all of  
56 which provide a large variety of environmental niches for different metabolic groups [8, 9]. The  
57 microbiology of the BSIs of some of the Red Sea brine pools has been explored with a  
58 combination of cultivation-dependent [10, 11] and molecular-based methods [12, 13]. Previous  
59 studies based on 16S rRNA gene sequences uncovered novel groups of Archaea and Bacteria  
60 inhabiting the BSI of Shaban Deep and Kebrit Deep of the Red Sea [6, 12].

61 Microbial community studies in the Mediterranean DHABs revealed that diverse  
62 biogeochemical processes apparently co-occur in the BSI [8]. Other investigations reported on  
63 the importance of methanogenesis and sulfur cycling in these environments [4, 14]. These  
64 findings were also corroborated by recent metagenomic studies, where pathways for  
65 methanogenesis and/or sulfate reduction were detected in brines from the Red Sea and in

66 DHABs in the Mediterranean Sea [15-17]. Additionally, unique microbial communities were  
67 found to thrive in the sediments of two brine pools in the Red Sea, and many of the reported  
68 microorganisms are hypothesized to play a dominant role in the methane and sulfur cycle, based  
69 on their phylogenetic affiliations [18].

70       Taken together, methanogenesis and sulfate reduction could thus be considered very  
71 important biogeochemical processes in deep-sea brines [4, 19]. However, the composition of the  
72 microbial communities involved in both processes is largely unknown for the Red Sea brine  
73 pools. Considering the extreme conditions of these environments and the unique combination of  
74 physicochemical features in each individual brine pool [1], we postulated the existence of novel,  
75 niche-adapted groups of methanogens and sulfate reducers in the BSIs. Moreover, despite the  
76 micro-oxic conditions present in the BSI [20], members of both groups are capable of tolerating  
77 minute amounts of oxygen [21-23] and could thus play an important role in these environments.  
78 Previous 454 amplicon data [20] uncovered interesting microbial communities in the sampled  
79 sites, but as many of the relatively short sequences stem from poorly characterized groups, we  
80 decided that nearly full-length 16S rRNA gene sequences would be important to provide better  
81 phylogenetic detail and resolution on members of these groups. Therefore, we analyzed the  
82 microbial communities in the BSIs of geochemically distinct brine pools of the Red Sea, using  
83 the canonical 16S rRNA gene, as well as functional marker genes encoding for the alpha  
84 subunits of methyl-coenzyme M reductase (*mcrA*) and dissimilatory sulfite reductase (*dsrA*) to  
85 uncover the main methanogenic and sulfate-reducing communities.

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## 89 2. Materials and methods

### 90 2.1. Sample collection

91 Water samples from the brine-seawater interfaces and the upper convective layers of the  
92 deep-sea brines were collected from the R/V *Aegaeo* during the 3<sup>rd</sup> KAUST Red Sea Expedition  
93 in November 2011 using a rosette sampler equipped with 10-l Niskin bottles and a CTD unit for  
94 monitoring salinity, temperature, transmission, oxygen, and pressure (Idronaut, Italy). Large  
95 volumes (ca. 200 l) of sample were collected from Atlantis II Deep BSI (Ai); first, second, and  
96 third upper-convective layer of Atlantis II Deep (labeled as A-UCL1, A-UCL2, A-UCL3,  
97 respectively), Discovery Deep BSI (Di), Erba Deep BSI (Ei), Kebrit Deep BSI (Ki), and Nereus  
98 Deep BSI (Ni) (Table 1). During sampling, we have avoided mixing between the seawater and  
99 the brine samples by carefully controlling the depth of the CTD and sampler when triggering the  
100 closure of each Niskin bottle to ensure sampling of desired layers. Furthermore, prior to sample  
101 collection on deck, we measured the salinities at the top and bottom of each individual Niskin  
102 bottle using a handheld refractometer (Master Refractometer, Atago, Japan) to confirm that the  
103 salinities of the samples matched the expected values of the targeted layers. Samples were then  
104 concentrated using a Tangential Flow Filtration (TFF) as described elsewhere [20]. Methane and  
105 carbon dioxide concentrations in the samples were determined via a commercial service provided  
106 by GEOMAR Helmholtz Centre for Ocean Research (Kiel, Germany, <http://www.geomar.de>).

### 107 2.2. DNA extraction, amplification, and sequencing of 16S rRNA genes

108 Nucleic acids were extracted as previously described [24] and the concentrations of the  
109 DNA were measured in a NanoDrop (Thermo Scientific, USA). Partial 16S rRNA genes were  
110 amplified by PCR by using combinations of the archaeal-specific primer 4F (5'-

111 TCCGGTTGATCCTGCCRG-3') [25], or the bacteria-specific primer 27F (5'-  
112 AGAGTTTGATCMTGGCTCAG-3') paired with the universal primer 1492R (5'-  
113 GGTTACCTTGTTACGACTT-3') [26]. The primers were chosen to produce sequences with  
114 maximum length. *In silico* testing using Silva-TestPrime ([http://www.arb-](http://www.arb-silva.de/search/testprime/)  
115 [silva.de/search/testprime/](http://www.arb-silva.de/search/testprime/) [27] with one allowed mismatch indicated a coverage for Bacteria of  
116 65.5% and a coverage for Archaea of 46.6%, while three allowed mismatches indicated a  
117 coverage for Bacteria of 71% and a coverage for Archaea of 53.1%. In addition, the above  
118 primers have a good coverage of major taxa reported in a previous study using 454 amplicon  
119 data [20]. The PCR conditions for archaeal 16S rRNA genes were: an initial denaturation of 5  
120 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1.5 min at 72 °C, and then a final  
121 extension step of 7 min at 72 °C. The conditions for bacterial PCR were 3 min at 94 °C, 35  
122 cycles of 1 min at 94 °C, 1 min at 53 °C, and 1.5 min at 72 °C, and then 7 min at 72°C. Purified  
123 PCR products were cloned into PCR@2.1 TOPO vectors (Invitrogen) according to the  
124 manufacturer's instructions. All clones with inserts from each library (856 and 1040 for archaeal  
125 and bacterial libraries, respectively) were selected for plasmid extraction and bi-directional  
126 sequencing on an ABI 3730 × 1 Capillary Sequencer at the Biosciences Core Laboratory at  
127 KAUST. Raw 16S rRNA gene sequences were quality checked, trimmed and assembled using  
128 Sequencher v.4.9 (Gene Codes Corporation).

### 129 2.3. Diversity and phylogenetic analysis

130 Assembled archaeal and bacterial 16S rRNA sequences were aligned and analyzed using  
131 mothur v.1.31, yielding operational taxonomic units (OTUs) grouped at 97% sequence identity  
132 level [28]. Potential chimeric sequences were removed using the Uchime 4.2 package [29], and  
133 diversity indices (Shannon) and estimated sample coverage (Good's coverage) were calculated



134 as implemented in mothur. Sequence alignments of the resulting representative OTUs (62 for  
135 Archaea and 281 for Bacteria) and closely related sequences recovered from GenBank using  
136 BLASTn [30], were done automatically using the SINA aligner ([http://www.arb-](http://www.arb-silva.de/aligner/)  
137 [silva.de/aligner/](http://www.arb-silva.de/aligner/)) against the SILVA SSU 115 database [31]. The SILVA-aligned sequences were  
138 then used to construct phylogenetic trees with a maximum likelihood algorithm using bootstrap  
139 analysis (1000 samples) to validate support for clades as implemented in ARB v.5.3 [32].

#### 140 2.4. Functional gene analysis

141 Partial *mcrA* and *dsrA* genes were amplified based on previously described methods [33,  
142 34]. Purification of PCR products, clone library construction and sequencing protocols were the  
143 same as the ones used for 16S rRNA genes described above. The OTUs of *mcrA* and *dsrA* genes  
144 were generated at a 6% distance cutoff using FunGene (<http://fungene.cme.msu.edu/>) [35].  
145 Deduced amino acid sequences of both genes were aligned with ClustalW [36]. The alignments  
146 of *mcrA* and *dsrA* genes were used for phylogenetic analyses with a maximum-likelihood  
147 algorithm (amino acids substitution model: LG for *mcrA*, and LG+G for *dsrA* genes) and 1000  
148 bootstraps as implemented in Geneious Pro version 7.1 (Biomatters Ltd.) and MEGA version  
149 6.06 [37], respectively.

#### 150 2.5. Quantification of gene copy numbers by real-time PCR

151 Copy numbers of total bacterial and archaeal 16S rRNA genes from each sample location  
152 were determined by quantitative real-time PCR (qPCR) using EXPRESS qPCR SuperMix  
153 (Invitrogen) and a two-step qPCR cycling program on an ABI 7900HT Fast Real-Time PCR  
154 System instrument (Applied Biosystem). The primers Bac518F and Bac786R for Bacteria and  
155 A519F and A727R for Archaea were used as described by Park et al. [38]. Standards were made  
156 from plasmids containing inserts of archaeal or bacterial 16S rRNA gene sequences. The

157 efficiency for archaeal 16S rRNA primers was 99.8% and that for bacterial 16S rRNA primers  
158 was 99.5%, as estimated based on the slope of the standard curve. To allow for better  
159 comparisons among the different samples, copy numbers of genomic DNA were normalized  
160 based on ng of genomic DNA.

### 161 *2.6. Nucleotide sequence accession numbers*

162 The 16S rRNA gene sequences from this study were deposited in GenBank under  
163 accession numbers KJ881441–KJ882283 (Archaea), and KM018335–KM019141, KP083299–  
164 KP083370 (Bacteria), while *mcrA* and *dsrA* gene sequences were deposited under accession  
165 numbers KJ880100–KJ880274 and KM241874–KM242055, respectively.

## 166 **3. Results and discussion**

### 167 *3.1. General microbial community structure*

168 The ratios of the copy numbers of bacterial to archaeal 16S rRNA genes as estimated  
169 using qPCR were used as a proxy for their abundances in each sample. These ratios ranged in our  
170 samples from 0.15 to 179.12 (Table 1), and Bacteria were more abundant than Archaea in six out  
171 of eight samples. The predominance of Bacteria over Archaea thus seems to be a general trend in  
172 the BSI (and brine bodies) of brine pools from the Red Sea ([5, 17]; this study) and the  
173 Mediterranean Sea [8, 19, 39, 40]; the exceptions being the BSIs of Kebrit Deep and Atlantis II  
174 Deep (this study) and the brine layer of the Urania DHAB in the Mediterranean Sea [19].  
175 Physico-chemical differences that might be either the reason for, or a result of, the high archaeal  
176 abundances are 1) they are highly sulfidic (in the case of Kebrit Deep and Urania DHAB; ~150  
177  $\mu\text{M}$  and 10 mM  $\text{H}_2\text{S}$ , respectively [9]), and 2) the BSIs of Kebrit and Atlantis II Deep have

178 dissolved oxygen concentrations ( $\text{DO}_2$ ) that are 4–9 times higher than those at the other  
179 locations, where  $\text{DO}_2$  is close to depletion (Table 1).

180 Phylogenetic analysis based on 16S rRNA gene sequences revealed a high microbial  
181 diversity in the brine interfaces of geochemically distinct brine pools of the Red Sea (Fig. 1). A  
182 total of 843 archaeal (>1000 bp length) and 960 bacterial (>1400 bp length) non-chimeric 16S  
183 rRNA gene sequences were obtained from the BSIs of the five brines and from the subsequent  
184 three upper convective layers of Atlantis II Deep (A-UCL1, A-UCL2, and A-UCL3). These  
185 sequences were clustered into 62 and 281 OTUs (at 97% sequences identity level) for archaeal  
186 and bacterial genes, respectively. Archaeal sequences were primarily affiliated with the phyla  
187 *Thaumarchaeota* (60%) and *Euryarchaeota* (37%), while the bacterial sequences encompassed  
188 diverse lineages. Our findings are in general consistent with previous studies [5, 20].

189 Hierarchical cluster analysis of the archaeal and bacterial 16S rRNA gene based on the  
190 Jaccard similarity index indicated similarities and differences among microbial communities of  
191 each location (Fig. 1A and 1B). The archaeal community in Kebrit Deep was clearly distinctive  
192 from those in the other locations (Fig. 1A). Archaeal communities in the three upper convective  
193 layers of Atlantis II Deep, BSIs of Nereus Deep and Erba Deep, and BSIs of Atlantis II Deep and  
194 Discovery Deep formed three separate clusters. In contrast with the results of the archaeal  
195 communities, we found no apparent clustering of the bacterial communities (Fig. 1B). Though, a  
196 highly stratified bacterial community profile was observed in the multi-layered Atlantis II Deep,  
197 concurrent with previous reports [5].

198 This is the first detailed phylogenetic study of nearly full-length prokaryotic 16S rRNA  
199 gene sequences from BSIs of Erba and Nereus Deep. Surprisingly, despite their salinity  
200 differences (9.8% vs. 15.4 %), they both harbor very similar microbial communities (Fig. 1). The

201 primary archaeal taxon was Marine Group I *Thaumarchaeota* (77%), while members of the class  
202 *Deltaproteobacteria* dominated their bacterial clone libraries (35.5% in Erba, and 27.9% in  
203 Nereus). In general, the microbial community compositions based on clone libraries were  
204 consistent with previous findings using an amplicon sequencing approach [20]. Additionally, six  
205 bacterial OTUs accounting for ~8% of all sequences were present in both brine pools, and are  
206 affiliated mostly with sulfate-reducing or sulfur-oxidizing taxa (e.g., *Deltaproteobacteria* and  
207 SAR324; Table S1).

### 208 3.2. Detailed analysis of archaeal communities

209 Both *Thaumarchaeota* and *Thermoplasmata* were ubiquitous in all five brine-seawater  
210 interfaces (BSI) and the three upper convective layers of Atlantis II Deep (A-UCL1, A-UCL2,  
211 and A-UCL3). This suggests that they are important components of deep-sea brine environments,  
212 and presumably possess adaptations to thrive in these gradient environments. *Thaumarchaeota*  
213 was the predominant group in archaeal clone libraries of all the five BSI samples (73–87%). The  
214 archaeal community composition in the Atlantis II BSI was different from the communities  
215 found in the subsequent convective layers. Clone libraries showed that the most abundant  
216 members in those convective layers belonged to the class *Thermoplasmata* (46–81%). Class  
217 *Methanomicrobia*-related sequences were also present (six out of eight sampled locations), but  
218 constituted only a small proportion of the archaeal communities (2.5%–11.4%; Fig. 1A).  
219 Additionally, a variety of archaeal lineages were found in the investigated Red Sea brine pool  
220 BSIs, albeit at low abundances, such as *Archaeoglobi*, *Halobacteria*, Marine Benthic Group A  
221 and D, Marine Group III, MSP41, CCA47 cluster and VC2.1 Arc6, Terrestrial Miscellaneous  
222 Group, SM1K20 group, South African Goldmine Euryarchaeotal Group (SAGMEG), Deep-Sea  
223 Euryarchaeotic Group, Miscellaneous Euryarchaeotic Group (MEG) and Terrestrial Hot Spring

224 Crenarchaeotic Group (THSCG) (Supplementary Figure S2). Many of the phylogenetic lineages  
225 of Archaea retrieved in this study are uncultured at present. A few of these lineages have been  
226 previously detected from the sediment samples of Atlantis II Deep and Discovery Deep of the  
227 Red Sea in a pyrosequencing approach using 16S rRNA genes [18].

228         Although we cannot rule out the possibility of reporting DNA sequences of non-  
229 metabolically active cells due to long-term preservation of DNA and cells in the deep-sea  
230 hypersaline environments, our recently published results [20] have shown that most of the  
231 thaumarchaeal sequences retrieved from the exact same sampled locations (BSIs) belonged to a  
232 Marine Group I phylotype that is absent in the overlaying water column. Still, this might not  
233 apply to all retrieved sequences. The cultivated species in the phylum *Thaumarchaeota* are  
234 autotrophic ammonia-oxidizing archaea [41], but a mixotrophic lifestyle in the dark ocean has  
235 also been suggested [42]. Considering their abundance and the halotolerant genomic features of  
236 thaumarchaeal single-cells from BSIs of Red Sea brines [20], we reconfirm previous findings  
237 that the BSI populations might play significant roles in the nitrogen and carbon cycles in the  
238 deep-sea brine interfaces with special adaptation to the hypersaline environments.

239         Detected members of the *Thermoplasmata* were mostly associated with the Candidate  
240 division MSBL1 (or Mediterranean Sea Brine Lakes Group 1) and MBGE (Marine Benthic  
241 Group E). Considerable proportions (5%–37%) of sequences related to MSBL1 were found in  
242 most clone libraries (six out of eight). In the Mediterranean DHABs, MSBL1 communities were  
243 commonly found in the lower interfaces and in the brines, and they have been assumed to be  
244 methanogenic due to a positive correlation between their abundances and the *in situ* methane  
245 concentrations [16, 43].

246 MBGE sequences were more abundant in the clone libraries of the upper convective  
247 layers of Atlantis II Deep than in other samples. This is consistent with previous reports  
248 indicating that they frequently occur at locations with higher temperatures (50 °C – 63 °C),  
249 correlating with the high G+C content of their 16S rRNA gene sequences [44]. They were often  
250 retrieved from deep-sea sediment, hydrothermal environments, chimney samples [45], and iron-  
251 rich habitats [46]. Therefore, we speculate that their increased abundance in the upper convective  
252 layers of Atlantis II is related to the increased temperature (52 – 65 °C) and iron concentration  
253 (24.5–70.5 µM). As no members of the MBGE clade and MSBL1 clade have been cultivated so  
254 far, their physiology and ecological roles in hypersaline deep-sea environments require future  
255 investigation.

### 256 3.3. Detailed analysis of bacterial communities

257 A wide diversity of Bacteria was observed in the BSIs of Atlantis II, Discovery, Kebrit,  
258 Nereus, and Erba Deep, corroborating previous reports on the microbial communities in the Red  
259 Sea brine pools [5, 6, 12]. The majority of bacterial 16S rRNA sequences were affiliated with  
260 *Proteobacteria* (relative abundance 37.5–89.4%), *Bacteroidetes* (0.8–13.2%), *Deferribacteres*  
261 (0.8–13.9%), and *Chloroflexi* (1.1–4.2%) (Fig. 1B). The remaining bacterial sequences belong to  
262 uncultured bacterial groups with unknown physiology such as Candidate division KB1, MSBL 2,  
263 and ST12-K34 (Fig S3). These uncultivated groups were previously reported from hypersaline  
264 brines in the Red Sea and DHABs in the Mediterranean Sea [6, 12, 43].

265 *Deltaproteobacteria* was the predominant bacterial class in the BSIs of colder brines such  
266 as Kebrit, Erba, and Nereus Deep (Fig. 1B). The high abundance of *Deltaproteobacteria* in the  
267 BSIs of these three brines is consistent with previous studies from the Mediterranean DHABs [4,  
268 40]. This group is also one of the most prominent metabolically active microbial groups thriving

269 in the chemocline of MgCl<sub>2</sub>-rich Discovery Basin [47], hypersaline Lake Kryos [39], and  
270 hydrothermal mud fluids of Urania DHAB, Mediterranean Sea [48]. These findings affirm the  
271 argument that sulfate reduction is one of the main metabolic processes occurring in the  
272 chemoclines of brine pools, which might be primarily performed by members of the  
273 *Deltaproteobacteria* [4, 19].

274 On the contrary, *Deltaproteobacteria* were less abundant in the clone libraries of the hot  
275 brines such as Discovery and Atlantis II Deep. The bacterial clone libraries of Atlantis II Deep  
276 shifted from being dominated by *Nitrospinae*-like bacteria in the BSI to being dominated by  
277 *Gammaproteobacteria* in the convective layers underneath (Fig. 1B). Similar patterns of  
278 gradually changing microbial communities were observed in a previous study based using  
279 pyrosequencing of the 16S rRNA genes in these two locations [5]. According to the same study,  
280 the combination of high temperature and salinity was presumed to shape the communities in both  
281 brines.

282 The majority of the OTUs in the class *Deltaproteobacteria* fell into four orders:  
283 *Desulfobacterales*, *Desulfurellales*, *Desulfovibrionales*, and *Syntrophobacterales* (Fig. 2). The  
284 remaining OTUs were assigned into four lineages with no cultured representatives, namely  
285 10bav-F6, DTB120, *Candidatus* Entotheonella, and SAR 324. A large fraction of  
286 *Deltaproteobacteria* (around 33.3%) in A-UCL1 could not be classified to any known family,  
287 based on the ARB-SILVA SSU 115 database.

288 Certain bacterial groups seemed specifically adapted to high salinities. For instance, the  
289 bacterial community of the deepest convective layer of Atlantis II (A-UCL3) was dominated by  
290 Candidate division KB1, a group with no cultured representatives. This group branches between  
291 the *Aquificales* and the *Thermotogales*, and is restricted to hypersaline conditions [49]. Members

292 of KB1 were initially retrieved from sediments in Kebrit Deep [12] and later obtained in  
293 enrichments of BSI with high salinities obtained from Lake Medee, the Mediterranean Sea [43].  
294 In this study, Candidate division KB1 was detected in interfaces with higher salinity such as A-  
295 UCL3, Di, and Ki (Fig. S3). The metabolic preferences of Candidate division KB1 remain  
296 unsolved although they seem to partially rely on reductive cleavage of the osmoprotectant  
297 glycine betaine, resulting in the formation of trimethylamine (TMA) and acetate [43].

### 298 3.4. Molecular diversity of *mcrA* genes

299 Up to now, all described methanogenic archaea fall into the seven orders in the phylum  
300 *Euryarchaeota*: *Methanococcales*, *Methanopyrales*, *Methanobacteriales*, *Methanosarcinales*,  
301 *Methanomicrobiales*, *Methanocellales*, and the recently proposed 7th order,  
302 *Methanomassiliicoccales* [50]. The reduction of CO<sub>2</sub>, the fermentation of acetate, and the  
303 dismutation of methanol or methylamines encompass the three major methanogenic pathways  
304 [51]. Although methanogens play an important role in the global carbon cycling in various  
305 environments, very little is known about the methanogenic players in the Red Sea brine pools.  
306 Methyl-coenzyme M reductase is unique to methanogens and catalyzes the last step in methane  
307 formation. Genes encoding the  $\alpha$  subunit of this enzyme (*mcrA*) have been employed as a  
308 specific marker to detect and differentiate methanogenic and anaerobic methanotrophic  
309 communities [52].

310 Figure 3 summarizes the diversity and phylogenetic analysis based on 199 *mcrA* gene  
311 sequences retrieved from the upper convective layers of Atlantis II Deep and the BSIs of Erba  
312 and Kebrit Deep. These *mcrA* gene sequences clustered into four OTUs at a 6% amino acid  
313 sequence distance cutoff. Two *mcrA* OTUs (OTU1 and OTU2, representing ~99% of the  
314 retrieved *mcrA* sequences) grouped together with cultured species of the genera



315 *Methanohalophilus* and *Methanococcooides*, along with clones from various hypersaline  
316 environments including the deep-sea hypersaline Lake Thetis [16], Lake Medee [43], Discovery  
317 Basin in the Mediterranean Sea [47], and Discovery Deep in the Red Sea [17]. The cultivated  
318 representatives of these genera utilize methylated compounds as methanogenic substrates and  
319 can produce methane in media with salinities of up to 4M NaCl [53]. This implies that the  
320 dismutation of methanol and methylamines is potentially the main methanogenic pathway in  
321 diverse hypersaline deep-sea basins. Previous studies have suggested that both methanogenesis  
322 and sulfate reduction are major energy-generating processes in the deep-sea hypersaline  
323 environment [4, 8]. However, members of methanogens are in competition with sulfate-reducing  
324 bacteria for their mutual substrates (H<sub>2</sub> and acetate). They are also more negatively affected by  
325 increased redox potential (e.g. the increase in DO<sub>2</sub>) and by the availability of other terminal  
326 electron acceptors (e.g., nitrate, iron, and sulfate); these conditions do exist in the brine pools.  
327 Thus, hydrogenotrophic or acetoclastic methanogens tend to be less common in hypersaline  
328 environments due to thermodynamic constraints [54]. Instead, methanogens in hypersaline  
329 habitats are thought to be restricted to non-competitive substrates such as methylated amines,  
330 which occur as derivatives of compatible solutes [55]. Our results are consistent with this notion.

331         The remaining two rare OTUs were present only in Kebrit BSI clone library. OTU3  
332 grouped with a previously unidentified cluster formed by clones recovered from rice field soil  
333 [56] and could not be assigned to any of the known methanogens. Interestingly, we found that  
334 OTU3 is related to *Candidatus* ‘*Methanoperedens nitroreducens*’ (78% similarity) and thus could  
335 represent an anaerobic methane-oxidizing microorganism. OTU4 clustered with cultivable  
336 species of *Methanomassiliicoccales* [50], but with low amino acid sequences similarities (63%–  
337 79%) [57]. *Methanomassiliicoccales* are rarely associated with deep-sea hypersaline locations,

338 and to our best knowledge, this is the first report of this phylotype from Red Sea brine pools,  
339 after their detection from Lake Kyros in the Mediterranean Sea [39]. Previously reported  
340 sequences associated with this order stemmed from various environments such as rumen, feces,  
341 hindguts, sludge, rice field soil, sediments, and anaerobic digesters. To date,  
342 *Methanomassiliicoccus luminyensis* is the only described species in this order [58]. Based on the  
343 physiology and the genome content of this isolate, this new order utilizes an H<sub>2</sub>-dependent  
344 methylotrophic pathway for methanogenesis.

345         High concentrations of methane gas seem to be a common trait of most DHABs that have  
346 been studied to-date, including those studied here (Table 1). However, the link between these  
347 geochemical data and the major methane-producing taxa in such locations is still obscure. Our  
348 study showed a low diversity of methanogens in our samples and a distribution seemingly  
349 restricted to certain layers. Several previous studies have hypothesized that members of the  
350 Candidate division MSBL1 could be the enigmatic methanogens in brine pools, given their  
351 numerical predominance among Archaea and occurrence in methane-containing layers [4, 43].  
352 So far, however, there is no conclusive evidence to support this hypothesis based on the  
353 following observations. In this study, we retrieved abundant MSBL1-related 16S rRNA genes  
354 from multiple samples, but except for a single phylotype from Kebrit BSI, no novel clusters of  
355 *mcrA* genes were detected. Thus, we assume that the Candidate division MSBL1 does not  
356 possess *mcrA* genes. Still, our study does not conclusively rule out the possibility that novel  
357 *mcrA* genes have been missed due to primer bias or undersampling of the present clone libraries.  
358 Additionally, the fact that the abundance of MSBL1 16S rRNA genes seems to be correlated  
359 with locations that possess high methane concentrations might be misleading, as the methane  
360 could either be produced abiotically [59] or biotically in deeper layers of the brine pool.

### 361 3.5. Molecular diversity of *dsrA* genes

362 Sulfate-reducing prokaryotes are a phylogenetically diverse group of anaerobes, with the  
363 majority of them belonging to the *Deltaproteobacteria* [60]. To yield energy, this group oxidizes  
364 hydrogen or small organic compounds and reduces sulfate to sulfide. The dissimilatory sulfite  
365 reductase (*dsr*), which contains two subunits (*dsrA* and *dsrB*), is the key enzyme in this process  
366 and is widely used as molecular marker to study the diversity of sulfate-reducing communities  
367 [61].

368 In the present study, *dsrA* clone libraries consisting of 220 sequences were constructed  
369 from the interfaces of five different brine pools in the Red Sea (Table 1). The *dsrA* gene  
370 sequences were clustered into 27 OTUs with a 6 % distance cutoff (Table S2). This is the first  
371 report on the diversity of sulfate-reducing bacteria (SRB) communities inhabiting the Red Sea  
372 brine pools. Phylogenetic analysis based on deduced amino acid sequences of *dsrA* clones  
373 revealed a high diversity of sulfate-reducing communities throughout the BSIs. Compared with  
374 the other brines in Mediterranean Sea [39], the Red Sea brine pools seem to possess a relatively  
375 higher diversity of SRB.

376 The majority of *dsrA* gene sequences in Erba Deep, Kebrit Deep, and Nereus Deep were  
377 affiliated with *Desulfohalobiaceae* and the members of *Desulfobacteraceae* such as  
378 *Desulfatiglans*, *Desulfosalsimonas*, *Desulfobacterium*, and *Desulfobacula* (Fig. 4). In contrast, in  
379 the warmer brines of Atlantis II and Discovery Deep, most of the OTUs formed distinct  
380 phylogenetic lineages clustering together with environmental sequences (Fig. 4). This result  
381 indicated a different composition of sulfate-reducing communities for each geochemically  
382 distinct brine pool. In addition, the diversity of sulfate-reducing communities based on *dsrA*

383 genes is in good agreement with the presence of sequences related to the members of  
384 *Desulfobacteraceae* and *Desulfohalobiaceae* in the 16S rRNA gene data (Fig. 2).

385 As shown in Figure 4, six different phylogenetic clades of *dsrA* gene sequences were  
386 found in the brine-seawater interfaces of the Red Sea brine pools. In Clade BSI I, two OTUs  
387 from Kebrit Deep BSI with a relative abundance of more than 30% were affiliated with genus  
388 *Desulfobacula*. They formed clusters with the *dsrA* clones from the interface of L'Atalante Deep  
389 and Lake Kryos in the Mediterranean Sea, suggesting that the sulfate-reducing groups in these  
390 environments are specifically adapted to the conditions in the BSIs [14, 47]. In Clade BSI II, an  
391 OTU from Erba and one from Kebrit Deep BSI were affiliated with *Desulfosalsimonas*  
392 *propionica*, a species of the *Desulfobacteraceae*. The sequences from Kebrit Deep in this clade  
393 were closely related to a clone obtained from the lower BSI of Lake Kryos [39]. The  
394 predominance of *Desulfobacteraceae* in the BSI of different brine pools might be explained by  
395 their wide range in nutritional diversity, oxygen tolerance, and metabolic plasticity [62].

396 Clade BSI III is constituted of an OTU recovered from Kebrit Deep BSI (18.2% salinity)  
397 that was distantly related to two isolated halophilic SRB species, *Desulfohalobium retbaense*  
398 [63] and *Desulfohalobium utahense* (both growing at salinities of up to 24%) [64]. This OTU  
399 was only present in Kebrit Deep, suggesting a preference for higher salinity environments. This  
400 finding is in a good agreement with previous analyses of *dsrAB* mRNA in the Discovery Basin of  
401 the Mediterranean Sea, which revealed that *Desulfohalobiaceae* dominated in the saltier section  
402 of the interface (from 1.60 up to 2.23 M MgCl<sub>2</sub>) [47].

403 The OTUs in Clade BSI IV were widely distributed in the Red Sea brine pools (Fig. 4,  
404 Table S2), especially in the ecosystems with higher sulfate concentration (Table 1). They were  
405 related to *Desulfatiglans anilini*, a sulfate-reducing bacterium that is capable of degrading a

406 variety of aromatic compounds including phenol [65]. Almost all halophilic and halotolerant  
407 strains of SRB isolated so far are incomplete oxidizers, which oxidize organic substrates to  
408 acetate [66]. Interestingly, sequences related to these groups are also found in Kebrit Deep  
409 (salinity 18%) and formed a cluster with a clone from the brine of L'Atalante Deep (salinity  
410 27%), which exceeds the maximum salt limit predicted for complete oxidizers (approximately  
411 13%) [67].

412 In Clade BSI V (Fig. 4), *dsrA* gene sequences from Erba and Nereus Deep were distantly  
413 related to *Thermodesulfatator atlanticus*, a chemolithoautotrophic SRB species within the family  
414 *Thermodesulfobacteriaceae* that was isolated from a hydrothermal vent [68]. Some of the OTUs  
415 in the hot brines Atlantis II and Discovery Deep formed three deeply branching evolutionary  
416 lineages (Clade BSI VI) that were different from any isolated sulfate-reducing bacteria (Fig. 4).  
417 As there are no cultivated representatives, the metabolism and physiology of this clade remains  
418 obscure. However, phylogenetic analysis indicates that they are related to organisms retrieved  
419 from similar environments in the Mediterranean Sea [14], implying that these phlotypes are  
420 specifically adapted to the DHABs. Considering the high abundance of deeply branching  
421 sequences in the Clade BSI VI (Fig. 4), we assume that the interfaces of the Atlantis II and  
422 Discovery Deep harbor specific sulfate-reducing communities that are quite different to known  
423 SRB.

424 In conclusion, the bacterial communities were very diverse and, based on 16S rRNA gene  
425 copy numbers, dominated over archaea in the majority of our samples. In the multi-layered  
426 Atlantis II Deep, archaeal and bacterial communities were stratified. Marine Group I  
427 *Thaumarchaeota*, MBGE and Candidate division MSBL1 *Thermoplasmata*, halophilic  
428 methanogens, and members of class *Deltaproteobacteria* were the most common microbial

429 groups associated with the chemoclines of the Red Sea brine pools. Methanogens were restricted  
430 to a few taxa in all studied locations, reiterating the harshness of these habitats. The sulfate-  
431 reducing communities were collectively diverse based on *dsrA* gene sequences, with the  
432 majority of the OTUs in Erba, Kebrit, and Nereus Deep being affiliated with genus  
433 *Desulfatiglans*. Additionally, the high-temperature Atlantis II and Discovery Deep harbor deeply  
434 branched lineage of *dsrA* gene sequences, which suggest that novel lineages of SRB reside in  
435 these environments. In the broader sense, these findings provide more insights on the ubiquity of  
436 methanogenic archaea and sulfate-reducers in hypersaline habitats, and should increase the  
437 impetus for future cultivation-attempts of the novel halophilic microorganisms.

#### 438 **Conflict of interest**

439 All authors declare no conflict of interest.

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648 **Table**

649 **Table 1.** Physical and geochemical parameters of the sampling locations, bacteria/archaea ratio,  
 650 number of clones, and alpha diversity  
 651

Sampling site <sup>a</sup>	Kebrit		Erba	Nereus	Discovery	Atlantis II			
	Ki	Ei	Ni	Di	Ai	A-UCL1	A-UCL2	A-UCL3	
<b>Latitude (N)<sup>b</sup></b>	24° 43.41'	20° 43.80'	23° 11.53'	21° 16.98'	21° 20.76'	21° 20.76'	21° 20.76'	21° 20.76'	
<b>Longitude (E)<sup>b</sup></b>	36° 16.63'	38° 10.98'	37° 25.09'	38° 3.18'	38° 4.68'	38° 4.68'	38° 4.68'	38° 4.68'	
<b>Depth (m)<sup>b</sup></b>	1467	2381	2432	2038	1998	2006	2025	2048	
<b>Thickness (m)</b>	3	10	12	35	4	10	12	20	
<b>Salinity (‰)<sup>b</sup></b>	18.2	9.8	15.4	13.8	5.6	8.6	11.2	15.4	
<b>H<sub>2</sub>S (μmol/l)<sup>b</sup></b>	149.8	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	
<b>CH<sub>4</sub> (ppmV)</b>	28955.5	15.5	23	53	b.d.l.	85.5	330	977	
<b>CO<sub>2</sub> (ppmV)</b>	103731	<400	<400	791.5	7878	18953	19288	12226	
<b>16S rRNA gene copy nr. (x10<sup>4</sup>)</b>	<i>Bacteria</i>	6.60	20.60	11.41	457.69	48.05	59.11	1.44	1.27
	<i>Archaea</i>	42.69	1.03	0.08	54.81	77.8	0.33	0.39	0.09
<b>Bacteria/Archaea 16S rRNA gene copy nr. ratio<sup>c</sup></b>	0.15	20.00	142.63	8.35	0.62	179.12	3.69	14.11	
<b>Bacteria/Archaea ratio after normalization<sup>d</sup></b>	0.07	9.07	64.07	3.79	0.28	81.26	1.67	6.40	
<b>Nr. of 16S rRNA gene clones [nr. of OTUs]</b>	<i>Bacteria</i>	250 [73]	189 [91]	104 [53]	121 [53]	92 [18]	181 [59]	- <sup>e</sup>	23 [12]
	<i>Archaea</i>	160 [22]	155 [18]	84 [9]	118 [9]	95 [7]	96 [6]	100 [12]	35 [12]
<b>Shannon index</b>	<i>Bacteria</i>	3.03	4.03	3.65	3.3	1.84	3.43	-	2.24
	<i>Archaea</i>	1.44	1.45	1.1	1.11	0.64	0.87	1.86	2.36
<b>Good's coverage</b>	<i>Bacteria</i>	0.71	0.52	0.5	0.56	0.8	0.67	-	0.48
	<i>Archaea</i>	0.93	0.97	0.96	1	0.97	1	0.99	0.94
<b>Nr. of clones of functional genes [OTUs]</b>	<i>mcrA</i>	12 [3]	152 [2]	-	-	-	-	20 [1]	15 [1]
	<i>dsrA</i>	42 [8]	112 [13]	31 [7]	18 [3]	5 [3]	12 [5]	-	-
<b>Good's coverage</b>	<i>mcrA</i>	0.75	0.99	-	-	-	-	0.95	0.93
	<i>dsrA</i>	0.81	0.88	0.77	0.83	0.5	0.58		

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653 <sup>a</sup> Abbreviations for sampling sites: Ki, Kebrit Deep BSI; Ei, Erba Deep BSI; Ni, Nereus Deep BSI; Di, Discovery Deep BSI; Ai,  
 654 Atlantis II Deep BSI; A-UCL1, A-UCL2, and A-UCL3, the first, second, and third upper-convective layer, respectively.

655 <sup>b</sup> For physicochemical data please refer to Ngugi et al. 2014

656 <sup>c</sup> To allow for a better comparison among the different samples, copy numbers of genomic DNA were normalized based on ng of  
 657 genomic DNA.

658 <sup>d</sup> Archaeal and bacterial abundance ratios were estimated based on the qPCR results and the average 16S rRNA gene copy (4.1  
 659 per cell in Bacteria and 1.86 per cell in Achaea (Lee et al. 2009).

660 <sup>e</sup> The bacterial library of A-UCL2 was not generated because of technical issues.

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662

663 **Figure legends**

664 **Figure 1.** Taxonomic classification and relative abundance of archaeal (A) and bacterial (B)  
665 communities in the brine-seawater interfaces of five different brine pools of the Red Sea. A total  
666 of 843 archaeal and 960 bacterial 16S rRNA gene fragments were classified using mothur based  
667 on SILVA database at 97% cutoff. The cluster dendrogram illustrates the linked hierarchical  
668 clustering of different environments based on the relative abundance of the OTUs in each  
669 sampling location. (**Ai**, Atlantis II Deep BSI; A-UCL1, A-UCL2, and A-UCL3, the first, second,  
670 and third upper convective layer, respectively; **Di**, Discovery Deep BSI; **Ei**, Erba Deep BSI; **Ki**,  
671 Kebrit Deep BSI; **Ni**, Nereus Deep BSI. MSBL1, Mediterranean Sea Brine Lakes Group 1;  
672 SAGMEG, South African Goldmine Euryarchaeotal Group).

673 **Figure 2.** 16S rRNA gene-based phylogenetic tree of the *Deltaproteobacteria* group, including  
674 the representative sequences from the Atlantis II, Discovery, Erba, Kebrit, and Nereus Deep. The  
675 topology of the tree is based on maximum-likelihood algorithm with 1000 bootstraps. The scale  
676 bar represents 0.10 fixed mutation per nucleotide position. Bootstrap values above 50% are  
677 shown.

678 **Figure 3.** Phylogenetic tree of *mcrA* genes showing the relationship of representative *mcrA*  
679 clones retrieved from the deep-sea brines of the Red Sea to known methanogens and  
680 environmental sequences. Taxonomy is based on FunGene (<http://fungene.cme.msu.edu>).  
681 Bootstrap values are based on 1000 replicates and values above 50% are shown. Percentages in  
682 parentheses indicate the relative abundance in each sample.

683 **Figure 4.** Phylogenetic tree based on deduced amino acid sequences of the *dsrA* clones from the  
684 brine-seawater interfaces of Red Sea brine pools, including sequences from Mediterranean  
685 DHABs. The topology of the tree is based maximum-likelihood method using 1000 bootstrap  
686 replicates. The scale bar represents 0.10 fixed mutation per nucleotide position and bootstrap  
687 values above 50% are shown. Percentages in parentheses indicate the relative abundance in each  
688 sample.

689

690 **Supplementary materials**

691 **Table S1.** Shared OTUs among the sampled locations and the relative abundance of archaeal and  
692 bacterial 16S rRNA genes.

693 **Table S2.** OTU classification and the relative abundance of *dsrA* genes in each sampled  
694 locations.

695

696 **Figure S1.** Sampling locations of five brine pools in the Red Sea.

697 **Figure S2.** Phylogenetic tree showing the affiliation of archaeal lineage detected from the  
698 interfaces of the Red Sea brine pools. The tree was constructed by maximum likelihood analysis  
699 using ARB. Taxonomy is based on SSURef\_115\_SILVA (<http://www.arb-silva.de>). Dots at  
700 nodes indicate bootstrap values above 50%.

701 **Figure S3.** Major lineages of Bacteria (excluding *Proteobacteria* phylum), harboring  
702 representative sequences from the interfaces of the Red Sea brine pools. Taxonomy is based on  
703 SSURef\_115\_SILVA (<http://www.arb-silva.de>), constructed by maximum likelihood analysis  
704 using ARB.

705 **Figure S4.** Rarefaction analysis of *mcrA* clone libraries.

706 **Figure S5.** Rarefaction analysis of *dsrA* clone libraries.

Figure 1

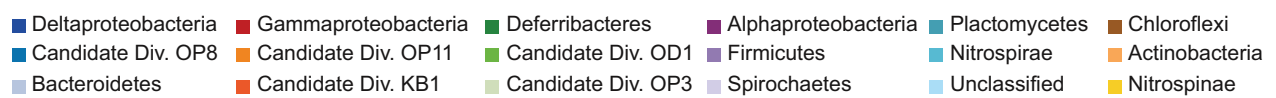
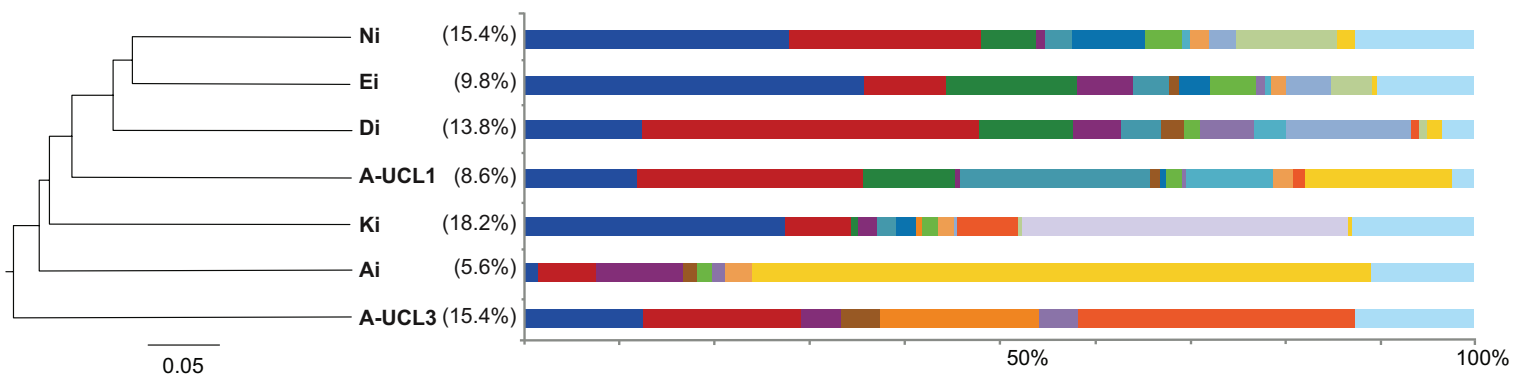
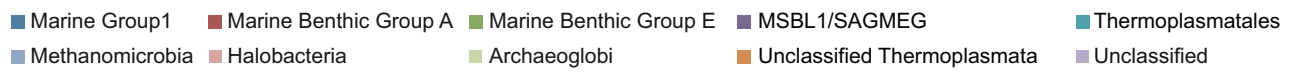
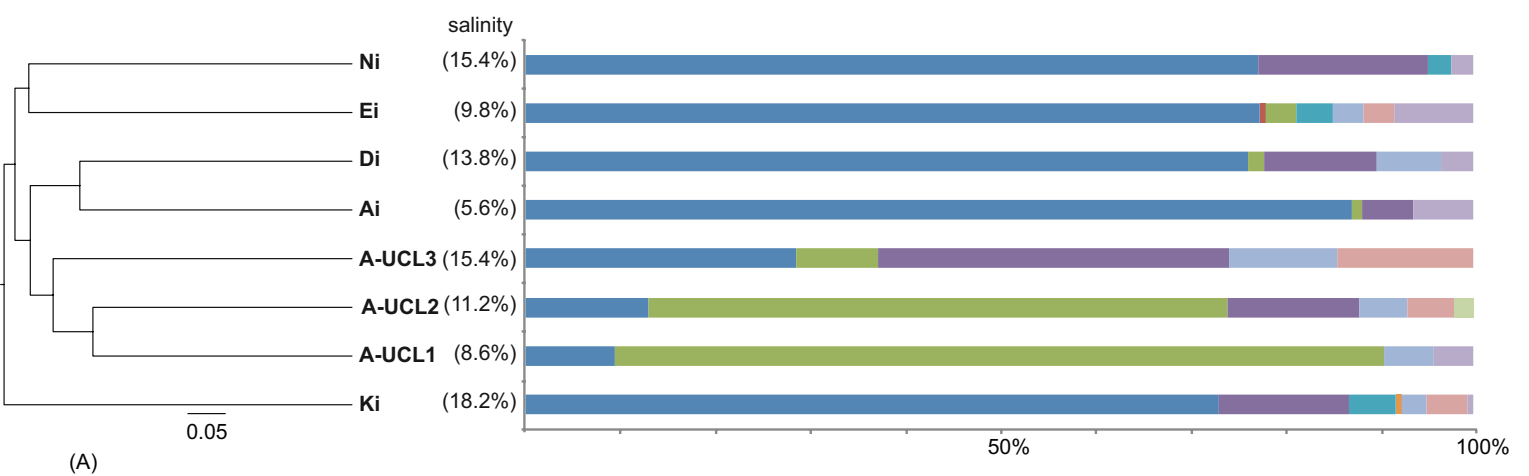
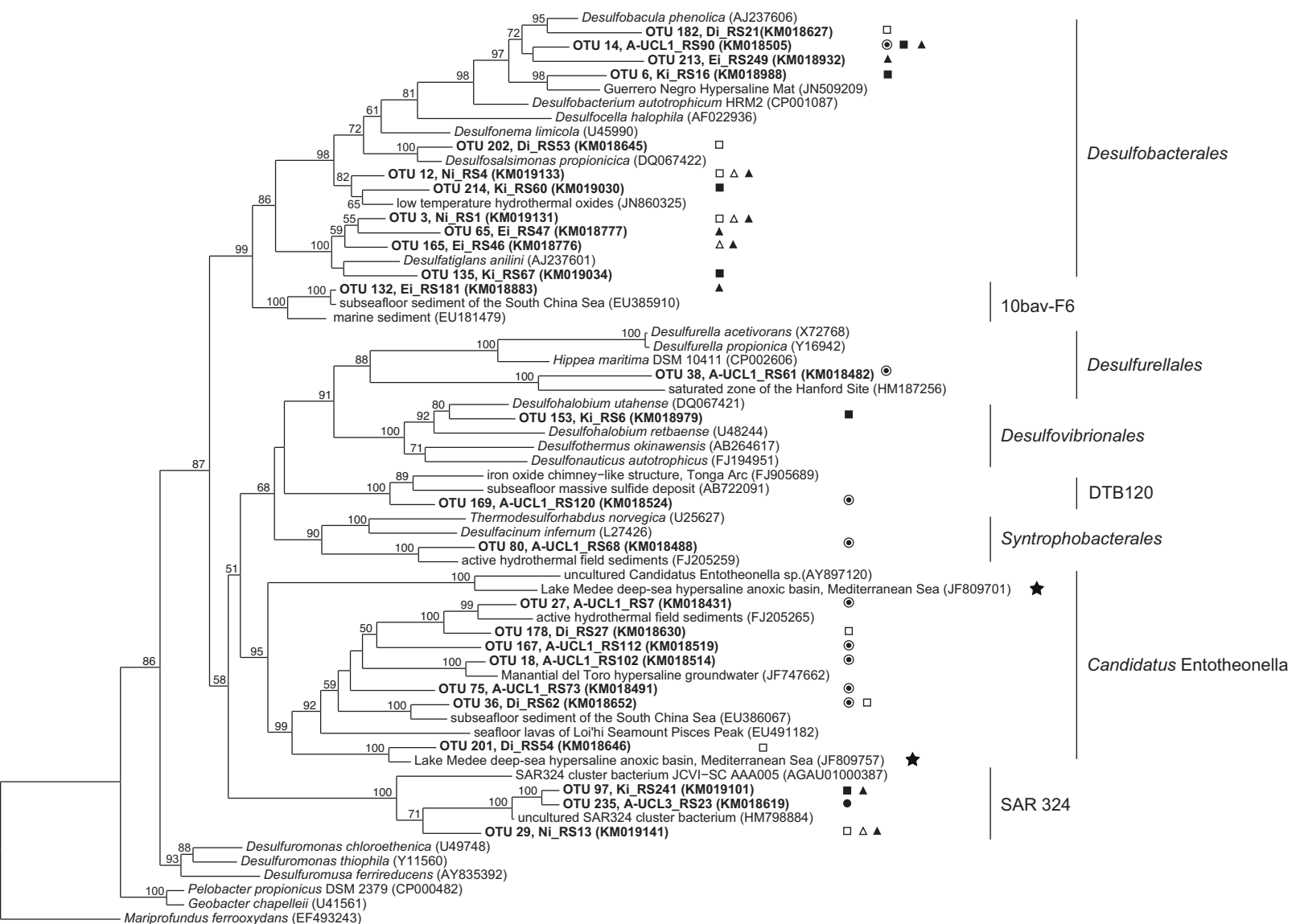


Figure 2



- Atlantis II Deep BSI      ● Atlantis II Deep UCL3      □ Discovery Deep BSI      ▲ Nereus Deep BSI
- ⊙ Atlantis II Deep UCL1      ■ Kebrit Deep BSI      ▲ Erba Deep BSI      ★ Mediterranean DHABs

Figure 3

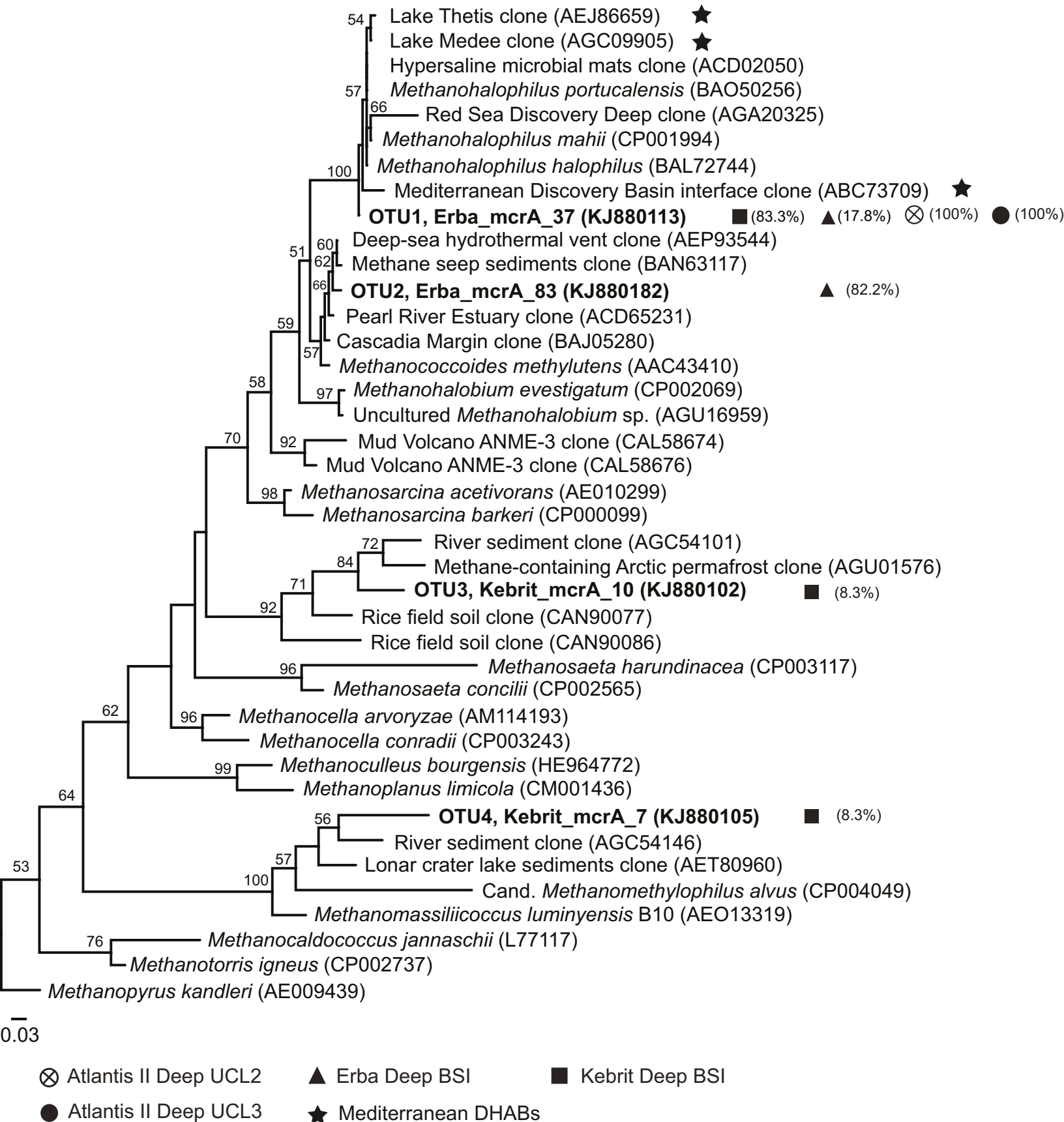
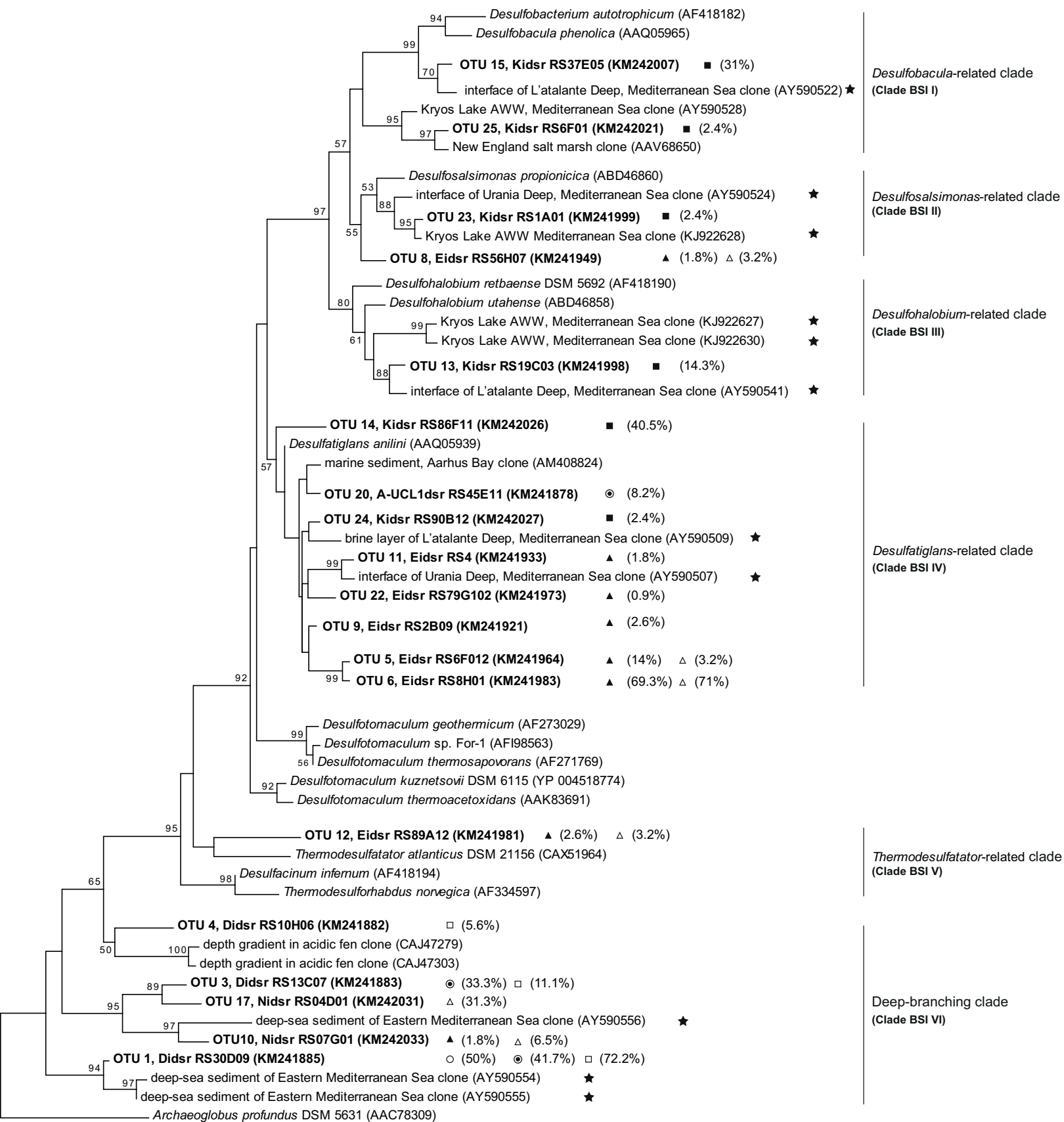


Figure 4



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○ Atlantis II Deep BSI   □ Discovery Deep BSI   ▲ Nereus Deep BSI   ★ Mediterranean DHABs  
 ⊙ Atlantis II Deep UCL1   ■ Kebrit Deep BSI   ▲ Erba Deep BSI



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