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Comparison of microbial communities associated with three Atlantic ultramafic hydrothermal systems

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Abstract :

The distribution of *Archaea* and methanogenic, methanotrophic and sulfate-reducing communities in three Atlantic ultramafic-hosted hydrothermal systems (Rainbow, Ashadze, Lost City) was compared using 16S rRNA gene and functional gene (*mcrA*, *pmoA* and *dsrA*) clone libraries. The overall archaeal community was diverse and heterogeneously distributed between the hydrothermal sites and the types of samples analyzed (seawater, hydrothermal fluid, chimney and sediment). The Lost City hydrothermal field, characterized by high alkaline warm fluids (pH>11; $T<95$ °C), harbored a singular archaeal diversity mostly composed of unaffiliated *Methanosarcinales*. The archaeal communities associated with the recently discovered Ashadze 1 site, one of the deepest active hydrothermal fields known (4100 m depth), showed significant differences between the two different vents analyzed and were characterized by putative extreme halophiles. Sequences related to the rarely detected *Nanoarchaeota* phylum and *Methanopyrales* order were also retrieved from the Rainbow and Ashadze hydrothermal fluids. However, the methanogenic *Methanococcales* was the most widely distributed hyper/thermophilic archaeal group among the hot and acidic ultramafic-hosted hydrothermal system environments. Most of the lineages detected are linked to methane and hydrogen cycling, suggesting that in ultramafic-hosted hydrothermal systems, large methanogenic and methanotrophic communities could be fuelled by hydrothermal fluids highly enriched in methane and hydrogen.

Keywords : *Archaea* ; hydrothermal vent ; Mid-Atlantic Ridge ; 16S rRNA gene ; Sediment ; ultramafic

Introduction

Deep-sea hydrothermal environments are characterized by intense physico-chemical gradients providing a large range of habitats for chemolithoautotrophic microorganisms (Kelley *et al.*, 2002). Most of the studies of microbial diversity associated with deep-sea hydrothermal environments have mainly investigated basaltic-hosted hydrothermal systems (Kelley *et al.*, 2002). However, a few studies showed that ultramafic-hosted hydrothermal systems contained specific microbial communities (Brazelton *et al.*, 2006; Perner *et al.*, 2007; Voordeckers *et al.*, 2008; Flores *et al.*, 2011). To date, only three ultramafic sites were fully described on the Mid-Atlantic Ridge (Rainbow, Lost City and Logatchev), and were characterized by high concentrations of methane and hydrogen, in contrast with basaltic-hosted hydrothermal systems (Kelley *et al.*, 2001; Charlou *et al.*, 2002; Schmidt *et al.*, 2007). Moreover, Ashadze, a novel hydrothermal site, was recently reported on the Mid-Atlantic Ridge (MAR) (Bel'tenev *et al.*, 2005; Charlou *et al.*, 2007; Fouquet *et al.*, 2007; Mozgova *et al.*, 2008; Bassez *et al.*, 2009; Charlou *et al.*, 2010). This part of the MAR is characterized by rock compositions indicating that anomalously enriched mantle domains are involved in the melting region (Dosso *et al.*, 1993), and also by numerous outcrops of serpentinized mantle-derived rocks (Bougault *et al.*, 1993; Cannat *et al.*, 1997). However, these ultramafic systems expelling fluids characterized by moderate to high temperatures are also probably linked to magmatic heating processes (Allen & Seyfried, 2004). Ultramafic hydrothermal fluids are

82 highly enriched in abiogenic methane and hydrogen as a result of serpentinization reactions
83 between the ultramafic rocks and seawater (Holm & Charlou, 2001; Charlou *et al.*, 2002;
84 Allen & Seyfried, 2004), and could therefore supply twice as much chemical energy as
85 basaltic-hosted hydrothermal systems (McCollom, 2007). Hence, most of the prokaryotes
86 found at these sites seemed to be related to methane and hydrogen cycling (Boetius, 2005;
87 Perner *et al.*, 2007; Voordeckers *et al.*, 2008; Flores *et al.*, 2011).

88 A large number of microbial communities from hydrothermal environments could be fuelled
89 by inorganic compounds (Amend & Shock, 2001). Although these microbial communities
90 occupy both aerobic and anaerobic habitats, anaerobic hyper/thermophilic *Archaea* are
91 reported to be usually associated with the hottest parts of these environments (Kelley *et al.*,
92 2002; Schrenk *et al.*, 2003; Takai *et al.*, 2004a), some of which could be entrained by
93 hydrothermal fluids from subsurface ecosystems (Deming & Baross, 1993; Holden *et al.*,
94 1998; Summit & Baross, 1998). Moreover, it was suggested that *Archaea* could encompass
95 up to 33-50% of the total microbial community in deep-sea hydrothermal environments
96 (Harmsen *et al.*, 1997; Nercessian *et al.*, 2003).

97 Although an increasing number of thermophilic prokaryotes are cultivated from hydrothermal
98 environments (Huber *et al.*, 2002; Miroshnichenko & Bonch-Osmolovskaya, 2006;
99 Reysenbach *et al.*, 2006; Wagner & Wiegel, 2008; Slobodkina *et al.*, 2009), molecular
100 phylogenetic approaches have revealed several new uncultivated lineages (Takai &
101 Horikoshi, 1999; Nercessian, 2003; Kormas *et al.*, 2006; Moussard *et al.*, 2006a).

102 Metagenomic approaches and functional gene analyses have also contributed to the
103 characterization of metabolic and physiological properties of these communities (Nercessian
104 *et al.*, 2005; Moussard *et al.*, 2006b; Moussard *et al.*, 2006c). However, to our knowledge,
105 rRNA-based molecular approaches have seldom been used to compare the microbial
106 diversity from multiple different hydrothermal sites (López-García *et al.*, 2003a; López-García
107 *et al.*, 2003b; Voordeckers *et al.*, 2008; Flores *et al.*, 2011).

108 In the present study, we characterized the molecular genetic diversity, using 16S rRNA gene
109 and functional genes of methanogens, methanotrophs and sulfate-reducers, associated with

110 three ultramafic-hosted hydrothermal sites: Rainbow, Lost City and Ashadze. As these
111 hydrothermal fluids are highly enriched in methane and hydrogen, these environments could
112 harbour specific prokaryotic communities possibly associated with potential subsurface
113 chemolithoautotrophic ecosystems. Hence, the aim of this study was to compare the
114 microbial communities of these ultramafic-hosted hydrothermal sites using molecular genetic
115 methods, in order to correlate their phylogeny with ecological niches.

116

117 **Materials and methods**

118 **Site location and sampling techniques**

119 Fluid, chimney and sediment samples were collected during the scientific cruises EXOMAR
120 (2005), SERPENTINE (2007) and MoMARDREAM-Naut (2007) conducted with the R. V.
121 “*L’Atalante*” and “*Pourquoi pas ?*” and using the ROV “*Victor 6000*” and DSV “*Nautilus*”. The
122 three hydrothermal fields explored, Rainbow (36°13’N; 33°54’W; ~ 2300 m depth), Lost City
123 (30°07’N; 42°07’W; ~ 750 m depth) and Ashadze 1 (12°58’N; 44°51’W; ~ 4090 m depth)
124 were all located along the Mid-Atlantic Ridge (MAR), although Lost City and Ashadze were
125 further from the axis (Fig. 1a).

126 Fluid samples from Rainbow, Lost City and Ashadze were collected respectively from the
127 “*thermitière*” chimney (36°13’76”N; 33°54’16”W; 2294 m depth, Fig. 1e), from a flange near
128 the EXOMAR 11 Marker (30°07’43” N; 42°07’16”W; 748 m depth, Fig. 1c), and from two
129 chimneys in the SE2 area at Ashadze 1 site (12°58’33”N; 44°51’78”W; 4097 m depth, Fig.
130 1f). Chimney samples were also retrieved from the two chimneys in the SE2 area of Ashadze
131 1 site where the fluids were previously collected. The sediment samples were retrieved from
132 the Rainbow site close to the active hydrothermal area (36°13’76”N; 33°54’04”W; 2287 m
133 depth, Fig. 1d) and from the immediate periphery of the Lost City site (30°07’57” N;
134 42°07’05”W; 752 m depth, Fig. 1b).

135 In order to describe the microbial communities from the surrounding seawater, the water
136 column from the Rainbow site (36°13’76”N; 33°54’06”W; 2291 m depth) was also sampled.

137 All fluid samples were collected using titanium syringes and analyzed as described
138 elsewhere (Charlou *et al.*, 2002). On board, the fluid samples were immediately removed
139 aseptically from the titanium syringes and stored at -80°C for molecular genetic analyses.
140 On board the sediment cores (~ 20 cm in length, 5 cm diameter) collected from the Rainbow
141 site, using a push-core device operated by the arm of the DSV "*Nautile*", were sectioned in
142 three equal samples and were designated as top, middle and bottom. The sediment surface
143 sample from the Lost City site was collected using PSDE system (Fig. 1b, Kato C.,
144 unpublished). The chimney fragments were collected in biobox and sediment samples were
145 stored aseptically at -80°C for molecular genetic analyses.

146 **DNA extractions and PCR amplification**

147 To avoid contaminations, all manipulations were carried out in a PCR cabinet (BiocapTM
148 RNA/DNA, erlab[®]), using Biopur[®] 1.5 mL Safe-Lock micro test tubes (EppendorfTM),
149 Rnase/Dnase Free Water (MP BiomedicalsTM) and UV-treated (>60 min) plasticware and
150 pipettes.

151 DNA extractions from fluids were performed from 50 mL of fluid left to thaw on ice prior
152 centrifugation (15000 g for 60 min). Supernatant was carefully discarded and DNA was
153 extracted from the pellet, following a modified FastDNA[®] Spin Kit for Soil (Bio101 Systems,
154 MP BiomedicalsTM) protocol (Webster *et al.*, 2003; Roussel *et al.*, 2009a). The DNA
155 extractions from sediments and chimney fragments were also performed using the modified
156 FastDNA[®] Spin Kit for Soil as described elsewhere (Roussel *et al.*, 2009a).

157 All amplifications were performed using a "GeneAmp PCR system" 9700[®] (Applied
158 BiosystemsTM). All PCR mixtures (50 μL) contained 5 μL of DNA template, 1X Taq DNA
159 polymerase buffer (MP BiomedicalsTM), 1 μL of dNTP (10 mM of each dATP, dCTP, dGTP
160 and dTTP), 10 μM of each primer and 0.5 μL of Taq DNA polymerase (MP BiomedicalsTM).
161 Negative controls were also carried out with DNA extractions performed without any sample.
162 For all controls, no PCR products were detected. Inhibition of PCR amplification by soluble

163 contaminants in the DNA extracts was also tested as described elsewhere (Juniper *et al.*,
164 2001).

165 Archaeal 16S rRNA gene amplification was conducted by nested PCR with combination of
166 primers A8f (5'-CGG TTG ATC CTG CCG GA-3') and A1492r (5'-GGC TAC CTT GTT ACG
167 ACT T-3') in the first round (Teske *et al.*, 2002; Lepage *et al.*, 2004), and with A344f (5'-AYG
168 GGG YGC ASC AGG SG-3') and A915r (5'-GTG CTC CCC CGC CAA TTC CT-3') in the
169 second round (Stahl & Amann, 1991; Sørensen *et al.*, 2004). PCR cycles for the first round
170 (A8f/A1492r), and for the second round (A344f/A915r) were as previously described
171 (Roussel *et al.*, 2009a). To minimize PCR bias, five independent PCR products from the first
172 round were pooled and purified (QIAquick PCR purification Kit; Qiagen™) and used as
173 template for the second round. This nested PCR was necessary to obtain visible PCR
174 products on a 0.8% (w/v) agarose gel stained with ethidium bromide.

175 A portion of the *mcrA* gene was amplified using the ME primers (Hales *et al.*, 1996) with the
176 following reaction conditions as described elsewhere (Roussel *et al.*, 2009a). A fragment of
177 the *pmoA* gene was amplified using the pmoA189-mb661 primer couple (Holmes *et al.*,
178 1995; Costello & Lidstrom, 1999) with the following reaction conditions: 1 cycle of 4 min at
179 92°C, 35 cycles of 1 min at 92°C, 1.5 min at 55°C and 1 min at 72°C, and 1 cycle of 9 min at
180 72°C. A portion of the *dsrA* gene was amplified using the DSR1F+ and DSR-R primers
181 (Kondo *et al.*, 2004) with the following reaction conditions: 1 cycle of 5 min at 94°C, 35 cycles
182 of 30 s at 94°C, 30 s at 54°C and 2.5 min at 72°C, and 1 cycle of 8 min at 72°C. For all
183 functional genes, two rounds with the previous reaction conditions were required to obtain
184 visible amplification products. An aliquot (5 µL) of three pooled PCR products of the primary
185 amplification was used as template for the second amplification round.

186 **CM-DGGE analysis**

187 In order to obtain the general archaeal 16S rRNA gene diversity associated with the
188 hydrothermal environment and to compare it with the seawater diversity, a preliminary CM-
189 DGGE analysis was performed as described elsewhere (Roussel *et al.*, 2009b).

190 After amplification of the nested PCR products, using two different fluorescent reverse
191 labelled (Cy3 or Cy5) primers from total DNA from either a hydrothermal sample or seawater,
192 these were pooled and loaded into the same lane. Archaeal 16S rRNA gene amplification
193 was performed with primers Saf-PARCH 519r, labelled with either Cy3 (hydrothermal
194 samples) or Cy5 (seawater), following touchdown PCR protocol as previously described
195 (Nicol *et al.*, 2003). All manipulations were performed in the dark. The PCR products were
196 analyzed by DGGE using a DCode Universal Mutation Detection System[®] (BioRad[™]) on a 1
197 mm thick (16 × 16 cm) 8% (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 40%, 37.5:1,
198 BioRad[™]) with a denaturant gradient between 30 and 70% prepared with 1 × TAE buffer (pH
199 8, 40 mM Tris Base, 20 mM acetic acid, 1 mM EDTA, MP Biomedicals[™]) and poured with a
200 "Gradient maker" (Hoefer SG30[®]). Electrophoresis was carried in 1 × TAE buffer at 60°C for
201 330 min at 200 V (initially at 80 V for 10 min). The gel was scanned using a Phospho
202 fluorimager Typhoon 9400[®] (Amersham Biosciences[™]).

203 **Cloning and sequencing**

204 Fourteen 16S rRNA gene, one *dsrA* gene, four *mcrA* gene, and eight *pmoA* gene clone
205 libraries were constructed. To minimize PCR bias (Polz & Cavanaugh, 1998), five
206 independent PCR products were pooled, purified (QIAquick PCR purification Kit; Qiagen[™]),
207 and cloned into *Escherichia coli* (XL10-Gold; Stratagene[™]) using the pGEM-T Easy vector
208 system I (Promega[™]) following the manufacturer's instructions. Positive transformants were
209 screened by PCR amplification of the insert using the vector-specific M13 primers. Plasmid
210 extraction, purification and sequencing of the insert were carried out by the sequencing
211 Ouest-Genepole platform[®] of Roscoff Marine laboratory (France).

212 **Phylogenetic analysis and statistical analyses**

213 Chimeras (Cole *et al.*, 2003) were excluded from the clone libraries and a total of 759
214 sequences (including those from the 16S rRNA gene and functional genes) were used for
215 further phylogenetical analysis. The phylogenetic placement was carried out using NCBI

216 BLAST search program within GenBank (<http://www.ncbi.nlm.nih.gov/blast>) (Altschul *et al.*,
217 1990). The 16S rRNA gene sequences (~553 bases) were then edited in the BioEdit 7.0.5.3
218 program (Hall, 1999) and aligned using CLUSTALW (Thompson *et al.*, 1994). The
219 phylogenetic trees were constructed by the PHYLO_WIN program ([http:// pbil.univ-lyon1.fr/](http://pbil.univ-lyon1.fr/))
220 (Galtier *et al.*, 1996) with Neighbour-Joining method (Saitou & Nei, 1987) and Jukes and
221 Cantor correction. The nonchimeric *mcrA* (~0.76 kb), *pmoA* (~0.51 kb) and *dsrA* (~0.22 kb)
222 sequences were translated into amino acids using BioEdit and then aligned using
223 CLUSTALW, and the PHYLO_WIN program with Neighbour-Joining algorithm, and PAM
224 distance (Dayhoff *et al.*, 1978) was then used for phylogenetic tree construction. For the
225 entire phylogenetic reconstruction, the robustness of inferred topology was tested by
226 bootstrap resampling (1000), values over 50% are shown on the trees. The richness from the
227 clone libraries was estimated, with the rarefaction curves at 99%, 97% and 95% sequence
228 identity levels, using the DOTUR program (Schloss & Handelsman, 2005). Operational
229 taxonomic units (OTUs), using a 95% or 97% sequence similarity, were generated with the
230 SON program (Schloss & Handelsman, 2006), and the percentage of coverage (Cx) of the
231 clone libraries was calculated by Good's method (Good, 1953) as described by Singleton
232 and colleagues (Singleton *et al.*, 2001). Statistical estimators, the significance of population
233 differentiation among clone libraries (F_{ST}) (Martin, 2002), and the exact tests of population
234 genetic differentiation (Raymond & Rousset, 1995), were calculated using Arlequin 3.11
235 (Excoffier *et al.*, 2005).

236 **Nucleotide sequence accession numbers**

237 The sequences are available from GenBank database under the following accession
238 numbers and names: 16S rRNA gene (FN650174 to FN650288), *mcrA* gene (FN650315 to
239 FN650322), *dsrA* (FN650289 to FN650291) and *pmoA* (FN650292 to FN650314).

240

241 **Results**

242 **Site description**

243 A total of 15 samples encompassing fluids, chimney fragments and sediments, were
244 retrieved from three Atlantic ultramafic-hosted hydrothermal sites: Rainbow, Lost City and
245 Ashadze (Fig. 1). The dilution of the hydrothermal fluid sample was estimated according to
246 pH measurements. Overall, the three sites had much higher hydrogen (<16 mM) and
247 abiogenic methane (<2.5 mM) concentrations than the MAR basaltic-hosted hydrothermal
248 sites (Charlou *et al.*, 2010).

249 All the hydrothermal fluid samples from the Rainbow site were retrieved from the “thermitière”
250 chimney group (Fig. 1e), except the “PP27 swarm” sample which was obtained in close
251 proximity to a shrimp swarm on the side of the PP27 chimney. The “thermitière” chimney
252 group was composed of both diffuse and black smoker venting. The Rainbow sediment
253 samples were retrieved nearby the hydrothermal chimneys and were predominantly made of
254 pelagic sediment (98% calcite) with a small amount of hematite, indicating a small
255 hydrothermal contribution (Fig. 1d). For this study, the maximum temperature measured at
256 Rainbow was 324°C, and the less diluted hydrothermal fluid analyzed had a pH of 3.40 (Fig.
257 2b), and high concentrations of hydrogen (>10 mM), carbon dioxide (17 mM), iron (>17 mM)
258 and methane (>1mM) (Charlou *et al.*, 2010).

259 The Lost City fluid samples were obtained from one of the hottest venting areas of this site,
260 which was located above a flange (Fig. 1c). To date, Lost City is a unique off-axis
261 hydrothermal site expelling fluids with a high pH (~ 11), as opposed to the other known
262 ultramafic environments that are acidic (Rainbow, Ashadze pH = ~ 3). The maximum
263 temperature recorded at Lost City (93°C) was lower than for Rainbow and Ashadze. The less
264 diluted hydrothermal fluid analyzed had a pH of 11.75 (Fig. 2a), and high concentrations of
265 hydrogen (>7 mM) and methane (0.9 mM).

266 Ashadze, a hydrothermal field that was recently explored for the first time during the French-
267 Russian Serpentine cruise (Fouquet *et al.*, 2008), is one of the deepest active black smoker

268 fields discovered so far (4100 m depth). Ashadze is characterized by an ultramafic rock
269 environment (Charlou *et al.*, 2007; Fouquet *et al.*, 2007; Fouquet *et al.*, 2008). Several
270 groups of active one to two meter high chimneys were observed at Ashadze 1 site. The fluid
271 and chimney fragments were obtained from two different active chimneys in a unique group
272 near the SE-2 marker (Fig. 1f). For this study, the maximum temperature measured at
273 Ashadze was 353°C. The less diluted hydrothermal fluid analyzed had a pH of 4.02 (Fig. 2b),
274 and high concentrations of hydrogen (>10 mM), carbon dioxide (>2.5 mM), iron (7.3 mM) and
275 methane (>0.80 mM).

276 **Archaeal 16S rRNA gene analyses**

277 *CM-DGGE*. All the 16S rRNA gene PCR products from all the samples were screened by
278 Co-Migration DGGE (CM-DGGE) prior cloning, in order to estimate the archaeal
279 phylogenetic diversity of each hydrothermal sample and to compare it directly with the
280 seawater diversity (Fig. 2a). Band pattern intensities from all Lost City samples, and from the
281 less diluted hydrothermal fluids, were weaker than for all the other samples, suggesting a
282 lower biomass and/or high concentration of PCR inhibitors (Fig. 2a). The archaeal seawater
283 CM-DGGE band pattern was different from all the hydrothermal fluid and chimney band
284 patterns (Fig. 2a), suggesting low levels of seawater contamination. The band patterns from
285 hydrothermal samples were mostly composed of DGGE fragments with higher melting
286 points, a probable consequence of higher GC content of the 16S rRNA gene. The high-GC
287 content of these 16S rRNA gene sequences indicates that the *Archaea* could be
288 hyper/thermophiles (Kimura *et al.*, 2006), as also suggested by the several putative
289 hyper/thermophilic lineages detected in the clone libraries from hydrothermal fluids and
290 chimneys (*Archaeoglobales*, *Methanococcales*, *Thermococcales*, *Methanopyrales*,
291 *Desulfurococcales*, *Nanoarchaeota*, DHVE; Fig. 2b).

292 *Clone libraries*. After technical optimization and removal of soluble PCR inhibitors and in
293 order to amplify sufficient PCR product for cloning, archaeal amplifiable DNA from all
294 samples was retrieved by nested PCR. However, no sufficient amplified PCR product was

295 obtained for cloning from the less diluted fluid samples (pH 11.75) and from the chimney
296 samples from Lost City. Fourteen different 16S rRNA gene clone libraries were constructed,
297 representing a total of 610 sequences. The coverage values for the 16S rRNA gene clone
298 libraries ranged from 68 to 97%, based on a 97% sequence similarity level (Fig. 2b). On the
299 whole, rarefaction curves were asymptotic for all clone libraries, based on a 95% sequence
300 similarity level, confirming sufficient sampling effort (Fig. S1).

301 The overall archaeal diversity analyzed was similar to previous studies (e.g. Brazelton *et al.*,
302 2006; Flores *et al.*, 2011) and very heterogeneously distributed between the sites (Lost City,
303 Rainbow, and Ashadze) and between types of samples (seawater, hydrothermal fluid,
304 chimney and sediment). The number of OTUs per clone library ranged from five to nineteen,
305 based on a 95% genus level of phylotype differentiation (Schloss & Handelsman, 2004), and
306 the Shannon-Wiener index of diversity ranged between 0.63 and 3.04 (Fig. 2b). The archaeal
307 diversity indices of all the samples were in the same range, except for the fluid associated
308 with Lost City which, as previously described (Schrenk *et al.*, 2004), displayed the lowest
309 detectable diversity (Fig. 2a and 2b). On average, the hydrothermal samples contained six
310 different lineages, except for Lost City (Fig. 2a and 2b), which is also in agreement with most
311 published studies on hydrothermal environments (e.g. Takai *et al.*, 2001; Nercessian *et al.*,
312 2003; Schrenk *et al.*, 2003; Schrenk *et al.*, 2004; Takai *et al.*, 2004b; Kormas *et al.*, 2006;
313 Page *et al.*, 2008; Nunoura *et al.*, 2010). All the 16S rRNA gene sequences obtained from
314 the clone libraries were assigned to 95 OTUs, based on a 95% sequence similarity level,
315 forming a total of 21 different phylogenetic lineages (Fig. 2b, 3a and 3b). On the whole, 16S
316 rRNA gene sequences were related to *Euryarchaeota* (51%), *Crenarchaeota* (48%) and
317 *Nanoarchaeota* (1%). The 16S rRNA gene clone libraries obtained from hydrothermal
318 samples (fluid and chimney) were dominated by sequences related to *Euryarchaeota* (69%),
319 whereas sequences related to *Crenarchaeota* were a majority in the sediment (92%) and
320 seawater samples (70%) (Fig. 2b). Seven of the fifteen *Euryarchaeota* lineages detected had
321 at least one known cultured representative, and six of these seven had known thermophilic
322 *Archaea* (*Halobacteriales*, DHVE2, *Archaeoglobales*, *Methanococcales*, *Thermococcales*,

323 *Methanopyrales*). Although three of the five *Crenarchaeota* lineages detected had at least
324 one cultured representative, only one was known to be thermophilic (*Desulfurococcales*).
325 Moreover, Marine Group I (MG-I) *Archaea* had the highest intra-lineage diversity
326 representing 25 OTUs based on a 95% genus level of phylotype differentiation.

327 **Functional gene clone libraries**

328 *Diversity of mcrA gene.* The operon coding for the MCR-I, which includes McrA subunit, is
329 found in all known methanogens (Reeve *et al.*, 1997). Four *mcrA* clone libraries were
330 obtained from sediment, fluid and chimney samples from Ashadze and Rainbow sites.
331 Although detected by previous studies (Kelley *et al.*, 2005), no *mcrA* gene sequences were
332 detected from Lost City samples. The diversity of the four *mcrA* libraries was limited to
333 sequences related to the H₂/CO₂ methanogens *Methanopyrales* and *Methanococcales*
334 orders (Fig. 4a), congruently with the 16S rRNA gene clone libraries (Fig. 3a). *mcrA* gene
335 sequences affiliated to *Methanopyrales* were only detected at Rainbow. Moreover, the *mcrA*
336 gene sequences from Rainbow and Ashadze matched the two groups of uncultured
337 methanogenic *Archaea* previously retrieved from Rainbow (Nercessian *et al.*, 2005).

338 *Diversity of dsrA gene.* Sequences coding for the *dsrA* gene were only retrieved from
339 Ashadze chimney 1 (Fig. 4b). *dsrA* gene sequences were previously detected in chimney
340 samples from Lost City (Gerasimchuk *et al.*, 2010), and in sediments from Rainbow
341 (Nercessian *et al.*, 2005). All *dsrA* gene sequences detected from Ashadze site were all
342 related to sequences from marine sediments and East-Pacific Rise hydrothermal vents, as a
343 probable consequence of a lack of *dsrA* gene sequences from the MAR in the databases.
344 *dsrA* gene sequences were mainly affiliated to sequences from the *Desulfobulbaceae* family
345 (Fig. 4b).

346 *Diversity of pmoA gene.* The *pmoA* gene was the most widespread functional gene detected,
347 as a PCR amplification was obtained on eight out of the fifteen samples tested (Fig. 2b and
348 4c). The phylogeny of the *pmoA* gene is usually poorly resolved, the bacterial *pmoA* gene
349 being distantly related to the ammonia monooxygenase subunit A (*amoA*) (Holmes *et al.*,

1995; Nicol & Schleper, 2006), as revealed by incongruence between tree topologies performed with different phylogenetic methods. However, two groups of *pmoA* sequences from Rainbow fluids and Ashadze chimney samples clustered (cluster *pmoA* 1 and cluster P-A) with sequences related to thermophilic methylophils (Inagaki *et al.*, 2003; Hirayama *et al.*, 2007)(Fig. 4c). Moreover, *pmoA* gene sequences from sediments from Rainbow grouped into two major clusters (cluster *pmoA* 2 and *pmoA* 3). Sequences from cluster *pmoA* 2 did not have any closely related sequences (Fig. 4c).

Community structures and distribution analyses

Although the seawater and the Rainbow sediment CM-DGGE band patterns were quite similar (Fig. 2a), all the sediment clone library community structures were indistinguishable from the combined communities and significantly different ($P < 0.001$) from the seawater (Fig. 2b). Insignificant F_{ST} and P tests ($P < 0.001$), based on an analysis at a 97% sequence similarity level, suggested that community structures from all the Rainbow hydrothermal fluids and Ashadze chimney 1 clone libraries were similar and indistinguishable from the combined communities (Fig. 2b). However, although the archaeal community structures from all the Rainbow hydrothermal fluids were also from similar lineage distributions, all the Ashadze chimney and fluid samples had significantly different population structures ($P < 0.001$; Fig. 2a and 2b). The archaeal diversity of all the other clone libraries was also significantly different from the seawater clone library ($P < 0.001$), showing that the hydrothermal vent archaeal communities are probably adapted to their environment. According to pH measurements, the archaeal diversity in the hydrothermal fluids was always the most reduced in the less diluted fluids (Fig. 2b). Moreover, a correlation was also observed between *Methanococcales* ($P < 0.001$) and *Thermococcales* ($P < 0.05$) lineages and the Rainbow fluids. Correlations were also shown between MG-I lineage and the hydrothermal sediments ($P < 0.01$), and between the unaffiliated *Methanosarcinales* cluster and the Lost City fluids ($P < 0.0001$).

376 Discussion

377 High diversity of putative chemolithoautotrophs

378 Overall, the analysis of the phylogenetic data showed a specific distribution of different
379 putative metabolic processes over the different MAR ultramafic-hosted hydrothermal
380 environments that were mainly dominated by putative chemolithoautotrophs.

381 *Putative ammonia-oxidizing Crenarchaeota*. Marine Group I (MG-I) was the most ubiquitous
382 lineage found in the MAR ultramafic-hosted hydrothermal environments, as sequences
383 related to the MG-I *Archaea* were detected in the majority of clone libraries (93%).
384 Interestingly, the archaeal community structure of the seawater clone library was dominated
385 by sequences related to MG-I (41%) and Marine Group II (48%), but was significantly
386 different from all the other clone libraries ($P < 0.001$). Congruently, Takai and colleagues
387 showed that the highest proportion of MG-I members in a hydrothermal environment from the
388 Central Indian Ridge, was found in the seawater adjacent to the hydrothermal emissions
389 (Takai *et al.*, 2004c). MG-I sequences also dominated the sediment 16S rRNA gene clone
390 libraries ($\geq 86\%$), as commonly found in marine surface sediments (Inagaki *et al.*, 2001;
391 Teske & Sorensen, 2008; Roussel *et al.*, 2009b). The highest diversity indices were also
392 observed in these sediment samples, as a consequence of the very high intra-phylum
393 diversity observed within the MG-I. However, all the MG-I 16S rRNA gene sequences from
394 the sediment clustered in phylogenetic groups different from the seawater MG-I, suggesting
395 that specific MG-I communities could be associated with sedimentary environmental
396 conditions (Roussel *et al.*, 2009b). Moreover, the G + C content of all the MG-I sequences
397 ranged between 48% to 52%, in opposition to the high-GC content of the 16S rRNA gene
398 sequences from hydrothermal fluids or chimneys, thus supporting the hypothesis that these
399 MG-I *Archaea* are probably adapted to the cooler ecological niches of the hydrothermal
400 environments (Kimura *et al.*, 2006; Ehrhardt *et al.*, 2007). Several studies also showed that
401 specific phylogenetic groups of MG-I *Archaea* appear to be endemic to basaltic crust

402 environments (Ehrhardt *et al.*, 2007; Mason *et al.*, 2007; Mason *et al.*, 2009). Some of these
403 specific subclades of MG-I *Archaea* could therefore be adapted to various environments as
404 they were also detected in aerobic and anaerobic basalt enrichment cultures and sediment
405 slurries (Mason *et al.*, 2007; Webster *et al.*, 2010). Hence, as the MG-I *Archaea* were
406 widespread in our hydrothermal fluid and chimney clone libraries, their presence could be the
407 result of the mixing of ambient seawater with cool niche water in the rocks of the
408 hydrothermal system.

409 MG-1 *Archaea*, regularly described as aerobic autotrophic ammonia oxidizers (Francis *et al.*,
410 2005; Konneke *et al.*, 2005; Hallam *et al.*, 2006), are commonly found in seawater and
411 marine sediments, forming several phylogenetic clusters with several cultured relatives (e.g.
412 Preston *et al.*, 1996; Konneke *et al.*, 2005). Moreover, based on the analysis of the first
413 sequenced genome of a cultured relative (*Cenarchaeum symbiosum*), the MG-1 were
414 proposed as a novel archaeal phylum named *Thaumarchaeota* (Brochier-Armanet *et al.*,
415 2008). Interestingly, moderate thermophilic ammonia-oxidizing crenarchaeotes were recently
416 isolated from hot springs (de la Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008) and may also
417 play a major role in the nitrogen cycle in these environments (Zhang *et al.*, 2008; Wang *et al.*,
418 2009). High ammonium concentration and removal rates were previously measured from a
419 Pacific hydrothermal system (Lam *et al.*, 2008), and a thermophilic origin for anaerobic
420 ammonium oxidation was also suggested (Canfield *et al.*, 2006). Hence, according to their
421 widespread dissemination in hydrothermal systems, as shown in several studies, and due to
422 the high mixing processes occurring in these dynamic systems (e.g. Takai *et al.*, 2004c),
423 MG-I may also play a role in ammonium oxidation in hydrothermal systems as previously
424 suggested for marine basalts (Mason *et al.*, 2007; Mason *et al.*, 2009).

425 *Putative hydrogen-oxidizing chemolithoautotrophs.* All the ultramafic hydrothermal fluids from
426 Rainbow, Ashadze and Lost city were highly enriched in abiogenic methane and hydrogen as
427 a result of serpentinization reactions between ultramafic rocks and seawater (Holm &
428 Charlou, 2001; Charlou *et al.*, 2002; Allen & Seyfried, 2004). Moreover, McCollom showed

429 that ultramafic-hosted hydrothermal systems could theoretically provide twice as much
430 chemical energy as comparable basaltic-hosted systems (McCollom, 2007). More than half
431 of the archaeal lineages detected from Rainbow, Ashadze and Lost City were related to
432 known cultured species, two thirds of which were involved in hydrogen or methane cycling
433 processes, supporting the theory that these ecosystems could be mainly fuelled by
434 hydrothermal fluids highly enriched in hydrogen and methane.

435 Methanogenesis was the most common putative hydrogen-oxidizing metabolism detected
436 among ultramafic hydrothermal fluids and chimney samples. Indeed, among all archaeal
437 lineages in the hydrothermal samples, *Methanococcales* was the most widespread, as it was
438 detected in a majority of the clone libraries (78%) obtained from hydrothermal fluids or
439 chimney samples. Interestingly, all the sequences related to *Methanococcales* from Rainbow
440 and Ashadze grouped with sequences previously detected at Rainbow (Nercessian *et al.*,
441 2005), suggesting that these could be long-term stabilized population in these chemically
442 slow evolving environments (Charlou *et al.*, 2002). *Methanococcales* Archaea are strictly
443 anaerobic autotrophic methanogens, using hydrogen and carbon dioxide or formate as
444 energy sources (Whitman *et al.*, 2001). Strains affiliated to *Methanocaldococcus infernus*
445 were successfully cultured from hydrothermal chimney samples from Rainbow and Ashadze
446 (Jeanthon C. and L'Haridon S. personal communication, respectively). Moreover, the
447 methanogenic potential of *Methanococcales* at Rainbow and Ashadze was also confirmed by
448 the detection of *mcrA* genes related to *Methanothermococcus thermolithotrophicus* (> 97%
449 similarity) and *Methanocaldococcus infernus* (> 97%). Although hyperthermophilic or
450 thermophilic members of the *Methanococcales* are commonly cultured and detected with
451 molecular tools from marine hydrothermal vent systems (e.g. Kelley *et al.*, 2002; Nercessian
452 *et al.*, 2003; Schrenk *et al.*, 2003; Takai *et al.*, 2004a; Perner *et al.*, 2007; Page *et al.*, 2008),
453 Schrenk and colleagues reported that *Methanococcales* encompassed a low proportion
454 (<5%) of the hydrothermal prokaryotic communities associated with the walls of a sulphide
455 chimney, whereas on different hydrothermal sites Takai and colleagues reported proportions

456 up to 76.5% (Schrenk *et al.*, 2003; Takai *et al.*, 2004a). These differences could be linked to
457 environmental factors such as the high hydrogen production from these ultramafic systems,
458 which fuel these communities.

459 Moreover, putative hyperthermophilic methanogens were also represented by
460 *Methanopyrales*. *Methanopyrales* were rarely detected on the MAR by molecular methods
461 (Flores *et al.*, 2011), probably as a consequence of technical biases or of the restricted
462 number of microbial studies of the MAR, though the first isolated member originates from a
463 hydrothermal system north of Iceland (Kurr *et al.*, 1991). Sequences related to
464 *Methanopyrales* were rarely detected elsewhere (Nercessian, 2003; Takai *et al.*, 2004a;
465 Ehrhardt *et al.*, 2007; Page *et al.*, 2008). However, in this study, eighteen sequences related
466 to *Methanopyrus kandleri* (>96% similarity) were detected from Rainbow and Ashadze fluids.
467 Interestingly, Takai and colleagues also reported recently an isolate related to *Methanopyrus*
468 *kandleri* capable of methanogenesis with H₂/CO₂ under elevated hydrostatic pressures and at
469 122°C (Takai *et al.*, 2008). As *mcrA* gene sequences related to *Methanopyrus kandleri* (88%
470 similarity) were also detected at Rainbow and as strains affiliated to *Methanopyrales* were
471 successfully cultured from Rainbow and Ashadze (Jeanthon and L'Haridon personal
472 communication, respectively), the *Methanopyrales* detected were probably capable of
473 methanogenesis, thus supporting the hypothesis that these sites may harbour large
474 methanogenic communities.

475 *Archaeoglobales*, another putative hydrogen-oxidizing archaeal lineage, was also found to be
476 widespread among hydrothermal samples from Rainbow and Ashadze. 16S rRNA gene
477 sequences closely related (> 96% similarity) to members of genus *Archaeoglobus*,
478 *Geoglobus* and *Ferroglobus*, were retrieved from Rainbow and Ashadze. Interestingly, a new
479 dissimilatory Fe(III)-reducing *Archaeoglobaceae* was also isolated from Ashadze and
480 reported as growing autotrophically on hydrogen (Slobodkina *et al.*, 2009). As several
481 *Archaeoglobales* are also iron-cycling *Archaea* (e.g. Kashefi *et al.*, 2002), the high
482 concentrations of iron (> 3 mM) released by acidic ultramafic-hosted hydrothermal
483 environments could possibly fuel specific members of these *Archaeoglobaceae* communities.

484 *Putative sulphur-cycling and methane-oxidizing communities.* Members of the
485 *Archaeoglobales* lineage also belong to the hyperthermophilic sulfate-reducing *Archaea*
486 (Miroshnichenko & Bonch-Osmolovskaya, 2006). Interestingly, putative sulphur-cycling
487 *Archaea* related to *Thermococcales* and *Archaeoglobales* lineages were detected in more
488 than half of the clone libraries (56%) obtained from hydrothermal samples (fluid or chimney)
489 and were always detected together, suggesting they could require similar environmental
490 conditions. However, contrary to all Rainbow hydrothermal fluid samples, *Thermococcales*
491 and *Archaeoglobales* at Ashadze were only detected from the Ashadze chimney 1 samples,
492 suggesting that all hydrothermal vents from Ashadze site did not share optimal conditions for
493 putative sulphur-cycling microorganisms. Interestingly, the first obligate piezophilic
494 hyperthermophilic microorganism, *Pyrococcus* CH1, was also recently isolated from the
495 Ashadze chimney 1 (Zeng *et al.*, 2009). Members of the *Thermococcales* order are mainly
496 characterized as thermophilic to hyperthermophilic anaerobic heterotrophs that ferment
497 peptides and sugars, and their growth can also be stimulated by sulphur reduction
498 (Miroshnichenko & Bonch-Osmolovskaya, 2006; Zeng *et al.*, 2009). However, some
499 members of the *Thermococcales* were also able to grow on acetate-utilising Fe(III) (Summit
500 & Baross, 2001) or capable of lithotrophic growth on carbon monoxide coupled with
501 hydrogen production (Sokolova *et al.*, 2004), thus matching the environmental conditions of
502 ultramafic-hosted hydrothermal systems. It has also been suggested that *Thermococcales*
503 and hyper/thermophilic members of the *Methanococcales* order could inhabit sub-seafloor
504 ecosystems (Summit & Baross, 1998; Summit & Baross, 2001; Kelley *et al.*, 2002; Takai *et*
505 *al.*, 2004a), and could be part of a hydrogen-driven subsurface lithoautotrophic microbial
506 ecosystem (Nealson *et al.*, 2005).

507 Within methane cycling communities associated with Rainbow, putative methanotrophic
508 ANME-2 sequences were detected, suggesting occurrence of anaerobic methane oxidation
509 communities associated with anoxic habitats below 90°C (Kallmeyer & Boetius, 2004).
510 Interestingly, *dsrA* gene sequences detected at Ashadze clustered with sequences

511 previously detected in methane-rich hydrothermal systems and related to the
512 *Desulfobulbaceae* family (Teske *et al.*, 2002; Nercessian *et al.*, 2005), indicating that these
513 putative sulfate-reducing bacteria could be linked to these specific environmental conditions.
514 Besides, some members of *Desulfobulbaceae* can live syntrophically with ANME-3 members
515 (Niemann *et al.*, 2006). However, although 16S rRNA gene sequences related to ANME-2
516 *Archaea* were detected, no ANME-3 *Archaea* were found. Nevertheless, the most
517 widespread functional gene (*pmoA*) detected in ultramafic-hosted hydrothermal
518 environments remained related to methanotrophic communities as a probable consequence
519 of the high methane concentration prevailing in these ultramafic-hosted hydrothermal
520 systems. Methanotrophic bacteria were also previously detected in *Bathymodiolus* species
521 and among the gill chamber of *Rimicaris exoculata* at the Rainbow hydrothermal field
522 (Duperron *et al.*, 2006; Zbinden *et al.*, 2008), suggesting that these symbionts could also be
523 present in seawater. However, no known sequences related to symbionts were detected in
524 the seawater, as a possible consequence of low cell concentrations or of a technical bias.
525 Moreover, the phylogenetic distribution of the *pmoA* gene was related to the habitat,
526 suggesting that different methanotrophic communities were specifically adapted to different
527 ecological niches (e.g. sediments and fluid/seawater mixing zones).

528 **Specific distribution and ecological niches**

529 The different environmental conditions (temperature, pH, hydrostatic pressure, metabolic
530 substrates) at the different MAR ultramafic-hosted hydrothermal sites generate diverse
531 microbial ecological niches (hydrothermal fluid, chimney, sediment, and seawater) that seem
532 to strongly select for specific communities.

533 *Site specific phylotypes.* Although molecular techniques (PCR and cloning) used to build
534 clone libraries are known to be inherently biased (Suzuki & Giovannoni, 1996; von
535 Wintzingerode *et al.*, 1997; Polz & Cavanaugh, 1998; Nocker *et al.*, 2007), we assumed that
536 the biases were equal for all samples as they were analyzed under the same strict conditions
537 (storage, DNA extraction, PCR amplification, cloning, sequencing) (von Wintzingerode *et al.*,

538 1997). However, comparisons of population structures from other studies using different
539 experimental conditions remain unreliable. For example, archaeal diversity from Rainbow
540 chimneys as described by Flores and colleagues (2011) was much higher than that
541 described by Voordeckers and colleagues (2008), suggesting either spatial and temporal
542 heterogeneity or a technical bias. Interestingly, archaeal diversity observed from the Rainbow
543 fluids in the present study was similar to that from the chimneys analysed using
544 pyrosequencing reported by Flores and colleagues (2011). The present study also shows
545 that some communities seemed to be site-specific and specifically adapted to different
546 ecological niches (e.g. sediments and fluid/seawater mixing zones).

547 Sequences related to *Nanoarchaeota*, for example, were only detected in the Rainbow
548 hydrothermal system, showing that the nanoarchaeal habitat extends to at least one of the
549 deep hot marine hydrothermal systems of the MAR. The recently discovered novel
550 *Nanoarchaeota* phylum has shown a wide distribution in high temperature ecosystems (Hohn
551 *et al.*, 2002; Huber *et al.*, 2002), and may represent pioneering communities in deep-sea
552 hydrothermal vents (McCliment *et al.*, 2006). *Nanoarchaeota* could also represent a fast-
553 evolving euryarchaeal lineage related to *Thermococcales* (Brochier *et al.*, 2005). Moreover,
554 the nano-sized *Nanoarchaeota* were previously described to have a symbiotic relationship
555 with *Ignicoccus hospitalis*, a member of the *Desulfurococcales* order isolated from the
556 Kolbeinsey Ridge, north of Iceland (Paper *et al.*, 2007). Interestingly, 16S rRNA gene
557 sequences with 94% similarity to the hyperthermophilic chemolithoautotrophic sulphur and
558 hydrogen-utilizing *Ignicoccus hospitalis*, were also retrieved exclusively from the same
559 Rainbow hydrothermal fluids, suggesting that a similar symbiotic relationship could also
560 occur between the *Nanoarchaeota* and specific *Desulfurococcales* from Rainbow.
561 Differences between the composition of archaeal communities associated with the two
562 hydrothermal chimneys from Ashadze could be probably linked to environmental factors as
563 the Ashadze chimney 3 has a higher copper concentration than chimney 1 (Charlou JL.,
564 Donval JP. and Konn C., unpublished). Moreover, 16S rRNA gene sequences related to

565 *Halobacteriales* were only detected from Ashadze, which is to date the deepest known
566 hydrothermal site. The highest similarity with a cultured relative was *Natronomonas*
567 *pharaonis* (98%), an extremely halo-alkaliphilic archaeon. The occurrence of halotolerant
568 prokaryotes in hydrothermal environments, growing at higher NaCl concentrations than most
569 marine microorganisms, was previously reported (Takai *et al.*, 2001). Due to phase
570 separation it is admitted that venting of a condensed vapor phase with low salinity will
571 generate a high salinity phase at depth. This phase may be venting later or be trapped in the
572 subsurface environments. In addition some authors have suggested a double diffusive
573 hydrothermal system where brines are trapped in the deepest part of the system and
574 exchange only heat with the upper convective system (Bischoff & Rosenbauer, 1989;
575 Fouquet *et al.*, 1993). If partially cooled, this deep high salinity reservoir may constitute an
576 extensive location for halotolerant prokaryotes. Hence, it was also suggested that these
577 communities could be associated with a sub-vent ecosystem, as well as with hydrothermal
578 chimneys (Kaye & Baross, 2000; Takai *et al.*, 2001).

579 As previously described, the off-axis Lost City hydrothermal system is remarkable by its
580 geological, geochemical and biological settings (Kelley *et al.*, 2005). The archaeal diversity
581 associated with hot and very alkaline Lost City hydrothermal fluid was limited to unaffiliated
582 *Methanosarcinales* and to MG-I sequences. The detected unaffiliated *Methanosarcinales*
583 sequences matched the Lost City *Methanosarcinales* cluster (99% similarity) described by
584 Schrenk and colleagues (Schrenk *et al.*, 2004), suggesting that these *Archaea* were involved
585 in methane cycling processes (Schrenk *et al.*, 2004; Boetius, 2005). However, no *mcrA* gene
586 sequences were detected at Lost City, as a likely consequence of low cell densities in the
587 Lost City fluids (Brazelton *et al.*, 2006). Members of the Lost City uncultured
588 *Methanosarcinales* cluster are probably endemic communities associated to cooler (<95°C)
589 and very alkaline habitats as they were not detected from any other hydrothermal sites. The
590 occurrence of molecular genetic evidences in hot and very alkaline fluids also suggests that
591 the Lost City *Methanosarcinales* have physiological potentials beyond the capacities of any
592 known cultured isolates (Mesbah & Wiegel, 2008).

593 *Specific ecological niches.* To summarize, besides some specific *Archaea* that seemed
594 endemic to some hydrothermal sites, the distribution of archaeal phylotypes and putative
595 metabolic processes was linked to different microbial niches (seawater, sediments,
596 macrofaunal communities, hydrothermal chimneys and fluids, Fig. 5). The cold and
597 oxygenated seawater (< 10°C) overlaying the hydrothermal systems probably represented
598 one of the largest microbial niches, and was characterized only by marine group lineages,
599 some of which could be aerobic ammonia oxidizers. These psychrophilic seawater
600 communities surrounding hydrothermal vents are most likely to benefit from high ammonium
601 inputs from the chemolithotrophic primary producer associated with the hydrothermal
602 structures. Another large ecological niche is probably the cold (< 10°C) and porous
603 sediments surrounding the hydrothermal systems, which may represent a stable environment
604 and suitable substrate for selection of specific seawater phylotypes (MG-I) and for
605 colonization by specific psychrophilic unaffiliated *Euryarchaeota* and methylotrophic bacteria.
606 The specific sedimentary microbial communities could be fuelled by the products from
607 organic matter degradation, but also by methane seepage from these ultramafic systems. In
608 contrast, the warm (~15°C) and relatively unstable mixing zones colonized by macrofaunal
609 communities were probably the most metabolically active microbial niche benefiting from
610 oxidized seawater compounds and from reduced compounds from the hydrothermal system.
611 Mixing zones between the adjacent ecological niches also occur as a result of steep physico-
612 chemical gradients characterizing these dynamic hydrothermal environments, therefore
613 resulting in exchanging microbial communities. Mesophilic to thermophilic methane-oxidizing
614 bacteria could dominate the moderate oxic habitats in the mixing environment as revealed by
615 the *pmoA* gene analyses. The detection of ANME-2 members suggests that moderate
616 thermophilic (<90°C) anaerobic methanotrophs could occur in probably restricted anoxic
617 habitats, as a consequence of the very steep oxygen and temperature gradients.
618 Methanotrophic archaeal communities fuelled by hydrogen and carbon dioxide could
619 probably dominate the more chemically reduced zones of this niche, which is closer to the
620 hydrothermal chimney. The thermophilic communities composed of *Methanococcales*,

621 *Methanopyrales*, *Thermococcales*, *Archaeoglobales*, and *Desulfurococcales* were in all
622 likelihood harboured by the hydrothermal chimneys and could mainly be composed of
623 hydrogen-oxidizing members. Although hydrothermal fluids from ultramafic systems such as
624 Rainbow do not have significant levels of hydrocarbons from biogenic origin,
625 methanogenesis could still be the dominant archaeal metabolic process, as the high
626 abiogenic methane concentration may mask the biogenic methane. Moreover, the
627 hyper/thermophilic methanogen *Methanococcales* order and the *Thermococcales* order could
628 be typical members of the hot anaerobic microbial ecosystem that could extend below the
629 Rainbow hydrothermal system seafloor.

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983

984 Captions

985 **Fig. 1.** (a) Location map of Atlantic ultramafic-hosted hydrothermal sites mentioned in this

986 study. (b) Photograph of the sediment sampling at Lost City hydrothermal field. (c)

987 Photograph of the fluid sampling using titanium syringe at Lost City hydrothermal field. (d).

988 Photograph of the sediment sampling using push-core devices at Rainbow hydrothermal

989 field. (e). Photograph of the fluid sampling using titanium syringe at Rainbow hydrothermal

990 field. (f). Photograph of temperature measurements at Ashadze 1 hydrothermal field.

991 **Fig. 2.** (a) Co-migration denaturant gradient gel electrophoresis (CM-DGGE) analysis of

992 archaeal 16S rRNA genes from seawater (blue) compared to Rainbow, Ashadze and Lost

993 City hydrothermal environments (red). The white arrows indicate the position of faint DGGE

994 bands. PCR products were amplified with the Saf-PARCH 519r*Cy5 (blue) or Saf-PARCH

995 519r*Cy3 (red) primer set and electrophoresis was performed using a gradient of 30–70%

996 denaturant. (b) Distribution of the archaeal phylogenetic communities based on 16S rRNA

997 gene from three ultramafic-hosted hydrothermal sites. The phylogenetic affiliation of each

998 clone sequence was determined by similarity analysis. For each phylogenetic affiliation, the

999 average G + C content of the detected 16S rRNA gene sequences is shown in brackets. The

1000 relative abundance of each phylotype was calculated and represented in a column diagram.

1001 Cx indicates coverage percentage for each clone library. OTU indicates the number of

1002 operational taxonomic units (95%) for each clone library. SW indicates the Shannon-Wiener

1003 index of diversity. *dsr*, *pmo* and *mcr*, respectively indicate positive amplification of the

1004 functional genes. ND: not determined. The asterisks indicate groups of clone libraries with

1005 insignificant ($P < 0.001$) differences between all the diversity indices (F_{ST} and the exact test

1006 method). ANME-2: *anaerobic methane oxidizers*, DHVE2: Deep-sea Hydrothermal Vent
 1007 *Euryarchaeota*, MBG-D: Marine Benthic Group D, MBG-A: Marine Benthic Group A, MG-1 (II,
 1008 III, IV): Marine Group 1 (II, III, IV), MBG-E: Marine Benthic Group E, UHE-1: Unaffiliated
 1009 Hydrothermal *Euryarchaeota*.

1010 **Fig. 3.** (a) Phylogenetic tree representing the *Euryarchaeota* 16S rRNA gene sequences.
 1011 Each phylotype is represented by one sequence with $\geq 97\%$ similarity grouping. The tree was
 1012 constructed using the Neighbor-Joining method with Jukes and Cantor correction. Bootstrap
 1013 values $< 50\%$ are not shown. Circles symbolize Ashadze clone libraries. Triangles symbolize
 1014 Rainbow clone libraries. Squares symbolize Lost City clone libraries. Underlined sequences:
 1015 seawater clone library. ANME: *anaerobic methane oxidizers*, DHVE: Deep-sea Hydrothermal
 1016 Vent *Euryarchaeota*, MBG-D: Marine Benthic Group D, MBG-E: Marine Benthic Group E,
 1017 SAGMEG: South African Gold Mine Euryarchaeotic Group, UHE-1: Unaffiliated Hydrothermal
 1018 *Euryarchaeota*. (b) Phylogenetic tree representing the *Crenarchaeota* 16S rRNA gene
 1019 sequences. Each phylotype is represented by one sequence with $\geq 97\%$ similarity grouping.
 1020 The tree was constructed using the Neighbor-Joining method with Jukes and Cantor
 1021 correction. Bootstrap values $< 50\%$ are not shown. Circles symbolize Ashadze clone libraries.
 1022 Triangles symbolize Rainbow clone libraries. Squares symbolize Lost City clone libraries.
 1023 Underlined sequences: seawater clone library. MCG: Miscellaneous Crenarchaeotal Group,
 1024 MBG-B: Marine Benthic Group B, MBG-A: Marine Benthic Group A.

1025 **Fig. 4.** Phylogenetic trees based on translated partial amino acid sequences of functional
 1026 genes (*mcrA*, *dsrA*, *pmoA*). The trees were constructed using the Neighbor-Joining method
 1027 using PAM distance (Dayhoff *et al.*, 1978). Bootstrap values $< 50\%$ are not shown. Circles
 1028 symbolize Ashadze clone libraries. Triangles symbolize Rainbow clone libraries. Squares
 1029 symbolize Lost City clone libraries. (a) *mcrA* gene. (b) *dsrA* gene. (c) *pmoA* gene.

1030 **Fig. 5.** Hypothetical model (not to scale) of microbial ecological niches in acidic Atlantic
 1031 ultramafic-hosted hydrothermal systems (Rainbow, Ashadze). Each ecological niche was
 1032 described by its average temperature, potential electron donors and acceptors metabolized

1033 by the microbial communities described in this study, and the distribution of these microbial
1034 communities. OM: Organic matter. *Lineage only detected from Ashadze.

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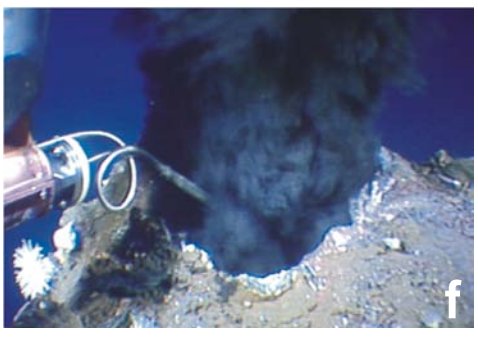
1037 **Supplementary material**

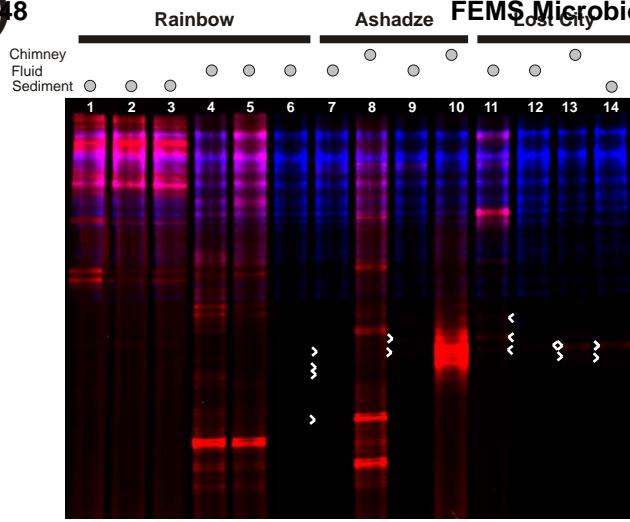
1038 **Fig. S1.** Rarefaction curves for the 16S rRNA gene clone libraries from the Fairway and New
1039 Caledonia Basin sites (Schloss and Handelsman, 2005). The sequence identity levels are
1040 represented in brackets.

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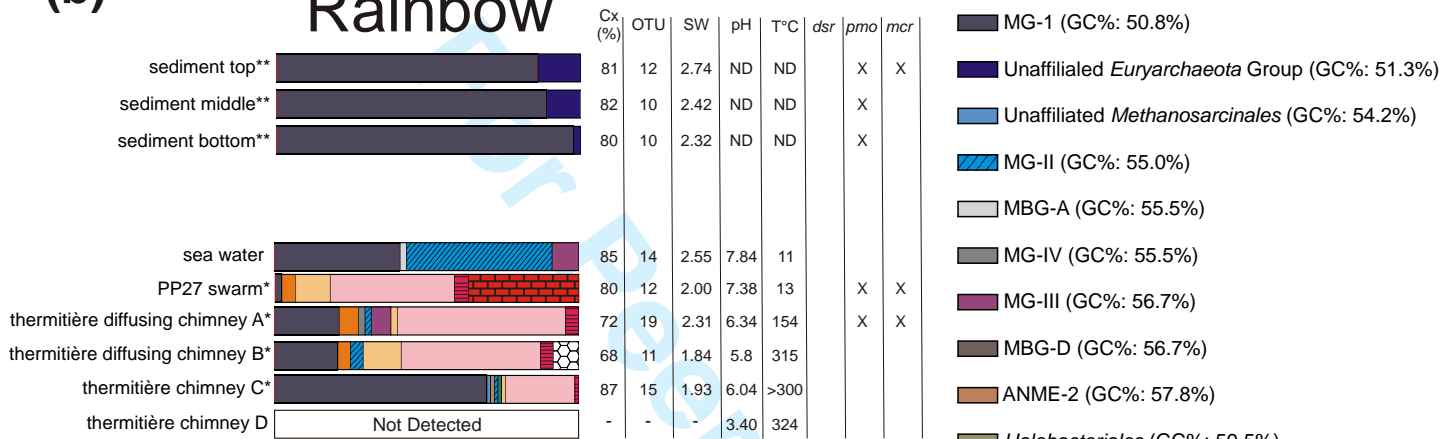




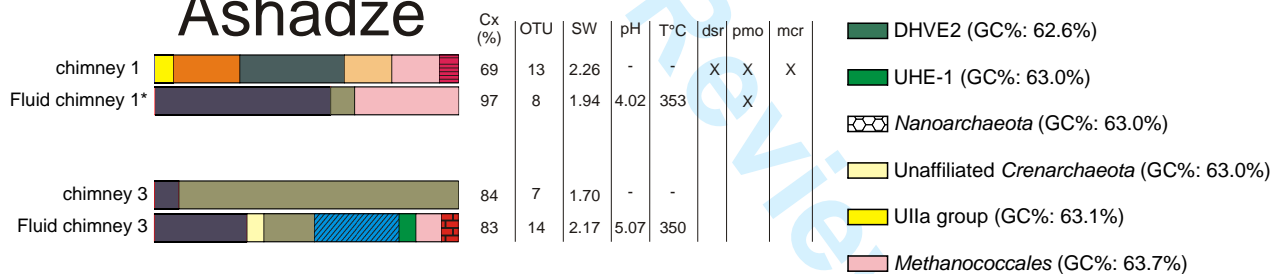
- 1 Rainbow, sediment top + Seawater
- 2 Rainbow, sediment middle + Seawater
- 3 Rainbow, sediment bottom + Seawater
- 4 Rainbow, PP27 swarm + Seawater
- 5 Rainbow, thermitière diffusing chimney A + Seawater
- 6 Rainbow, thermitière diffusing chimney B + Seawater
- 7 Ashadze, Fluid chimney 1 + Seawater
- 8 Ashadze, chimney 1 + Seawater
- 9 Ashadze, Fluid chimney 3 + Seawater
- 10 Ashadze, chimney 3 + Seawater
- 11 Lost City, Fluid (pH 10.66) + Seawater
- 12 Lost City, Fluid (pH 11.75) + Seawater
- 13 Lost City, chimney + Seawater
- 14 Lost City, sediment + Seawater

(b)

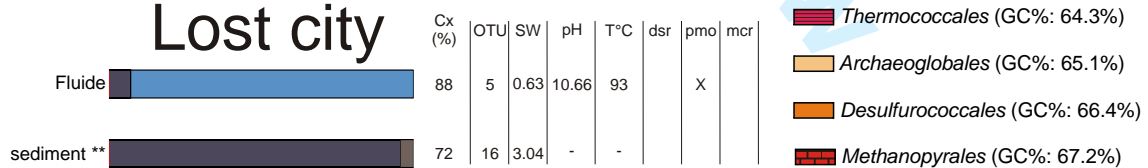
Rainbow

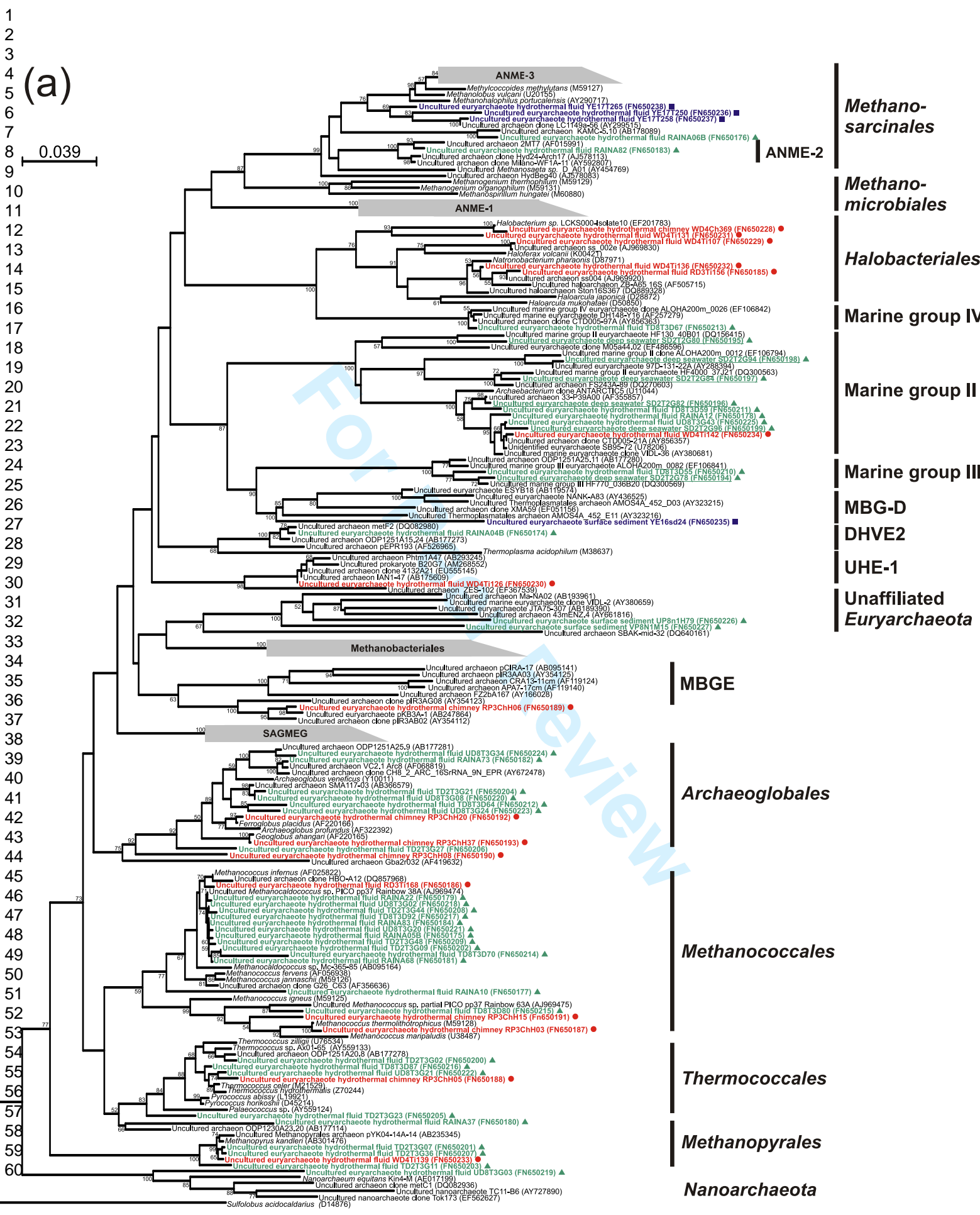


Ashadze



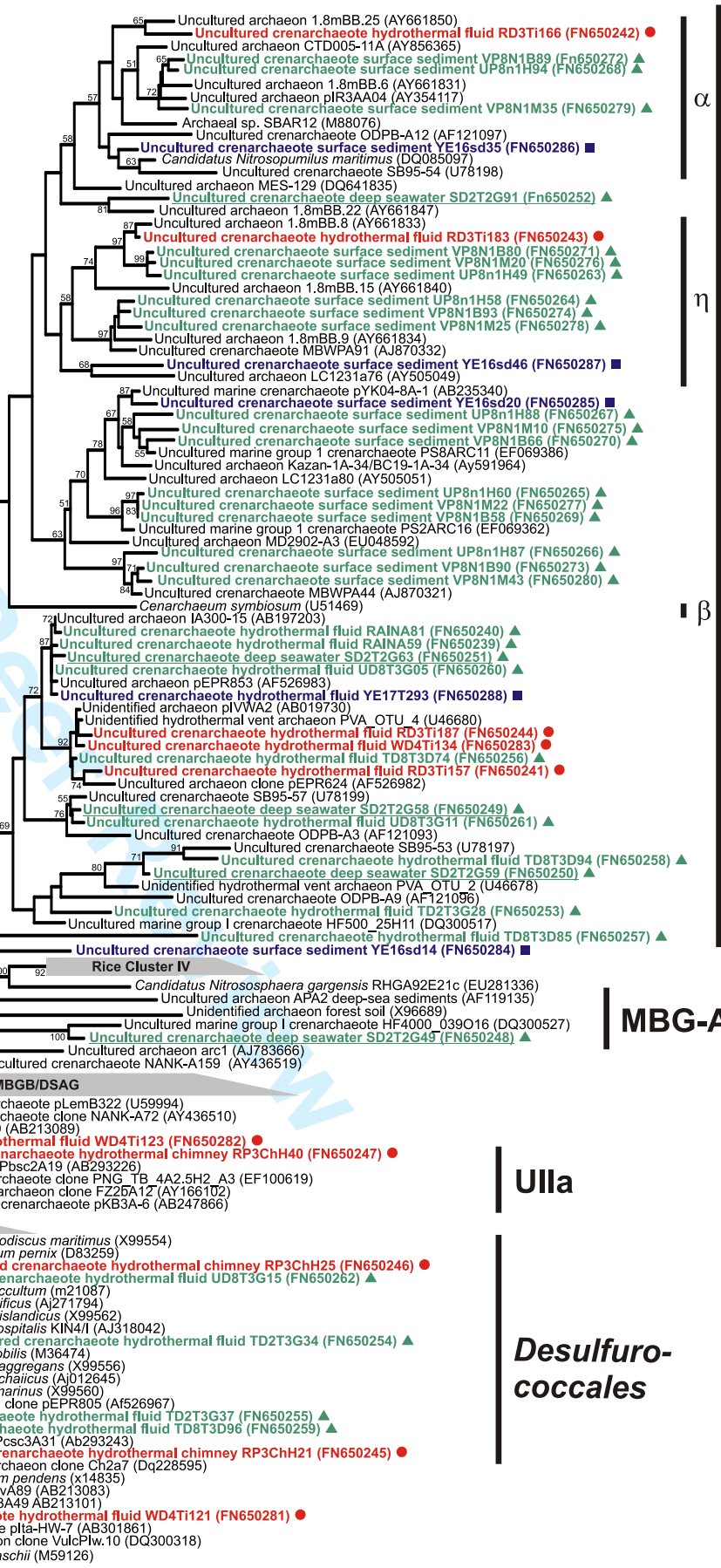
Lost city





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(b)



Marine Group 1

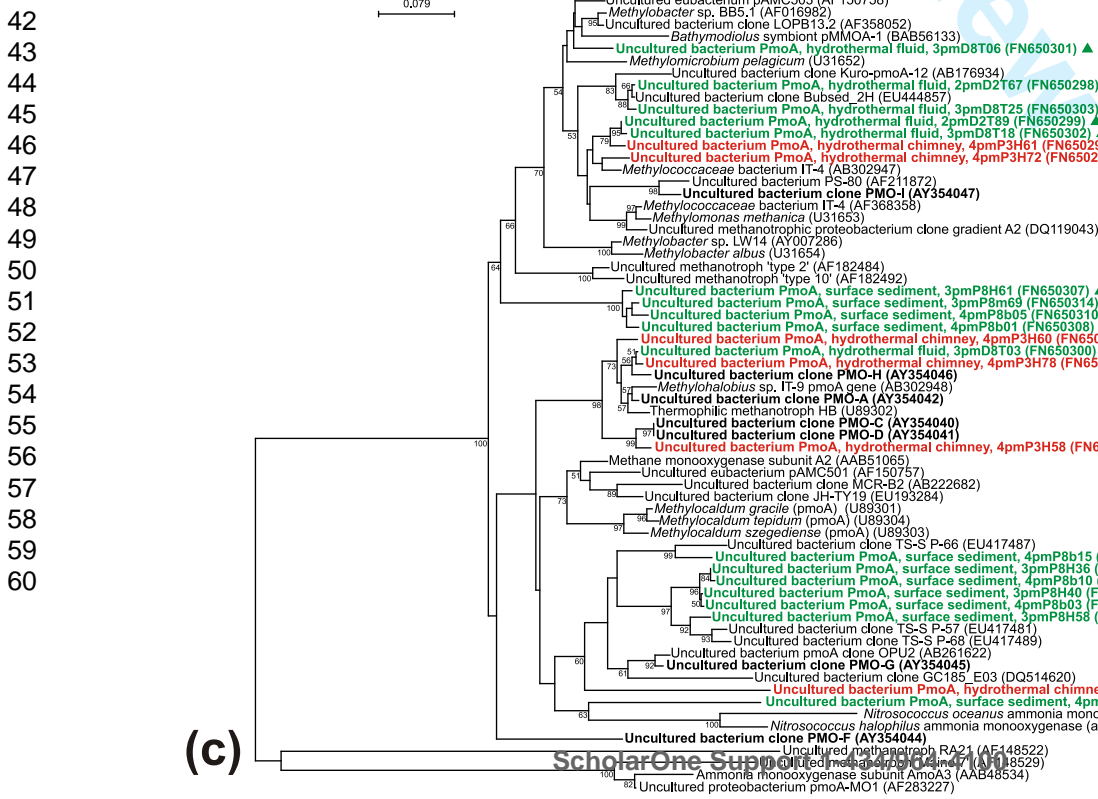
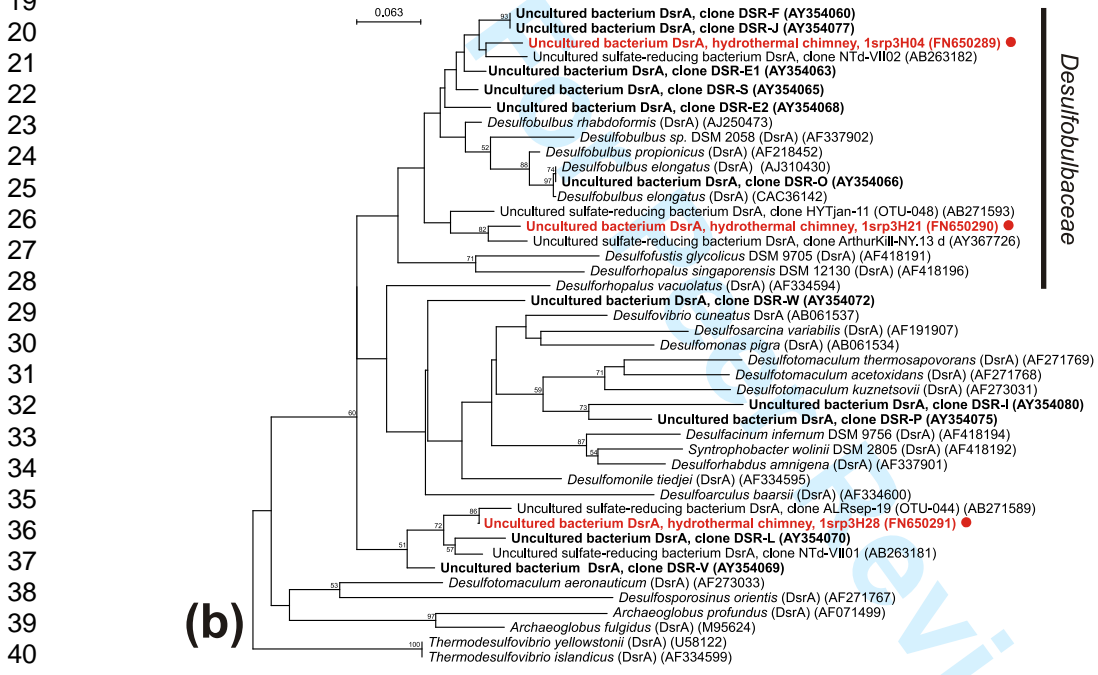
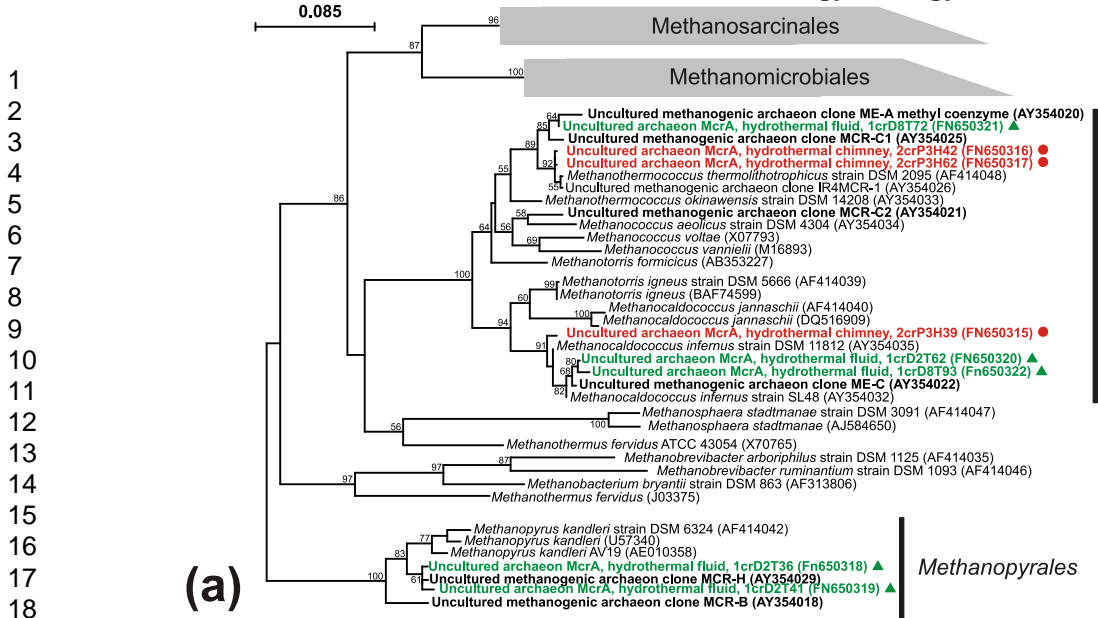
α

β

MBG-A

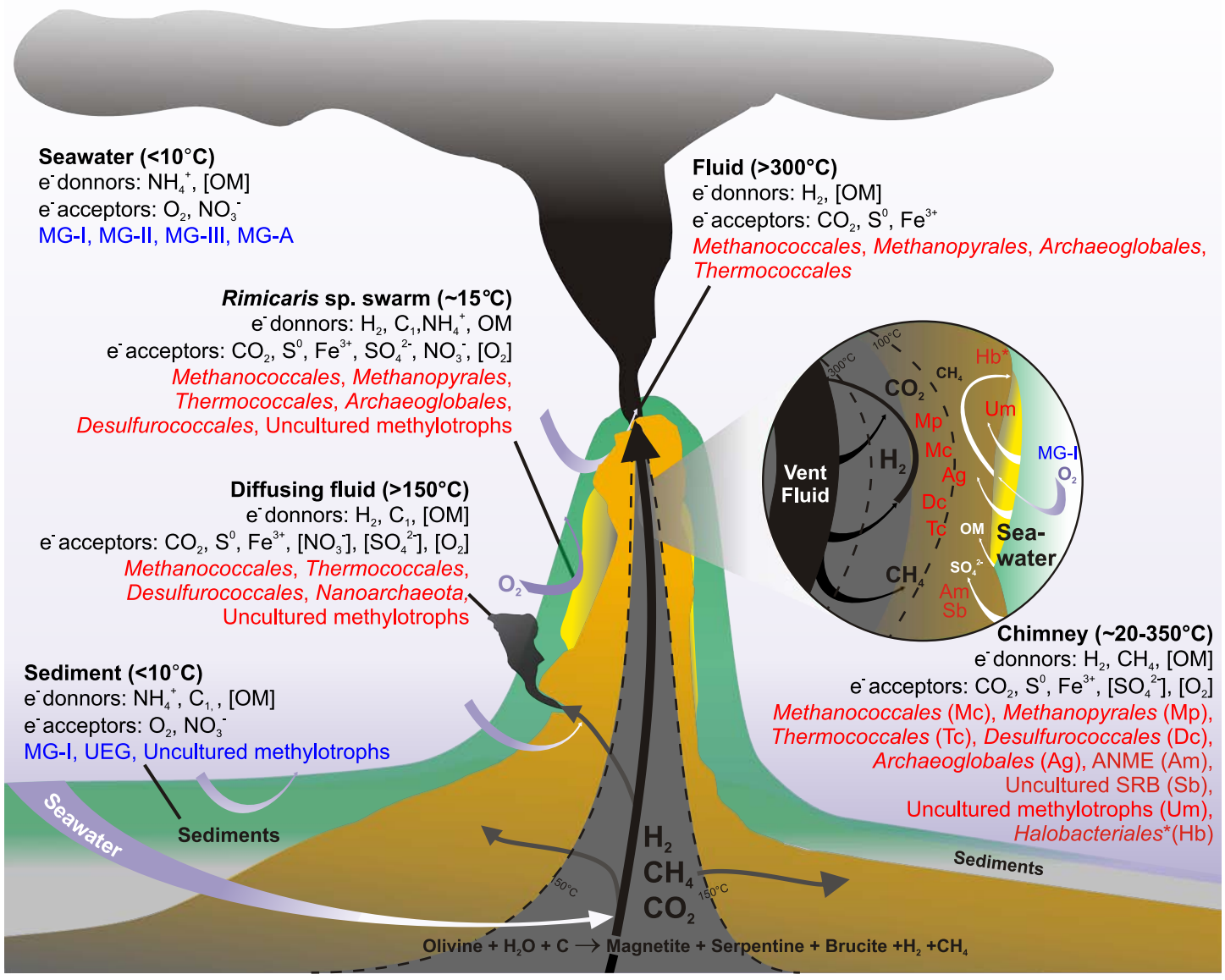
Ulla

Desulfurococcales



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- Macrofaunal communities**
- Anaerobic Hydrogen-oxidizing communities**
- Aerobic methane-oxidizing communities**

- Psychrophilic communities**
- Mesophilic to hyperthermophilic communities**
- [Variable concentration]**

Supplementary material

Rainbow

Ashadze

Lost City

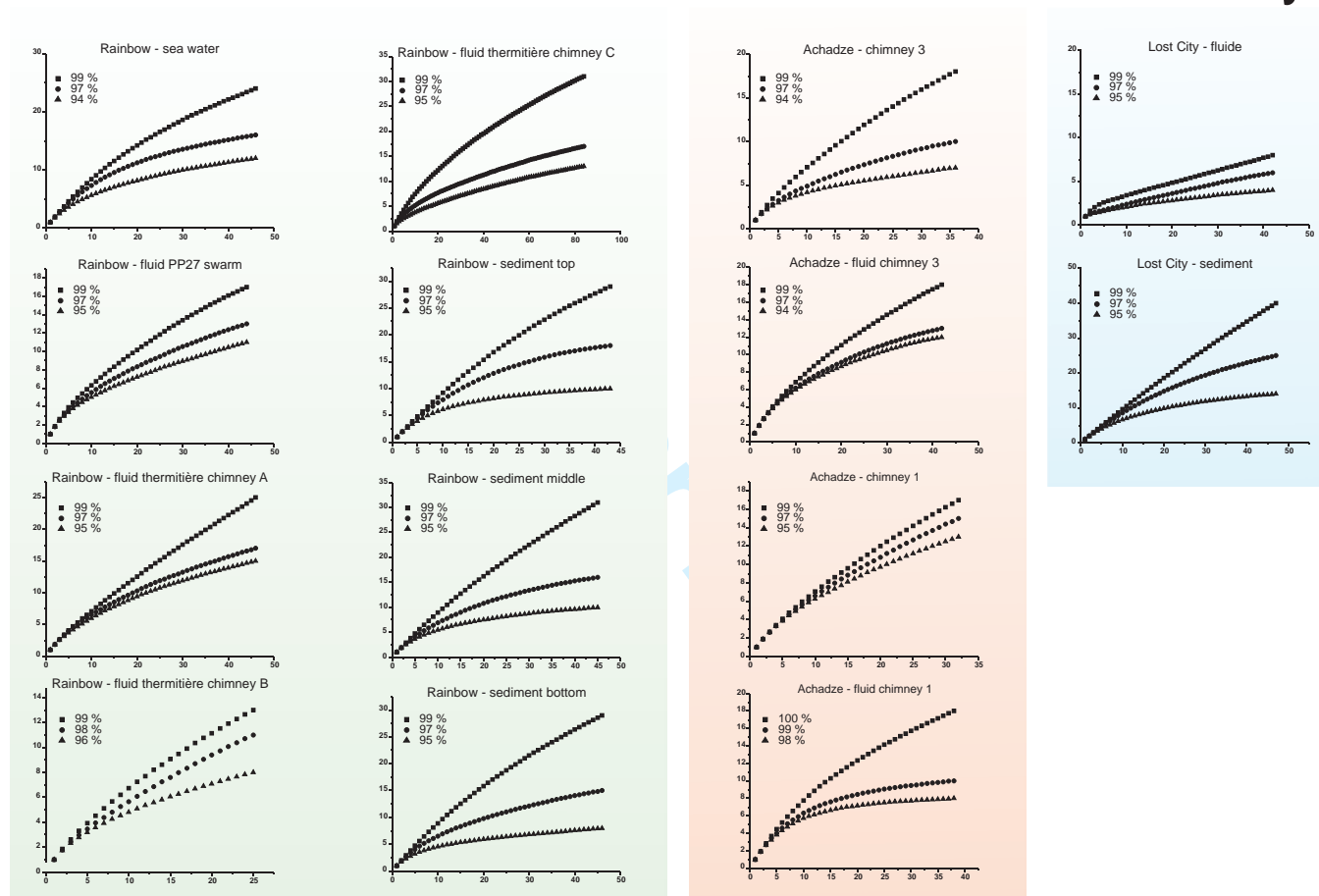


Fig. S1. Rarefaction curves for the 16S rRNA gene clone libraries from the Fairway and New Caledonia Basin sites (Schloss and Handelsman, 2005). The sequence identity levels are represented in brackets.