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Consortia cultivation of the *Desulfobacterota* from macrophyte periphyton: tool for increasing the cultivation of microorganisms involved in mercury methylation

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32 **Abstract** (entre 150 et 250 mots)

33

34 Invasive macrophytes are a persistent environmental problem in aquatic ecosystems. They also cause
35 potential health issues, since periphyton colonizing their aquatic roots are hot spot of mercury
36 methylation. Because periphytons are at the base of the trophic chain, the produced methylmercury
37 is bioamplified through the food webs. In this work, a consortia cultivation approach was applied in
38 order to investigate methylators in the periphyton of *Ludwigia sp.*, an invasive macrophyte. Five
39 growth conditions were used in order to favor the growth of different sulfate reducers, the major
40 mercury methylators in this periphyton. A total of 33 consortia containing putative Hg methylators
41 were obtained. Based on the amino acid sequences of HgcA (essential enzyme for Hg methylation), the
42 obtained consortia could be subdivided into five main clusters, affiliated with *Desulfovibrionaceae*,
43 *Desulfobulbaceae* and *Syntrophobacteraceae*. The main cluster, related to *Desulfovibrionaceae*,
44 showed the highest sequence diversity; notwithstanding most of the sequences of this cluster showed
45 no close representatives. Through the consortia approach, species thus far uncultivated were
46 cultivated. The successful cultivation of these species was probably possible through the metabolites
47 produced by other members of the consortium. The analysis of the microbial composition of the
48 consortia uncover certain microbial interactions that may exist within this complex environment.

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59 **Keywords:** Periphyton; Consortia cultivation; Microbial diversity; *Desulfobacterota*; *hgcA*
60 gene

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64 **Introduction**

65 Mercury (Hg) can be found under different speciation forms in the environment (Streets
66 et al. 2011). The methylmercury (MeHg) is one form of Hg (Bastos et al. 2006) known for its
67 bio-accumulation and bio-amplification through the trophic chain (Lebel et al. 1997; Morel et
68 al. 1998). The consumption of MeHg-contaminated fishes (Bastos et al. 2006) is threatening to
69 human, since the consumption of high concentrations of MeHg causes several neurological
70 diseases (Risher et al. 2002; Díez 2009). Hg methylation is mainly mediated by anaerobic
71 microorganisms. In aquatic environments, the prevailing Hg methylators are the sulfate-
72 reducing bacteria (SRB) affiliated with the *Desulfobacterota* (Compeau and Bartha 1984, 1985;
73 Gilmour et al. 1992). Other *Desulfobacterota* participate to the Hg methylation process, such
74 as iron-reducing bacteria (Fleming et al. 2006; Kerin et al. 2006; Si et al. 2015) and syntrophs
75 (Gilmour et al. 2013; Bae et al 2014), as well as other prokaryotes, such as methanogenic
76 Archaea (Wood 1968; Hamelin et al. 2011). Since the discovery of the genetic determinisms
77 for Hg methylation (*hgcAB* genes) (Parks et al. 2013), other microorganisms were identified
78 through metagenomic data analysis as potential Hg methylators (Gilmour et al. 2013; Jones et
79 al. 2019; Capo et al. 2020; Gionfriddo et al. 2020; Azaroff et al. 2020; Vigneron et al. 2021).

80 Although MeHg production is performed by anaerobic microorganisms, Hg methylation
81 in oxic environments was observed (Achà et al. 2005; Gentès et al. 2013, 2017; Bouchet et al.
82 2018; Gallorini and Loizeau 2021; Wang et al. 2022), including in periphyton of aquatic
83 macrophytes. Periphytons are communities of microorganisms (microalgae, fungi,
84 macroinvertebrate and prokaryotes) attached to dead or living substrates in the aquatic
85 environments (Costerton et al. 1995). These biofilms are embedded in a matrix of secreted
86 extracellular polymeric substances (EPS) (Costerton et al. 1995) that allow the adaptation of
87 the microbial community to environmental changes (Davey and O'toole 2000). The aquatic
88 plant periphyton is a biofilm that grows in oxic environments (i.e., the water) on living surfaces
89 (i.e., the plants). The periphyton contains anaerobic microorganisms due to the anoxic micro-
90 niches existing within this biofilm that are the consequence of an oxygen gradient (Achà et al.
91 2005; Desrosiers et al. 2006) or transient anoxic conditions (Costerton et al. 1995). There is a
92 correlation between the plant architecture and the development of the microbial community
93 within the periphyton. Indeed, microbial communities are dependent on the plant architecture;
94 the more complex the plant structure is, the more complex the microbial community will
95 become (dos Santos et al. 2013).

96 The periphyton growing on the roots of aquatic macrophytes is known to be a Hg
97 methylation hot spot (Guimarães et al. 1998, 2000; Cleckner et al. 1999; Liu et al. 2009;
98 Alanoca et al. 2016; Gentès et al. 2017; Bouchet et al. 2018). Since it is at the base of the food
99 webs, the periphyton is an important way of introducing MeHg in the trophic chain (Cremona
100 et al. 2009; Molina et al. 2010). First studies on Hg methylation in periphyton were mostly
101 conducted through chemical analysis (Coelho-Souza et al. 2011) by using environmental tracers
102 to detect the activity of Hg methylation. Few recent data are available about microbial diversity
103 (based on 16S rDNA diversity) and activity of aquatic plant's periphyton. These data suggest
104 the importance of sulfate-reducing microorganisms in the Hg methylation in this environment
105 (Gentès et al. 2013, 2017; Bouchet et al. 2018). On the other hand, studies on the isolation of
106 pure strains from Hg methylator periphytons are very scarce. Those studies lead to the isolation
107 of representatives of the genus *Desulfovibrio*, an easy cultivated group of SRB (Gentès et al.
108 2017, 2020). Like many other studies related to Hg methylation, isolations from environmental
109 samples mostly lead to the isolation of a wide variety of representatives of *Desulfovibrio*
110 species, and explains the abundance of *Desulfovibrio*-related HgcA sequences in the databases
111 (Gionfriddo et al. 2021).

112 In Sanguinet Lake, a temperate freshwater lake in the southwest of France, high levels of
113 MeHg were found in carnivorous fishes. This lake is covered partially by the invasive
114 macrophyte *Ludwigia sp.* The periphyton growing on this macrophyte showed high Hg
115 methylation rates and was identified to be one of the most important Hg methylation spots in
116 the lake (Gentes et al, 2013). Microbial studies identified SRB affiliated with the
117 *Desulfobacterota* phylum, as the main actors of Hg methylation in this environment (Gentès et
118 al. 2013). However, species belonging to this phylum are rare in the periphyton from Sanguinet
119 Lake (about 1%) (Gentès et al. 2020), and therefore difficult to isolate. Only few strains
120 affiliated with the *Desulfovibrionaceae* and able to methylate Hg, could be isolated from this
121 periphyton (Gentès et al. 2017). In this work, we applied different cultural and molecular
122 approaches than the earlier studies to describe the methylating community in the periphyton
123 from *Ludwigia sp.* found in the Sanguinet lake. We first analyzed the composition of the
124 microbial community in this periphyton by NGS. We developed a protocol of consortia
125 cultivation and their diversity identification (NGS), in order to increase the diversity of putative
126 Hg methylators through increasing their growth capacity. The consortia carrying
127 microorganisms involved in Hg methylation were identified via the presence of *hgcA* gene,
128 used as marker for Hg methylation.

129 **Material and methods**

130 Site and Sampling

131 The Sanguinet lake (58 km²) is located in the Landes area (southwest of France). The
132 overall characteristics of the lake are given in Table 1. Its average depth is 9 m and the
133 watershed surface is about 200 km². The average water residence time is 1587 days. Nitrogen
134 concentrations are found up to 0.58 mg. L⁻¹ for ammonium and 0.44 mg. L⁻¹ for nitrate,
135 characteristics of a mesotrophic lake. However, phosphate concentrations are found up to 0.023
136 mg. L⁻¹ and chlorophyll concentrations are varying between 1.5 and 4.9 µg. L⁻¹, characteristics
137 of an oligotrophic lake (Gentès et al. 2013; Moreira et al. 2016; Canredon et al. 2019).

138 *Ludwigia sp.*, an invasive macrophyte, covers partially the shores of the lake. Samples of
139 sediments and *Ludwigia* floating roots covered by periphyton were collected on February, 9th,
140 2020 and on September, 11th, 2020 in Ispe Bay (Ip) (sampling site GPS coordinates (DMS
141 type): 44° 26' 22.126" W 1° 11' 27.397"). Samples were collected, put in sterile falcons and
142 stored at 4°C for cultivation experiments and -20°C for diversity analyses.

143

144 Taxonomic diversity of periphyton through MiSEQ 16S rDNA sequencing and analysis

145 DNA from *in situ* samples was extracted using the DNeasy UltraClean Microbial Kit
146 (QIAGEN, Germany) according to the manufacturer's instructions and stored at -20°C.
147 Extracted DNA were quantified using QUBIT dsDNA high sensibility (HS) Assay Kit
148 (ThermoFisher Scientific, Oregon, USA) according to the manufacturer's protocol. PCR was
149 performed using specific primers, 515F (GTG YCA GCM GCC GCG GTA) and 928R (CCC
150 CGY CAA TTC MTT TRA GT) to amplify V4-V5 region of the 16S rDNA gene, tagged with
151 MiSEQ adaptors. PCR was performed using ampliTaq Gold 360 master mix (Applied
152 Biosystems, CA, USA), 0.4 µM of each primer and between 10 to 15 ng of extracted DNA were
153 used. PCR cycles were applied as following: 10 min of initial denaturation at 95°C, 30 cycles
154 of 30 s of denaturation at 95°C, 30 s annealing at 65°C, 40 s of elongation at 72°C with 7 min
155 final elongation at 72°C. The PCR products were sequenced in Genome Transcriptome
156 Platform of Bordeaux (PGTB), using MiSEQ technology. The data have been deposited in
157 GenBank under accession number PRJNA906750.

158

159 Sequence analysis was done with the pipeline FROGS from the Galaxy portal of the
160 Toulouse Midi-Pyrenees bioinformatics platform (Escudié et al. 2018). After a preprocessing
161 step including quality filter, read trimming and read assembly, sequences were clustered with
162 Swarm (Mahé et al. 2014) with an aggregation distance of 3 and a denoising clustering step.
163 Operational taxonomic units (OTU) with abundance lower than 0.005% were removed
164 (Bokulich et al. 2013). The SILVA database 128 was used to perform the OTU affiliations
165 (Quast et al. 2013).

166

167 Consortia cultivation and taxonomic identification

168 The cultures were conducted in a minimal medium containing the following (in g.L⁻¹,
169 unless otherwise indicated): NH₄Cl, 0.25; NaCl, 1; KH₂PO₄, 0.2; MgSO₄, 0.4; MgCl₂, 0.4;
170 CaCl₂.2H₂O, 0.1; KCl, 0.5; Hepes, 10 mM; trace element SL12 (modified from Overmann et
171 al. 1992), 1 mL.L⁻¹; selenite/tungstate (Widdel and Bak 1992), 1 mL.L⁻¹. The pH of the culture
172 medium was adjusted to 7.0. Oxygen in the culture medium was depleted using N₂ gas-flow
173 right after the autoclaved and filtered V7 vitamin solution (Pfennig and Trüper 1992) was
174 added. Specific substrates were added in the culture medium to favor the growth of different
175 bacterial populations: i) Na₂SO₄, 10 mM and lactate-Na, 10 mM for *Desulfovibrio*, ii) Na₂SO₄,
176 10 mM and propionate-Na, 10 mM for *Desulfobulbaceae*, iii) Na₂SO₄; 10 mM and acetate-Na,
177 10 mM for *Desulfobacteaceae*, iv) Propionate-Na, 20 mM and Fe₂O₃, 1 mM for
178 *Syntrophobacter*, v) Crotonate, 10 mM and Fe₂O₃, 1 mM for *Syntrophus*. Culture media
179 containing Na₂SO₄ were supplemented with FeSO₄ (1 mM) to follow the growth of SRB by the
180 development of a black iron sulfide precipitate (FeS). Each culture medium was supplemented
181 with 100 µM of Na₂S₂O₄ to reduce the medium.

182 The protocol used for the selection and growth of the consortia is modified from Colin et
183 al, (Colin et al. 2017). Briefly, 2 g of sediment or biofilm were added to 20 ml of culture
184 medium. Then, successive dilutions were performed from 10⁻¹ to 10⁻⁵ in sterile penicillin
185 bottles. The content of the bottles was distributed in 96-wells plates in an anoxic chamber.
186 Plates was sealed with Alumaseal CS TM film and incubated in Gas-Pack pouches in the dark,
187 at 18°C for three weeks to three months. When growth was observed, the wells were randomly
188 selected and diluted for a second time in the 96-wells plates, from 10⁻¹ to 10⁻⁸ and incubated in
189 similar way. The consortia that grew in the most diluted wells were selected and inoculated in
190 6 ml of culture medium, in vacuette tubes. For some dilution series, two or three wells were

191 recultivated because a late growth in more diluted wells from the one already recultivated was
192 observed.

193 Consortia have stored at -80°C whereas DNA from consortia was extracted using DNeasy
194 UltraClean Microbial Kit (QIAGEN, Germany) according to the manufacturer's instructions
195 and stored at -20°C. Identification of consortia composition was done by 16S rDNA sequencing
196 using the same protocol as performed for *in situ* identification of the periphyton community
197 (see above). The data were deposited in GenBank under accession number PRJNA910502.

198

199 Isolation of pure strain and identification through 16S rDNA sequencing

200 Two methods for isolation of pure strains from consortia were applied: a liquid medium
201 technique and a deep agar dilution procedure. For the isolation attempt in liquid medium, the
202 culture media were unchanged and prepared as described before. Consortia were diluted in
203 microplates and incubated at 30°C for 14 days. The content of three wells, among the highest
204 dilutions where growth was detected, were sub-cultured in a new 96-well microplates in the
205 same conditions. Once the growth was observed, cultures were regrown in higher volume and
206 stored at -80°C with 25% of glycerol. For deep agar dilutions series, the protocol described by
207 Pfennig (1978) was used (Pfennig 1978). Isolated colonies were picked and recultured in liquid
208 medium (as described above).

209 For isolates identification, wells showing cell growth and isolates from deep agar tubes
210 were resuspended in 100 µL of water. PCR amplification of the 16S rDNA gene was performed
211 using universal primers (63F 5'-
212 CAGGCCTAACACATGCAAGTC3'; 1387F 5'-GGGCGGWGTGTACAAGCC-3')
213 (Marchesi et al. 1998), the ampliAq Gold 360 master mix (Applied Biosystems, CA, USA),
214 0.5 µM of each primer and 1 µL of cellular suspension. PCR cycling was performed following
215 manufacturer's instructions, with annealing temperature of 58°C. 16S rDNA amplicons were
216 quantified by QUBIT dsDNA high sensibility (HS) Assay Kit (ThermoFisher Scientific,
217 Oregon, USA) according to the manufacturer's protocol before Sanger sequencing (Eurofins,
218 Germany). Quality of sequences were verified using Unipro UGENE Software (Okonechnikov
219 et al. 2012) and a Blast search was performed to identify the closest sequences in NCBI
220 database. The sequences of the pure strains were deposited under numbers from OQ053276 to
221 OQ053279.

222

223 Functional identification through *hgcA* cloning

224

225 Identification of consortia that carry *hgcA* gene was performed by PCR amplification
226 using *hgcAB* primers ORNL-HgcAB-uni-32R (CAG GCN CCG CAY TCS ATR CA) and
227 ORNL- HgcAB-uni-F (AAY GTC TGG TGY GCN GCV GG) (Gionfriddo et al. 2020), the
228 AmpliTaq Gold 360 master mix (Applied Biosystems, CA, USA), 0.5 μ M of each primer and 1
229 ng of extracted DNA. A touchdown PCR protocol was set up, as follow: after 2 min of initial
230 denaturation at 95°C, 5 touchdown cycles of 30 s denaturation at 95°C, 30 s of annealing at
231 68°C (decreasing 1°C at each cycle), and 30 s of elongation at 72°C. PCR was followed by 30
232 cycles of 30 s of denaturation at 95°C, 30 s annealing at 63°C, 1 min of elongation at 72°C with
233 7 min final elongation at 72°C. The higher annealing temperatures reduce nonspecific PCR
234 products and promote specific amplifications at the start of the PCR.

235 PCR products were purified using Illustra™ GFX PCR DNA and Gel Band Purification
236 kit (GE Healthcare, Chicago, Illinois, US) according to the manufacturer's instructions. Purified
237 amplicons were quantified by QUBIT dsDNA high sensibility (HS) Assay Kit (ThermoFisher
238 Scientific, Oregon, USA) according to the manufacturer's protocol. Because the sequencing of
239 these PCR products was unsuccessful when performed on the purified PCR products, a cloning
240 step was necessary to obtain isolated DNA fragments accessible for sequencing. Purified PCR
241 products were ligated into a TOPO vector with TOPO™ TA Cloning™ Kit (Invitrogen,
242 Carlsbad, CA, USA), used to transform One Shot™ TOP10 competent *E. coli*. Transformed
243 cells were grown on LB broth agar plates with 50 μ g. mL⁻¹ ampicillin, 40 mg. mL⁻¹ X-gal and
244 100 mM IPTG. Colony-PCR was performed with M13 primers: M13F (GTA AAA CGA CGG
245 CCA G) and M13R (CAG GAA ACA GCT ATH AC). AmpliTaq Gold 360 master mix
246 (Applied Biosystems, CA, USA), 0,2 μ M of each primer were used to carry out the PCR
247 following manufacturer's instructions, with annealing temperature of 54°C. PCR products with
248 the expected sizes were sequenced (Eurofins Genomics, Germany). The resulting sequences
249 obtained were translated into amino acid sequences with ExPASy, and aligned with a HgMATE
250 database containing known *hgcA* sequences (Gionfriddo et al. 2021). Finally, a HgcA
251 sequences-based phylogenetic tree was constructed using MegaX (Molecular Evolutionary
252 Genetics Analysis) with the Neighbor-Joining (NJ) method. The sequences were deposited
253 under numbers from OQ282917 to OQ282952.

254

255

256 **Results**

257 The Sanguinet lake is defined as oligo-mesotrophic lake (OCDE 51982) based on its main
258 physical and chemical characteristics (Table 1). Sulfate concentration in the lake is low,
259 although the existing concentration is enough for sulfate reduction to occur (Gilmour et al.
260 1992).

261 Diversity of sediments and *Ludwigia*'s periphyton of through MiSeq 16s rDNA sequencing

262 The microbial diversity was determined in sediments and periphyton from winter and late
263 summer sampling campaigns based on 16S rDNA MiSEQ sequencing. Alpha diversity metrics
264 (Table 2) revealed that the richness in the sediment was more important than the richness in the
265 biofilm for both campaigns. The global richness was higher in late summer than in winter both
266 in periphyton and sediment samples, with Chao1 index ranging from 606 to 875 for the biofilms
267 and from 886 to 1182 species for the sediments. The comparison of Hill numbers (Chao et al.
268 2014) revealed that the distribution (the relative abundance of individuals) was different
269 between campaigns and between periphyton and sediment. Periphyton from the winter
270 campaign had the lowest value for the highest Hill number (2D : inverse Simpson) with $7.18 \pm$
271 2.25 , highlighting a very low evenness. Sediment from late summer campaign exhibited the
272 highest evenness, probably because of the environmental parameters favoring a higher global
273 activity and thus more active populations than in winter.

274 The *Proteobacteria* and the *Bacteroidetes* dominated the sediment and the periphyton
275 microbial communities in both sampling periods. They both represented up to 76% of the
276 relative abundance (Figure 1). The relative abundance of the firmicutes decreased in the
277 summer (less than 0.08%) compared to the winter (up to 20% of the relative abundance).
278 Cyanobacteria represented near 6% of the relative abundance in the samples of summer
279 campaigns and winter periphyton samples, but their relative abundance was much less (0.4%)
280 in the winter sediment samples. The *Desulfobacterota* represented less than 3% of the overall
281 community and only 0.7% in biofilm sample in summer campaign (Figure 1). Among the
282 *Desulfobacterota*, populations affiliated with the *Desulfovibrionaceae*, the
283 *Desulfobacteraceae*, the *Desulfobulbacaea*, the *Geobacteraceae* and the *Syntrophobacteraceae*
284 were detected (data not shown). The sulfate reducers affiliated with the *Desulfobacterota* (i.e:
285 *Desulfovibrionaceae*, *Desulfobacteraceae*, and *Desulfobulbacaea*) represented between
286 0.014% (summer periphyton sample) and 1.25% (winter periphyton sample). The
287 *Desulfovibrionaceae* were dominant in the winter campaign representing 100% of the sulfate

288 reducing community whereas the *Desulfobacteraceae* and the *Desulfobulbaceae* accounted for
289 60% of the sulfate reducing community in summer.

290

291 Consortia cultivation and *hgcA* screening

292 Previous studies demonstrated that sulfate reducing microorganisms from the
293 *Desulfobacterota* were the main mercury methylators in Sanguinet lake (Gentès et al. 2013). In
294 order to investigate these microorganisms in the *Ludwigia sp.* periphyton and the sediments,
295 consortia were cultivated using different specific substrates to favor the selection of different
296 metabolic groups among the *Desulfobacterota*. A total of 142 consortia (Table 3) were first
297 obtained. PCR using DNA from 81 of these consortia resulted in a positive amplification (out
298 of the 142) using specific primers for *hgcA* gene. To confirm the presence of *hgcA* genes in the
299 consortia, PCR fragments were cloned and sequenced. Only 36 from the 81 consortia were
300 confirmed to contain the *hgcA* gene (Table 3).

301 HgcA amino acid sequences from consortia were translated and a phylogenetic tree was built
302 using the Hg-MATE database (Gionfriddo et al, 2020) (Figure 2). Among these 36 consortia,
303 three (BSL262, BP321 and BP322) were not considered because they were redundant (same
304 dilution series and same *hgcA* sequence) to BSL261 and BP323 (see Material and Methods,
305 Consortia cultivation and taxonomic identification). Among the 33 remaining sequences, six
306 different clusters were identified. The sequence of the first one was related to HgcA from
307 *Syntrophorhabdus aromaticivorans* UI DSM 1771. The sequence of the second group was
308 related to the *Pseudodesulfovibrio hydrargyri* BerOc1. The sequence of the third group was
309 related to HgcA sequence of *Desulforhopalus singaporensis* DSM 12130. The fourth group was
310 a group of four sequences close to the sequence of *Desulfobulbus propionicus* DSM 2032. The
311 sequence of the fifth group was close to HgcA sequence of *Desulfohalovibrio reitneri* L21-Syr-
312 AB. Lastly, the sixth group contained the greatest number of sequences, and the closest match
313 was the HgcA amino acid sequences found in *Fundidesulfovibrio putealis* DSM 16056.
314 However, based on phylogenetic tree clustering, this group of sequences could be divided in
315 three subgroups and only the sequence from the first sub-group (from the consortium named
316 SSL37) was very close to the HgcA sequence of *F. putealis*. The other two sub-groups were, to
317 some extent, distant to this reference sequence. Among the two other sub-groups, the one
318 containing 14 sequences could further be subdivided into four parts and the one containing 13
319 sequences could be subdivided into two parts (Figure 2). The sixth group was thus highly

320 diverse with no known representatives. While each of these different sub-parts seems to contain
321 sequences distantly related to *F. putealis*, the exact species or strains, to which these sequences
322 were related, could not be determined.

323

324 Microbial composition of *hgcA*-containing consortia

325 The richness in the consortia varied from two to 44 OTUs. All the consortia contained
326 populations belonging to different groups of the *Desulfobacterota* phylum (Figure 3), expected
327 to carry *hgcA* gene. More importantly, other microorganisms such as representatives of the
328 genera *Dechloromonas*, *Aeromonas*, *Paludibacter* or *Pseudomonas* were frequently detected in
329 the consortia, with relatively high abundances. The composition of consortia cultivated from
330 sediments were different from consortia cultivated from biofilms. Indeed, we found strict
331 anaerobes, such as *Paludibacter*, mainly in the sediments. Conversely, microorganisms with
332 aerobic or facultative anaerobic lifestyles, such as *Stenotrophomonas* or *Acinetobacter*, were
333 commonly found in consortia from biofilms.

334 *Desulfobacterota* diversity obtained in this work using consortia cultivation was
335 compared with the previous *Desulfobacterota* diversity obtained by Gentes et al. (Gentès et al.
336 2017, 2020) using isolation approaches (Table 4). Ten new populations were obtained in this
337 work and were not closely related to the obtained strains in both other studies. Four populations
338 found in the other studies were not obtained in this work. Thanks to the consortia culture, a
339 broader *Desulfobacterota* diversity was attained. From, the *Desulfobacterota* sequences
340 retrieved in the *in situ* samples in this study, only four populations could not be retrieved in
341 consortia (Table 4). A phylogenetic tree was built using 16S sequences from Gentes et al
342 (Gentès et al. 2017, 2020) and sequences related to the *Desulfobacterota* obtained in this work
343 (consortia and *in situ* samples) (Supp data S1). Consortia containing HgcA sequence close to
344 *Syntrophorhabdus aromaticivorans* UI DSM 1771 contain a 16S sequence close to
345 *Syntrophobacter fumaroxydans* found in the other studies. Consortia containing HgcA
346 sequences close to *Desulfobulbaceae* contain 16S sequences close to *Desulfobulbus elongatus*
347 or *Desulfobulbus propionicus*. Consortia containing HgcA sequence close to
348 *Pseudodesulfobivrio hydrargyri* BerOc1 contain a 16S sequences close to the same strain,
349 thoroughly invariable between both affiliations. In contrast, the consortia containing HgcA
350 sequences close to *F. putealis* did not all contain the 16S sequences corresponding to *F.*
351 *putealis*. Indeed, while the HgcA sequences of BP23, BSA33, SP32, SSP271, BSP341, BP332,

352 BP33, BC36, BSA17, SP272, BP31, BP323-2-1 and BSA321 consortia showed HgcA of *F.*
353 *putealis* as the closest relative, the 16S analysis showed the absence of *F. putealis* in the
354 populations present in these consortia. Instead, populations contained in these consortia
355 affiliated with the *Desulfobacterota* phylum were close to *Solidesulfovibrio aerotolerans* DvO5
356 AY46987, *Solidesulfovibrio burkinensis* HDv AF053752, *Solidesulfovibrio carbinolicus* DSM
357 3852 or *Humidesulfovibrio articus* B15 whose HgcA sequences are not known.

358

359 Isolation and identification of pure strains

360 Attempts to isolate strains from consortia containing different populations involved in the
361 methylation of Hg were performed. For that, consortia were selected based on the presence of
362 *hgcA* gene and their position on the phylogenetic tree. The focus was on consortia close to
363 members of *Desulfobulbus*, *Dechloromonas* and *Syntrophobacter* genera, because few relatives
364 have been already isolated. Additionally, some consortia that formed an important group at the
365 bottom of the tree and contained other well-represented microbial groups were also selected for
366 isolation. The isolation attempts were done on the following consortia: SP11; SSA241.
367 BSA232; BSL262; BSP32; SSP271; BSA33; BP462; SSL37 and BSA321. Only *F. putealis*
368 was isolated as pure strain from a restricted number of consortia.

369

370 **Discussion:**

371 The level of nutritional elements (nitrate, phosphate) found in Sanguinet lake allows the
372 development of microorganisms, including primary producers as shown by the chlorophyll
373 content (Table1). The presence of cyanobacteria and microalgae in sediments from summertime
374 illustrate their photosynthetic activity, characteristic of such environments. Like all the lakes
375 from this area, Sanguinet lake was very well studied, because of the presence of invasive aquatic
376 plants. Among the invasive plants, *Ludwigia* species are found in the lake (Zuazo et al. 2019).
377 Interestingly, periphytons colonizing the floating roots of *Ludwigia* plants are known to be a
378 hotspot of mercury methylation (Gentès et al. 2013), at a higher rate in the late summer
379 compared to springtime (Carrier 2016). The difference in the mercury methylation rates
380 between seasons is probably caused by the increase of primary production in summertime,
381 classically observed in periphytons (Lázaro et al. 2019). The methylation in the periphyton is
382 mainly due to sulfate reducers belonging to the *Desulfobacterota* as shown by inhibition

383 experiments with molybdate (Gentès et al. 2013). Sulfate in aquatic environment allows the
384 presence of SRB, and consequently the production of MeHg, especially in the lakes from the
385 south west of France (Canredon et al. 2019). The sulfate concentration in Sanguinet lake (about
386 100 μ M) corresponds to the concentration where the maximum of methylation activity occurs
387 for sulfate reducers as demonstrated in Gilmour et al. (1992).

388 In the present study, we investigated microbial diversity in *Ludwigia* periphyton and
389 sediments. Differences in the diversity and evenness observed between the sediment and
390 biofilm can be explained by the fact that the biofilm is colonized by microbial populations
391 originated from the sediments but also from the water column. The cyanobacteria colonizing
392 the periphyton provide primary production within the biofilms, which also benefit from exudate
393 of the plants as source of organic carbon (Srivastava et al. 2016), generating thus a specific
394 microecosystem. *Proteobacteria* and *Bacteroidetes*, the most abundant phyla, includes
395 heterotrophs able to use a wide range of carbonaceous substrates. The presence of anaerobic
396 bacteria is explained by the cohabitation of different microbial communities and the formation
397 of anoxic micro-niches via the consumption of oxygen by aerobes and facultative anaerobes
398 (Ito et al. 2002). Previous studies showed the presence of the same dominant phyla i.e.,
399 *Proteobacteria*, *Cyanobacteria* and *Bacteroidetes* in the periphyton from the same lake (Gentès
400 et al. 2017, 2020) as well as from periphyton found in lake Titicaca (Bouchet et al. 2018).

401 Previous studies, aiming to determine the main mercury methylators in periphyton,
402 showed that sulfate reducing bacteria and methanogens are the main players in Hg
403 transformations in periphytons (Hamelin et al. 2011; Gentès et al. 2013; Bouchet et al. 2018).
404 Microbial community composition is thus an important factor controlling Hg transformations.
405 In our study, no methanogenic Archaea known for their Hg methylating activity could be found
406 in the periphyton, in agreement with the key role of sulfate reducers in Hg(II) methylation
407 already observed in this biofilm (Gentès et al. 2017). However, the anaerobic sulfate reducing
408 bacteria belonging to the *Desulfobacterota* in the microbial community of the periphyton are
409 minor populations. Their presence is explained by the sulfate found in this lake, which they use
410 as electron acceptor in sulfate respiration (Gilmour et al. 1992). Thus, the Hg transformations
411 in periphyton is mediated by SRB, but these transformations are also driven by others
412 parameters such as the microbial community present in the periphyton (primary production and
413 global activity), the environmental parameters (temperature, light, ...) or nychthemeral
414 parameters (Frohne et al. 2012).

415 In order to broaden the cultivated SRB involved in HgII methylation in the periphyton, a
416 consortia culturing approach coupled to the detection of *hgcA* gene was applied. The
417 phylogenetic tree built based on HgcA amino acid sequences found in the consortia showed a
418 dispersion of HgcA sequences that does not strictly follow the 16S rDNA-based taxonomy
419 (Figure 2). Based on this phylogenetic tree, six main clusters of HgcA sequences could be
420 obtained. The cluster containing a sequence close to *Syntrophorhabdus aromaticivorans* UI
421 DSM 1771 from *Syntrophorhabdaceae* family showed the importance of consortia cultivation
422 approach, since representatives of this syntrophic species do not grow in pure culture (Qiu et
423 al. 2008). Although the HgcA sequence is known, the ability to methylate mercury of
424 *Desulforhopalus singaporensis* was not investigated until now. The sequences found close to
425 *Pseudodesulfobivibrio* are in agreement with this well studied genus known to be involved in
426 HgII methylation (Gilmour et al. 2011; Goñi-Urriza et al. 2015; Barrouilhet et al. 2022) but are
427 in disagreement with the halophilic character of these organisms. Another sequence was also
428 found closely related to the halophilic *Desulfohalovibrio reitneri* organism. Some
429 *Desulfobulbaceae* from the genus *Desulfobulbus*, known to be involved in HgII methylation
430 (Benoit et al. 2001), were cultivated in this work. However, some of the HgcA amino acid
431 sequences obtained in this work that belong to the *Desulfobulbaceae* family, belong to
432 populations never been cultivated before, highlighting again the advantage of consortia
433 cultures.

434 Up to 26 from 33 HgcA sequences were retrieved from a quite diverse cluster that could
435 be separate in two subclusters. One was close to HgcA sequence from *F. putealis* while the
436 second subgroup appeared more distant to any known sequences. *F. putealis* is a sulfate
437 reducing bacterium from *Desulfovibrionaceae* family constantly found in biofilm samples from
438 this lake (Gentès et al. 2017, 2020). This species contain *hgcA* gene but the implication of the
439 strains affiliated with this species in HgII methylation was never studied. Regarding HgcA
440 sequences from the sub-group distantly related to *F. putealis*, the data were not sufficient to
441 assert that the amino acid sequences of this sub-group were linked to *F. putealis*. Our results
442 pointed out that the catalogue of HgcA amino acid sequences did not match all the HgcA
443 sequences found in the obtained consortia. For example, it seemed that some HgcA sequences
444 close to *F. putealis* come from *Humidesulfobivibrio articus* or *Solidesulfobivibrio aerotolerans*.
445 However, these species are not identified as mercury methylators and their genomes has not
446 been sequenced. Further genomic analyses is necessary to investigate the strains from the
447 consortia containing these categories of HgcA amino acid sequences.

448 The results obtained in this work showed that a wide variety of microorganisms involved
449 in HgII methylation could be obtained using consortia cultivation compared to pure culture
450 isolation (Gentès et al. 2017, 2020). Here, we identified microorganisms from
451 *Desulfovibrionaceae* but also from *Desulfobulbaceae* and syntrophic organisms, difficult to
452 obtain in pure culture. The growth of these microorganisms was possible due to the presence of
453 other microorganisms from different groups, whose metabolisms and growing factors are
454 probably the key parameters to the production of substrates allowing the cooperation (Bryder
455 1981) and the maintenance of the populations of interest. This also explains why some
456 *Desulfobacterota* found in consortia are not those expected when using specific substrates (Fig.
457 3) for consortia selection. In the attempt to isolate new microorganisms from selected consortia,
458 only *F. putealis* affiliated strains were isolated so far, confirming that the culture in consortia
459 protect and maintain microorganisms for which the pure strain approach is extremely difficult,
460 if not impossible, to reach.

461 Consortia composition analysis also revealed the presence of some aerobes or facultative
462 anaerobic microorganisms participating to the biogeochemical cycles in these environments.
463 These microorganisms are more abundant in the biofilm, which is an oxic environment. The
464 analysis of global diversity supports the presence of these aerobic microorganisms promoted
465 by the presence of cyanobacteria capable of producing oxygen and organic matter suitable for
466 heterotrophic microorganisms. These microorganisms consume the oxygen coming from the *in*
467 *situ* production or diffusion of oxygen from the water, favoring the appearance of anaerobic
468 micro-niches. The facultative anaerobic microorganisms present in the consortia such as
469 representatives of the genera *Aeromonas*, *Pseudomonas* or *Dechloromonas* may also switch to
470 a fermentative or denitrifying metabolism (Silby et al. 2011; Taubert et al. 2022) favoring the
471 production of electron donors such as acetate, ethanol, lactate or even the production of
472 hydrogen used by other microorganisms including members of the *Desulfobacterota*. As shown
473 by Matheron and Baulaigue (1976) cooperation can take place between different micro-
474 organisms through the production of substrates by fermentation that can be used by SRBs. The
475 sulfide produced by the SRBs can be re-oxidized by the anoxygenic phototrophic bacteria. Our
476 *in situ* analysis showed the presence of purple non-sulfur phototrophic bacteria known to have
477 a versatile metabolism and able to oxidize the sulfide produced by SRBs. These anoxygenic
478 phototrophic bacteria are also able to reduce HgII (Grégoire and Poulain 2016) affecting thus
479 mercury methylation and transformations. Our consortia were not cultivated for the purpose to
480 isolate anoxygenic phototrophic bacteria (absence of light) but demonstrated once again the

481 advantages of the consortia cultivation in terms of increasing the diversity and enhancing the
482 cooperation/interaction between microorganisms.

483 Recent studies using metagenomic approaches in several environments (Gionfriddo et al.
484 2016; Capo et al. 2020; McDaniel et al. 2020; Vigneron et al. 2021) discovered several
485 Metagenomic Assembled Genomes (MAGs) carrying *hgcA* genes (with no cultivated relatives)
486 that are not considered to date as implicated in Hg methylation. Those studies are important
487 since they increase the diversity of putative Hg methylators but remain quite descriptive. It will
488 be interesting to determine if representative of these MAGs are capable to methylate Hg. In this
489 regard, a cultivable approach is necessary, and consortium culture approach could be a way to
490 overcome culture limitations.

491

492 **Author contributions**

493 MG was in charge of the funding, RG collected the samples, DS and CG generated the dataset, DS and
494 GC analyzed the data, DS wrote the manuscript with contributions from MSG, RG and BKH.

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498 **Competing Interests**

499 Authors declare no competing interest

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503 **References**

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729 **Table 1 : Main characteristics of Cazaux Sanguinet lake.**

Watershed surface (km ²) ^{b,c}	200
Area (km ²) ^{a,b,c}	58
Max depth (m) ^{b,c}	23
Average depth (m) ^b	9
Volume (million of m ³) ^{b,c}	500
Average water residence time (day) ^b	1587
T°C ^{a,b}	6 – 25
pH ^{a,b}	7.5 – 8.0
Ammonium (mg.L ⁻¹) ^c	0 – 0.58
Nitrate (mg.L ⁻¹) ^c	0 – 0.44
Phosphate (mg.L ⁻¹) ^a	0.023
Sulfate (mg.L ⁻¹) ^{b,c}	7.7 11.5
Chlorophyll a (µg.L ⁻¹) ^c	1.5 – 4.9

730 a : Gentès, et al. (2013), b : Canredon, et al. (2019), c : Moreira, et al. (2016).

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733 **Table 2 : Alpha diversity metrics from periphytic biofilm and sediment**

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	February 2020		September 2020	
	Periphyton	Sediment	Periphyton	Sediment
Chao1	607 ± 43	886 ± 75	875 ± 1	1183 ± 12
Observed species richness	416 ± 49	735 ± 71	738 ± 26	1049 ± 23
¹ D ^(a)	28 ± 9	102 ± 42	134 ± 32	284 ± 23
² D ^(b)	7 ± 2	25 ± 11	40 ± 12	60 ± 11

735 (a), ¹D is the exponential of Shannon entropy index ; (b), ²D is the Inverse of Simpson index

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739 **Table 3 : Number of consortia obtained from February and September campaigns from periphyton**
740 **and sediments, and number of positive amplicons for *hgcA* gene and confirmed by amplicon**
741 **sequencing**

Substrate	Winter			Summer		
	Consortia obtained	Positive amplicons (<i>hgcA</i>)	Confirmed by sequencing ^(a)	Consortia obtained	Positive amplicons (<i>hgcA</i>)	Confirmed by sequencing
Sulfate + Lactate	28	18	7 (2)	12	8	2
Sulfate + Propionate	17	6	3 (2)	17	12	2
Sulfate + Acetate	4	3	2 (1)	14	7	7
Propionate	23	16	10 (4)	15	7	2
Crotonate	5	1	1	7	3	0
Total	77	44	23 (9)	65	37	13

742 a: Numbers in brackets indicate consortia from sediment

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745 **Table 4 : *Desulfobacterota* (closest relative) obtained in the consortia and the *in situ* analyses**
 746 **compared to those obtained by Gentes et al., 2017, 2020**

Closest relative taxonomic name (strain number)	Populations		Strains	
	This study (<i>In situ</i> samples)	This study (Consortia culture)	Gentes et al 2017	Gentes et al 2020
<i>Fundidesulfovibrio putealis</i> (DSM16056)	X	X	X	
<i>Desulfovibrio butyraliphilus</i> (BSY)		X		
<i>Solidesulfovibrio alcoholivorans</i> (DSM5433)		X		
<i>Solidesulfovibrio burkinensis</i> (HDv)	X	X	X	X
<i>Solidesulfovibrio carbinolicus</i> (DSM3852)	X			
<i>Solidesulfovibrio aerotolerans</i> (DvO5)	X	X		
<i>Desulfovibrio carbinoliphilus</i> (FW-101-2B)	X		X	X
<i>Humidesulfovibrio mexicanus</i> (DSM13116)	X	X	X	
<i>Desulfovibrio vulgaris</i> (Hildenborough M34399)				X
<i>Desulfovibrio desulfuricans</i> (ATCC 29577)			X	X
<i>Desulfocapsa thiozymogenes</i> (Bra2)		X		
<i>Desulfopila aestuarii</i> (MSL86)		X		
<i>Oryzomonas japonica</i> (Red 96)	X	X		
<i>Syntrophobacter fumaroxidans</i> (MPOB)	X	X		
<i>Desulfomonile tiedjei</i> (DSM6799)				X
<i>Desulfobulbus elongatus</i> (FP)	X	X		
<i>Desulfobulbus propionicus</i> (DSM 2032)	X	X		X
<i>Humidesulfovibrio articus</i> (B15)	X	X		
Unidentified SRB		X		X
Unidentified SRB		X		
<i>Desulfatirhabdium sp.</i> (SVa 0081)	X			
<i>Desulfocastanea catecholica</i> (NZva20)	X			

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749 **Figure Captions**

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751 **Figure 1:** Relative abundance of phyla detected in the periphyton of aquatic roots of *Ludwigia*
752 *sp.* and sediments of Sanguinet lake in (A) February 2020 and in (B) September 2020. The data
753 show the mean of three replicates.

754

755 **Figure 2:** Phylogenetic trees based on HgcA sequences retrieved in consortia. Blue: sequences
756 from consortia obtained from biofilms in winter. Red: sequences from consortia obtained from
757 biofilm in summer. Green: sequences from consortia obtained from winter sediments.

758 Blue: sequences from consortia obtained from biofilms in winter. Red: sequences from consortia
759 obtained from biofilm in summer. Green: sequences from consortia obtained from winter sediments.
760 The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown.
761 The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test
762 (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the
763 same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary
764 distances were computed using the Maximum Composite Likelihood method and are in the units of the
765 number of base substitutions per site. This analysis involved 171 nucleotide sequences. Codon positions
766 included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair
767 (pairwise deletion option). There were a total of 1038 positions in the final dataset. Evolutionary
768 analyses were conducted in MEGA X (Felsenstein 1985; Saitou and Nei 1987; Tamura et al. 2004;
769 Kumar et al. 2018).

770

771 **Figure 3:** Heat map of the microbial composition of consortia. B: Biofilm, S: Sediment. C:
772 Crotonate, P, Propionate, SA: sulphate and acetate, SL: sulphate and lactate, SP: sulphate and
773 propionate. C1: winter samples, C2: summer samples. Consortia in red are consortia used for
774 isolation attempts and stars represents the consortia whose isolation has given results (pure
775 strains).

776 **Supplementary data:** Phylogenetic tree based on 16S rDNA from this study and study from
777 Gentes 2017 and 2020 (Clones and strains).

778 The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown.
779 The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test
780 (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the
781 same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary
782 distances were computed using the Maximum Composite Likelihood method and are in the units of the
783 number of base substitutions per site. This analysis involved 171 nucleotide sequences. Codon positions
784 included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair
785 (pairwise deletion option). There were a total of 1038 positions in the final dataset. Evolutionary
786 analyses were conducted in MEGA X (Felsenstein 1985; Saitou and Nei 1987; Tamura et al. 2004;
787 Kumar et al. 2018).

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789 Figure 1

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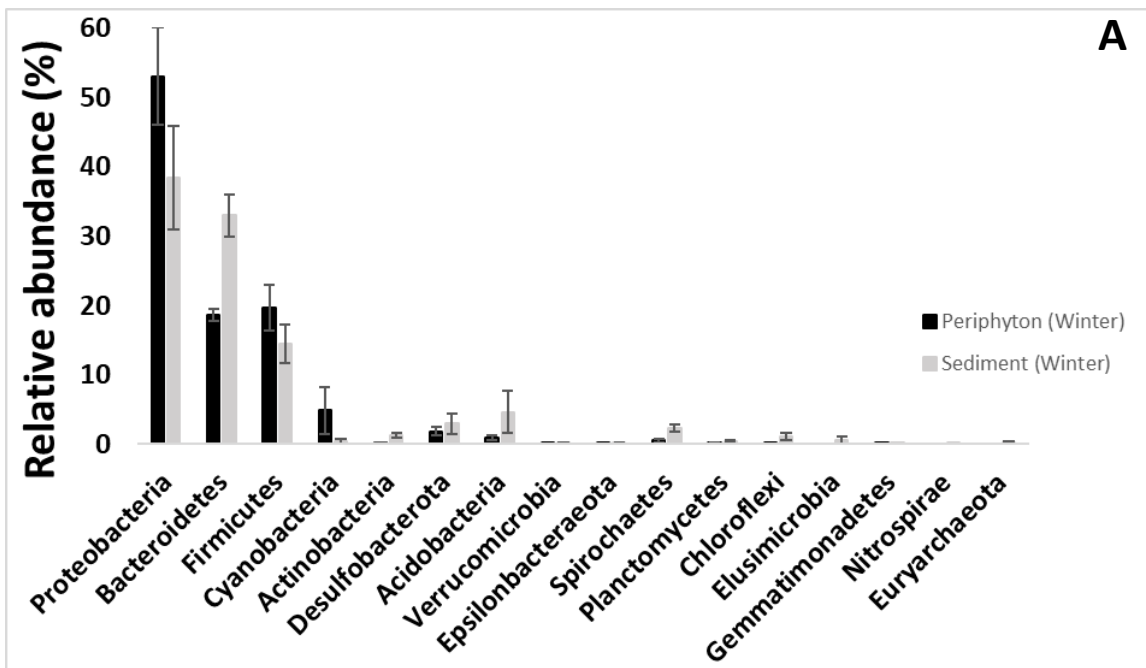
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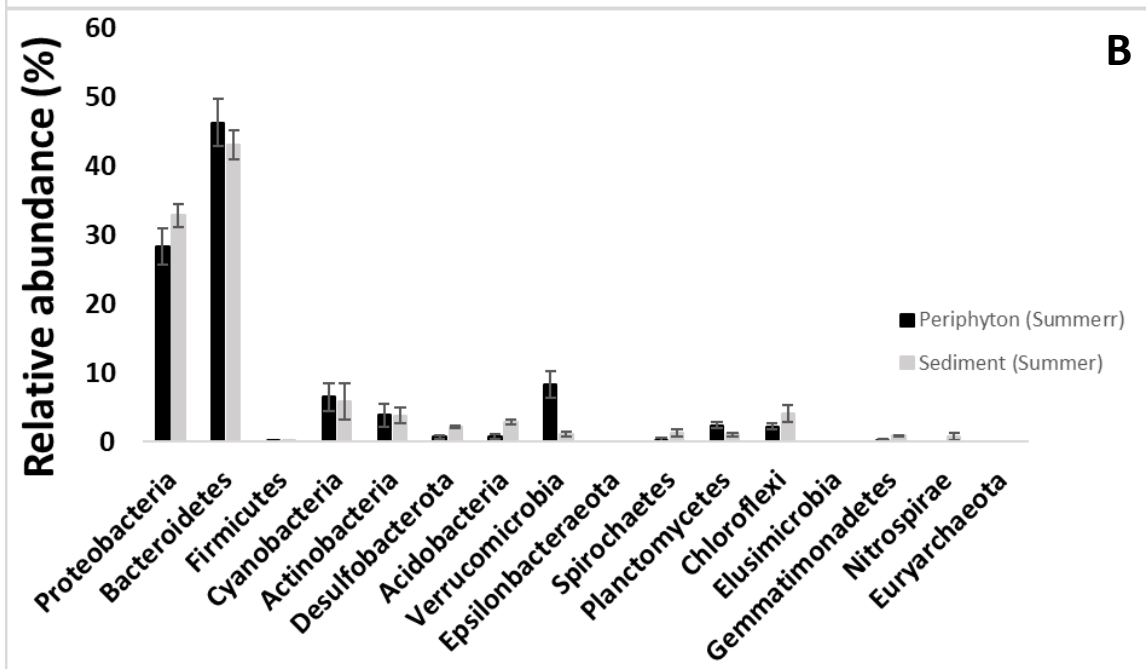
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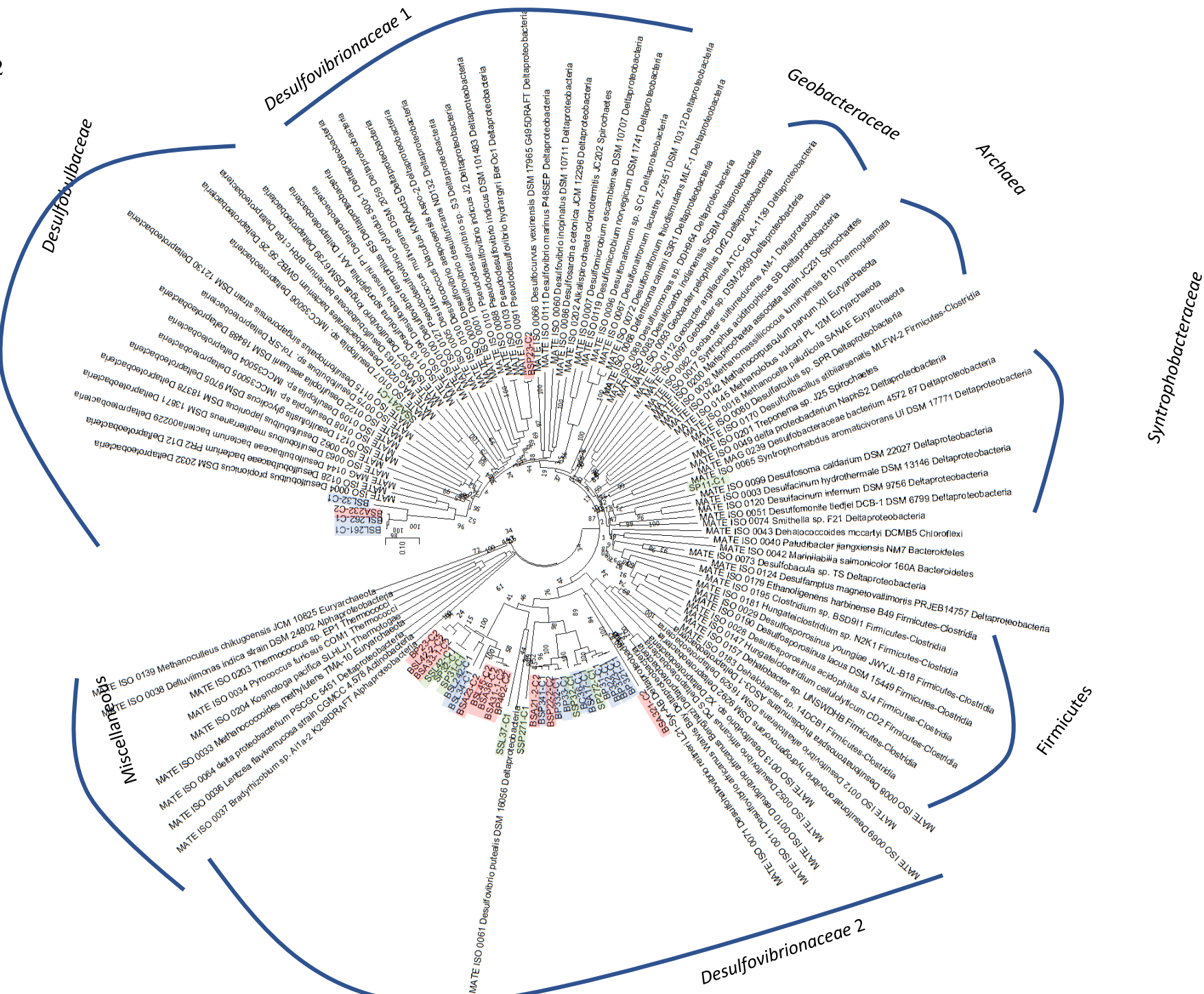
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817 Figure 2

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837 Figure 3

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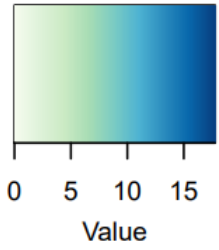
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