1 # Structure of bacterial communities along a hydrocarbon contamination
2 gradient in coastal sediment

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26 ABSTRACT

27 The bacterial diversity of chronically oil polluted retention basin sediment located in
28 the Berre lagoon (Etang-de-Berre, France) was investigated. This study combines chemical
29 and molecular approaches in order to define how *in situ* petroleum hydrocarbon
30 contamination level affects bacterial community structure of subsurface sediment.
31 Hydrocarbon content analysis revealed clearly a gradient of hydrocarbon contamination in
32 both water and sediment following the basin periphery from the pollution input to the lagoon
33 water. The nC17 and pristane concentrations suggested alkane biodegradation in the
34 sediments. These results combined with those of Terminal-Restriction Fragment Length
35 Polymorphism (T-RFLP) analysis of the 16S rRNA genes indicated that bacterial
36 communities structure was obviously associated with the gradient of oil contamination. The
37 analysis of bacterial community composition revealed dominance of bacteria related to the
38 *Proteobacteria* phylum (*Gamma*-, *Delta*-, *Alpha*-, *Epsilon*- and *Betaproteobacteria*),
39 *Bacteroidetes* and *Verrucomicrobium* groups, and *Spirochaetes, Actinobacteria* and
40 *Cyanobacteria* phyla. The adaptation of the bacterial community to oil contamination has not
41 as consequence dominance of known oil-degrader bacteria. Predominance of populations
42 associated to sulphur cycle is observed. The input station presented particular bacterial
43 community composition revealing the adaptation of this community to the oil contamination.

44 **INTRODUCTION**

45 Oil pollution represents a major threat to marine life and ecosystems, and with the world
46 demand for oil increasing, it is likely to remain one of the biggest threats to the marine
47 environment for years to come. Consequently there is growing interest in reclaiming polluted
48 marine and coastal sites by using oil-degrading bacteria (Head & Swannell, 1999).
49 Mediterranean coastal regions are particularly exposed to oil pollution due to extensive
50 industrialization and urbanisation and transport of crude and refined oil to and from refineries.
51 The *Etang de Berre*, a brackish lagoon bordering the Mediterranean Sea in the South of
52 France, constitutes an important industrial area receiving seawater from the Gulf of Fos and
53 freshwater from a few natural tributaries. For decades, the lagoon has received huge amounts
54 of hydrocarbons from refineries, petrochemical plants and transportation systems, in addition
55 to biogenic inputs (Jacquot *et al.*, 1999). Despite efforts since the 1970s to reduce oil spills
56 and waste entering the lagoon by using water-treatment plants and retention basins, *Etang de*
57 *Berre* remains a heavily contaminated ecosystem.

58 The fate of spilled oil in coastal ecosystems depends largely on a wide variety of
59 physical, chemical and biological processes, among which biological degradation or
60 bioattenuation is the principal removal route (Leahy & Colwell, 1990). Recently, several
61 studies have demonstrated the efficiency of estuarine and sediment oil-degrading microbial
62 communities in microcosms (Coulon *et al.*, 2007; McKew *et al.*, 2007; Röling *et al.*, 2002).
63 However, little information on the response *in situ* of the indigenous microbial communities is
64 available (Macnaughton *et al.*, 1999). This information is crucial to better understand the fate
65 of oil in marine systems and for the development of knowledge-based strategies to accelerate
66 the ecological repair process.

67 In oil-polluted marine intertidal flats and coastal lagoons, the development of microbial
68 mats dominated by cyanobacteria, colourless and purple sulphur bacteria and sulphate-

69 reducing bacteria is frequently observed (Van Gemerden, 1993). We have described the
70 structure of pristine hypersaline microbial mat communities of Salins-de-Giraud (Fourçans *et*
71 *al.*, 2004), their dynamics (Fourçans *et al.*, 2006) and their capacity to degrade crude oil when
72 maintained in microcosms (Bordenave *et al.*, 2004). Recently, we started to characterize
73 microbial mats developed in restricted oil input area suggesting the possible effects of
74 petroleum on microbial community composition (Hernández-Raquet *et al.*, 2006). To further
75 investigate the potential impact of petroleum hydrocarbons on microbial community structure
76 and diversity and hence provide a better understanding of natural attenuation process in
77 coastal environment, chemical, molecular and multivariate analyses were combined together.
78
79 **MATERIALS AND METHODS**
80 **Site and Sampling procedure.** The Berre lagoon (France) has a long history of oil
81 pollution, the north eastern area being described as the most contaminated of the lagoon,
82 particularly in the ten upper centimetres of the sediments (Jacquot *et al.*, 1999). Located in
83 this area (43°29'05''N; 5°11'17''E), the retention basin studied collected hydrocarbon
84 charged water from a petrochemical industry since several decades (Fig 1). Highly used up to
85 the 70's, it remains chronically supplied with contaminated rainfall water that flow from the
86 industry to the primary sedimentation tank and from the sedimentation tank to the retention
87 basin by overflowing system.
88 Samples were collected in May 2006 from nine stations screening the waste input
89 (EDB1), the basin periphery (EDB2 to EDB8), and water out of the basin (EDB9) (Fig. 1).
90 The station EDB1 is located in a restricted area limited by a floating barrier that retains the
91 floating hydrocarbons. Water samples were first collected at the nine stations for hydrocarbon
92 analysis. A thin beige mat of few millimetres cover the subsurface of black anoxic sediments.
93 This mat and sub-surface sediments were sampled together, quickly homogenized and frozen

94 in liquid nitrogen for both hydrocarbon and molecular analysis. Samples were stored at –80

95 °C until analysis.

96

97 **Chemical analysis.** Sediment samples (2 g) were chemically dried with 2 g of

98 anhydrous sodium sulphate (Na_2SO_4). Dried samples were extracted with 6 mL of

99 hexane:dichloromethane (1:1) by horizontal shaking at 150 oscillations per min over 16 h and

100 finally sonicated for 30 min at 20 °C. After centrifugation (5897 *g* for 20 minutes), extracts

101 were cleaned on Supelclean™ solid phase extraction (SPE) tubes as recommended by the

102 manufacturer (SPE Supelclean Envi™-18, Supelco Bellefonte, USA). Extracts were diluted

103 10 times for gas chromatography analysis.

104 Seawater samples (40 mL), collected in glass vials washed in acid solution and rinsed with

105 hexane, were extracted using SPE tubes as recommended by the manufacturer (SPE

106 Supelclean Envi™-18, Supelco Bellefonte, USA). After transfer of the water samples into the

107 SPE tubes, extracts were eluted with 5 mL of hexane: dichloromethane (1:1) and then

108 evaporated to 0.7 mL over an ice bath to minimize loss of semi-volatile low molecular weight

109 polycyclic aromatic hydrocarbons (PAH).

110 Deuterated alkanes (C_{10}^{d22}, C_{19}^{d40} and C_{30}^{d62}) and PAH (naphthalene^{d8}, anthracene^{d10},

111 chrysene^{d12} and perylene^{d12}) internal standards were added to the extracts at 0.5 µg mL^{-1} and

112 0.4 µg mL^{-1}, respectively. Aliphatic hydrocarbons and PAH were identified and quantified by

113 GC-MS using a Thermo Trace GC gas chromatograph coupled to a Thermo Trace DSQ®

114 mass spectrometer as described previously (Coulon *et al.*, 2007).

115 For quality control, a 2 ng µL^{-1} diesel standard solution (ASTM C_{12}-C_{60} quantitative, Supelco)

116 and a 1 ng µL^{-1} PAH Mix Standard solution (Supelco) were analyzed every 15 samples. The

117 recovery percentages of the extraction method used for sediment and seawater samples were

118 85% and 89%, respectively. The variation of the reproducibility of extraction and

119 quantification of samples were determined by successive extractions and injections (n = 6) of
120 the same sample and estimated to +/- 8% in both cases.
121
122 **DNA extraction.** Total genomic DNA extractions from environmental samples were
123 performed in triplicate using the alternative lysis method of the UltraClean™ Soil DNA
124 isolation kit (MOBIO Laboratories Inc., USA). The manufacturer's instructions were
125 followed except for the initial step of lysis where 500 µL of each sediment sample were
126 vortexed horizontally during 20 minutes. Then, purified DNA was suspended in 50 µL of
127 sterile water and examined by agarose gel electrophoresis. All extracted genomic DNA
128 samples were stored at –20 °C until further processing.
129
130 **PCR and T-RFLP analysis.** The fluorescently labelled primers used for PCR amplification
131 of bacterial 16S rRNA gene were TET 8F (5'-tetrachlorofluorescein phosphoramidite-
132 AGAGTTTGATCCTGGCTCAG-3') (Lane, 1991) and HEX 1489R (5'-hexachlorofluorescein
133 phosphoramidite-TACCTTGTTACGACTTCA-3') (Weisburg, 1991). PCR and T-RFLP
134 analysis were carried out as described previously (Bruneel *et al.*, 2006) using the Taq DNA
135 polymerase (Eurobio). The fluorescent PCR products were viewed on 1% w/v agarose gels,
136 cleaned with PCR purification kit (GE Healthcare) and 10 µl of purified product digested
137 separately with 3 U of enzyme *Hae*III, *Hin*fI or *Hpa*II for 3 h at 37°C (New England Biolabs).
138 1 µl of restriction digests were then mixed with 20 µl of deionized formamide and 0,5 µl of a
139 TAMRA labelled Genescan 500 bp internal size standard (Applied Biosystems), denatured for
140 5 min at 95°C, and immediately transferred to ice. Triplicate samples were loaded onto an
141 ABI PRISM 310 automated genetic analyser (Applied Biosystems). T-RFLP profiles were
142 aligned by identifying and grouping homologous fragments, and normalized by calculating
143 relative abundances of each T-RFs from height fluorescence intensity. Combining data from
144 each restriction enzyme, we compared normalized T-RFLP profiles. For statistical analysis,

145 the averages of height of terminal restriction fragments (T-RFs) detected in triplicates with
146 each endonuclease and each primer were used. In order to identify similarities between the
147 bacterial communities at the different sites, two-dimensional non-metric multidimensional
148 scaling ordination (2D-nMDS) based on presence/absence transformation and Bray Curtis
149 similarity of all treated T-RFLP data were carried out with Primer6 (Plymouth Routines In
150 Multivariate Ecological Research, version 6.1.6). Canonical Correspondence Analysis (CCA)
151 was performed to examine the influence of environmental variables (salinity, pH and
152 conductivity of water, hydrocarbon concentrations and distributions in water and sediment) on
153 the structure of bacterial communities using MVSP software (Multi-Variate Statistical
154 Package 3.12d, Kovach Computing Services, 1985-2001, UK).
155
156 **Clone library.** To characterize the bacterial populations inhabiting three stations of
157 the retention basin (EDB1, EDB2, EDB3), 16S rRNA genes were amplified and cloned using
158 unlabeled 8F and 1489R primers. The PCR products were cloned in *Escherichia coli*
159 TOP10F' (Invitrogen) using the pCR2.1 Topo TA cloning kit (Invitrogen, Inc.). Cloned 16S
160 rRNA gene fragments were amplified using M13 primers surrounding the cloning site. Inserts
161 were sequenced using the Big Dye® Terminator v3.1 cycle sequencing kit (Applied
162 Biosystems). Sequences (about 1400 bp) were first analysed using the CHECK CHIMERA
163 program on the RDP Database Project website (http://rdp8.cme.msu.edu/html/) (Cole *et al.*,
164 2003). Then the sequences were compared with the GenBank nucleotide database library by
165 BLAST on-line searches (Altschul *et al.*, 1997). Multiple sequence alignment of clones was
166 performed by using CLUSTALX (Thompson *et al.*, 1997) and PROcessor of SEQuences
167 v2.91 (Filatov, 2002). Phylogenies were constructed with the Molecular Evolutionary
168 Genetics Analysis v3.0 program (Kumar *et al.*, 2004) using Kimura two parameters model
169 and Neighbour-joining algorithm. Significance of branching order was determined by

170 bootstrap analysis with 1000 resampled data sets. PAST (PAleontological Statistics v1.60)
171 software from http://folk.uio.no/ohammer/past/ website was used to perform rarefaction
172 analysis and calculate diversity indices for each clone library with clone phenotype similarity
173 defined at 97% 16S rRNA sequence similarity. In order to determine the significance of
174 differences between the clone libraries, LIBSHUFF method was applied (Singleton *et al.*,
175 2001).
176
177 **Nucleotide sequence accession numbers.** The sequences determined in this study have
178 been submitted to the EMBL database and assigned Accession Nos. **AM882511** to
179 **AM882649**.
180
181 **RESULTS**
182 **Hydrocarbons distribution in the retention basin.** Total extractable hydrocarbon
183 content (THC) in water and sediment samples ranged between 25 and 109 µg L^{-1} and between
184 42 and 286 mg kg^{-1}, respectively (Fig. 2). About 70 compounds were found within the sub-
185 surface sediment of the different stations sampled including 29 alkanes ranging from C_{10} to
186 C_{36}, 38 PAHs and 3 conservative biomarkers, e.g. C_{30}-hopane. Except EDB1 station where
187 THC concentrations in sediment were lower than expected, the chemical analyses showed a
188 clear gradient of petroleum hydrocarbons contamination in water and sediment following the
189 basin periphery from EDB2 to EDB8 (Fig. 2). THC in water from EDB8 were similar to those
190 found in EDB9 showing the efficiency of this basin in hydrocarbon trapping, as well as the
191 possible influence of the lagoon water in the basin. For all stations, oil concentrations in
192 sediment were at least 1000 times higher than in water and hydrocarbon concentration
193 sediment/water ratios were five-fold higher at EDB2 than at EDB7 or EDB8.

194 The hydrocarbon composition in the different stations showed that alkanes represented
195 78 to 92% of the total hydrocarbon in water against 10 to 38% in sediment (data not shown).
196 Conversely, PAHs represented 8 to 22% of total hydrocarbon concentration in water against
197 61 to 89% in sediment (data not shown). Alkyl-homologues of phenanthrene and pyrene were
198 representing together more 80% of the PAHs within the sediment samples. Overall, the
199 hydrocarbon fingerprint analysis showed that THC concentration within the lagoon correlated
200 with the distance from the inlet where sedimentation and accumulation of the hydrocarbons
201 were clearly observed between the stations EDB2 and 8. Except to the station 8, the
202 diagnostic weathering ratio ∑chrysenes/∑phenanthrenes was unchanged at all stations
203 indicating that no weathering process was occurring within the sediment samples. However,
204 significant losses occurred in low molecular weight n-alkanes, as shown by the distribution
205 change of nC17 and pristane concentration in station EDB1 and EDB2 (Fig. 3).
206
207 **Bacterial community analysis by 16S rRNA gene T-RFLP fingerprinting.** T-RFLP
208 patterns analysis (Fig. 4) showed eight different bacterial community structure forming three
209 clusters with similarity up than 66%: A grouping EDB1 to EDB4, B grouping EDB5 to
210 EDB7, and C including only EDB8 community. Canonical correspondence analysis (CCA)
211 combining T-RFLP data and different environmental factors such as salinity, pH, conductivity
212 of water revealed no clear influence of these parameters in the bacterial community structure
213 of the samples (data not shown). Nevertheless, CCA analysis realised with T-RFLP data and
214 hydrocarbon concentrations in water and sediment indicated that water and sediment
215 hydrocarbon contents are the main measured variables that explain 32% of the bacterial
216 community distribution (Fig. 5A). The pattern of bacterial communities structure from EDB2
217 to EDB8 followed the oil sediment concentration axis while the bacterial community structure
218 of EDB1 seemed to be influenced by others environmental factors as oil content in water. The

219 impact of oil content on the bacterial community structure assessment have already been
220 suggested in coastal Mediterranean sediments of Lavera highly polluted by refinery effluents
221 (Mille et al., 1985).

222 When variables (T-RFs = OTUs operational Taxonomic Unit) were plotted on the CCA
223 (Fig. 5B), they fell into four clusters. Cluster 3 was related to the hydrocarbon distribution in
224 sediment (e.g. based on the alignment of variables along the THC sediment axis), and cluster
225 4 was probably influenced by others environmental factors. The OTUs of cluster 1 are all
226 specific to the EDB1 station and represent 15% of the total diversity and 5% of relative
227 abundance of this sample. In the same way, the OTUs of cluster 2 are all specific to the EDB2
228 site and represent about 4.5% of the total diversity of this sample and 1.2% of the relative
229 abundance.

230

231 **Composition of bacterial communities.** Hydrocarbon distribution and concentration as
232 well as bacterial community structure analysis showed that station EDB1 is particular and
233 different from the rest of the stations. Thus phylogenetic analysis of 202 clones of 16S rRNA
234 genes of the stations EDB1, EDB2 and EDB3 was performed in order to obtain a more
235 precise picture of the bacterial community structure. The rarefaction analysis was performed
236 in order to determine if the number of clones analysed was representative of the diversity.
237 Although the curves did not reach a plateau (data not shown), the homologous curves
238 obtained by comparison of EDB1, EDB2 and EDB3 clone libraries with LIBSHUFF method
239 (data not shown) indicated that libraries are representatives of the most abundant populations
240 in the original communities (high homologous coverage at evolutionary distance up to 0.05).
241 The comparison of the clone libraries with the LIBSHUFF method reveals also that 16S
242 rRNA gene sequences of EDB1 compared with those of EDB2 were composed of
243 significantly different phylotypes (XY_{12} p-value=0.010; YX_{12} p-value=0.323), while those of

244 EDB2 and EDB3 libraries were not significantly different (XY_{23} p-value=0.928; YX_{23} p-
245 value=0.075). The Smith and Wilson eveness diversity index was 3.724, 3.628 and 3.707 for
246 EDB1, EDB2 and EDB3 respectively, indicating the same level of complexity of the three
247 community stations despite the different and high concentrations of oil in these sediments.
248 These bacterial communities appeared adapted to the environmental stress.

249 The phylogenetic analysis of clone sequences obtained from the three libraries revealed
250 dominance of bacteria belonging to the *Proteobacteria* (*Gamma*-, *Delta*-, *Alpha*-, *Epsilon*-
251 and *Betaproteobacteria*), presence of bacteria belonging to *Bacteroidetes*, *Spirochaetes*,
252 *Verrucomicrobium*, *Actinobacteria*, *Cyanobacteria*, and the presence of diatoms (Fig. 6 and
253 Table 1). All these sequences were closed related to those found either in coastal water,
254 seawater or in microbial mats, polluted or not with petroleum hydrocarbons (Abed *et al.*, 2006
255 ; Brakstad & Lødeng, 2005 ; Coulon *et al.*, 2007 ; Gentile *et al.*, 2006 ; Van Gemerden,
256 1993). Approximately 1.5% of the sequences were affiliated with uncultured clones of
257 unclassified bacteria (candidate division JS1), 2.5% were defined as chimera and therefore
258 excluded of all analysis.

259 Clone sequences were mainly distributed in the *Gamma*- (37%) and
260 *Deltaproteobacteria* (33%), but in different proportions between the different sites (Fig. 6).
261 *Gammaproteobacteria* represented 29% of EDB1 total sequences whereas they represented
262 42 and 41% in EDB2 and EDB3 respectively. Conversely, *Deltaproteobacteria* represented
263 40% of EDB1 sequences while they were estimated to 30 and 31% in EDB2 and EDB3
264 respectively. Whatever the station, most of the acquired sequences from *Gamma*- and
265 *Deltaproteobacteria* were related to sulphur-oxidizing (*Olavius algarvensis* sulphur-oxidizing
266 endosymbiont, *Thyasira flexuosa* gill symbiont, *Alkalispirillum mobile*, *Thiobaca spp.*,
267 *Thiocapsa spp.*, *Halochromatium spp.* and *Amoebobacter spp.*) and sulphate-reducing
268 bacteria (*Desulfobacterium*, *Desulfosarcina*, *Desulfococcus*, *Desulfotignum*, *Bacteriovorax*

269 and *Anaeromyxobacter*) (Fig. 7A-7B). Sequences related to oil-degrading sulphate-reducing
270 bacteria (Fig. 7B) and *Spirochaeta smaragdinae* isolates from oil-polluted systems (Table 1)
271 were also found in the mat studied here. Few clone sequences related to well-known oil-
272 degrading bacteria under aerobic condition such as *Marinobacter spp.* were found.
273

274 **DISCUSSION**

275 Chemical analysis of water and sediment of the basin indicated hydrocarbon concentrations
276 similar to those previously found in this site (Hernández-Raquet *et al.*, 2006) and comparable
277 to those found in well-established polluted environment like in the Arabian gulf coasts one
278 year after the gulf war (20 to 369 mg kg^{-1} of dry sediment) (Al-Thukair, 2002), or those found
279 in the Prince William Sound (at historical industrial sites, i.e. not after the Exxon Valdez oil
280 spill; 1 to 151 mg kg^{-1} of PAHs) (Page *et al.*, 2006). The alkane/PAHs ratios in the basin
281 sediments are the converse of those previously found in the lagoon (Jacquot *et al.*, 1999),
282 suggesting a particular pollution of this basin different from the Berre lagoon pollution.
283 Hydrocarbon compounds were widely accumulated in all sediment stations, particularly
284 PAHs. It is well known that hydrocarbons, particularly PAHs, have low aqueous solubility
285 and high solid-water distribution ratios, which prevent their utilization by planktonic bacteria
286 and promote their accumulation in the solid phase of the terrestrial environment (Johnsen *et*
287 *al.*, 2005). However, the lower ratio of nC17/pristane observed in sediment at EDB1 and
288 EDB2 stations suggested that alkanes biodegradation process occurred. Although the purpose
289 of this retention basin is to increase sedimentation of oil effluent in order to limit oil diffusion
290 and facilitate abiotic and biotic degradation, PAH weathering diagnostic ratio indicated that
291 no degradation was occurring. PAHs might be sorbed and sequestered within the sediment
292 and not exposed either to abiotic loss or microbial attack. It is well known that cyanobacterial
293 mat grow extensively building thick laminated mats on the oiled sediment surface and sealing

294 the surface (Barth 2003, Bordenave *et al*., 2007). This phenomenon is also observed in the
295 retention basin (Fig. 1C). Consequently, oil is trapped in an anaerobic milieu in which oil
296 transformation can occur but at very slow rate (Garcia de Oteyza et Grimalt 2006). The
297 chemical analysis pointed also out that oil concentration in the sediment at EDB1 station was
298 lower than expected, while this station constituted the place of hydrocarbon input and was
299 limited by a floating barrier supposed to prevent hydrocarbon scattering.

300 Oil content of sediments of the retention basin provides an opportunity to
301 investigate how microbial communities differ along a hydrocarbon gradient in a coastal
302 ecosystem by T-RFLP analysis of 16S rRNA genes. Based on CCA, the amount of
303 hydrocarbon in the sediment has an effect on the microbial community structure. The
304 phylogenetic analysis of three analysed stations showed clones related to previously identified
305 oil-related SRBs, *Marinobacter spp.* and others. However, the CCA explains only 32% of the
306 data, others factors like fine sediment or organic matter carried out by the effluent are likely to
307 be involved in the assessment of the bacterial community structure either if sedimentation of
308 these kind of particles are supposed in the sedimentation tank (Fig. 1A). Previous study
309 concerning bacterial composition of the station EDB1 using DGGE and ARISA showed
310 specific microbial community associated to oil contamination level (Hernandez et al., 2006).
311 Enrichment culture of EDB1 sediment has permitted the identification of species involved in
312 oil degradation (Hernandez *et al*., 2006; Ranchou-Peyruse *et al*., 2004) although this
313 populations represent a minor fraction of the mat communities in the sediment of the Etang de
314 Berre retention basin (Hernandez *et al*., 2006). McKew *et al*. (2007) demonstrated in
315 microcosms experiment the quick activity of oil-degraders from environmental sample while
316 these organisms could not be detected in their environment. Mazella *et al*. (2005) have
317 already suggested that hydrocarbon-degraders able to degrade petroleum are present in low
318 proportion within the sediment. The impact of oil in the bacterial community structure is not

319 only associated to biodegradation (use oil as carbon source) but it can be associated to others
320 phenomena as toxicity, physical chemical changes, or others (Berge *et al*., 1987; Cochran *et*
321 *al*., 1998). Indeed, the sediments of the retention basin contain many other organic matter that
322 can be used easier than oil as carbon source by bacteria.

323 Bacteria populations associated to sulphur cycle has been found abundant as usually
324 in this kind of environments. Van Gemerden (1993) have already demonstrated the
325 importance of colourless sulphur bacteria, purple sulphur bacteria and sulphate-reducing
326 bacteria in promoting oxygen and sulphide microgradients in mats. Oil pollution is well
327 known to stimulate the sulphur cycle significantly (Kleikemper *et al.*, 2002 ; Lovley, 1997).
328 During the last decade, studies have shown the potential of coastal marine sediment for
329 anaerobic hydrocarbon degradation under sulphate-reducing conditions (Coates *et al.*, 1997 ;
330 Townsend *et al.*, 2003 ; Widdel & Rabus, 2001). Moreover, with the exception of the
331 uppermost layer, the bulk of organic matter-rich marine sediments contaminated by petroleum
332 hydrocarbons are anoxic (Canfield, 1993). Sequences related to oil-degrading sulphate
333 reducing bacteria found in the mat studied here reinforced the possible role of these
334 microorganisms in anaerobic oil degradation. Indeed, in marine reduced sediments,
335 hydrocarbon degradation coupled to sulphate-reduction is considered as the most relevant
336 metabolism among the different anaerobic processes (Rothermich *et al.*, 2002).

337 In conclusion the present study demonstrated a gradient of hydrocarbon contamination
338 in the retention basin and indicated that structure of the bacterial communities were correlated
339 with the hydrocarbon contamination level. The oil pollution constitute therefore a selection
340 pressure for these bacterial communities which seems furthermore well-adapted because of
341 their level of complexity. Nevertheless, this selection pressure doesn’t induce increase of oil
342 degrading bacteria as no obvious degradation process or many clones related to known-
343 degrading bacteria were identified. The statement of a specific bacterial community structure

344 associated to low hydrocarbon concentration in EDB1 sediment station was clearly displayed.
345 The role of this bacterial community in the unexpected low total hydrocarbon concentration
346 station should be clarified.
347

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354

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465 **TABLE LEGEND**

466 **Table 1:** Phylogenetic sequences other than *Gamma*- and *Deltaproteobacteria* identified in

467 16S rRNA gene libraries.

Phylogenetic group	Closest match	Accession No of closest organism	Sequence identity (%)
Alphaproteobacteria	*Shingomonas* sp. 86 [O]	AY177366	85
	Hyphomicrobium vulgare ATCC27500	Y14302	90
	Pseudoruegeria aquimaris SW-255	DQ675021	93
Betaproteobacteria	Uncultured *Acidovorax* sp. clone DS137	DQ234219	92
Epsilonproteobacteria	*Sulfurimonas paralvinella* [An]	AB252048	90-92
	Sulfurospirillum sp. SM-5	U85965	99
	Uncultured epsilon XME15	EF061977	93-98
Bacteroïdetes	*Cytophaga* sp. BHI80-3	AJ431238	94-95
Spirochaeta	*Spirochaeta smaragdinae* [O/An]	U80597	90-96
Actinobacteria	Uncultured *Rubrobacteraceae clone Elev_16S_1016*	EF019514	91
Verrucomicrobia	Uncultured *Verrucomicrobia* bacterium clone LD1-PA15 [An]	AY114312	80-97
Cyanobacteria	*Synechococcus* sp. HO	AF448075	93
Diatoms chloroplast	*Nitzschia frustulum*	AY221721	99
	Odontella sinensis	Z67753	98
	Phaeodactylum tricornutum	EF067920	99
Unclassified bacteria	Uncultured hydrocarbon seep bacterium GCA025 [O]	AF154106	82-99

[O] : isolated from hydrocarbon polluted system [An] : anaerobic bacteria

468

469 Table 2: composition of the bacterial communities of the stations EDB1, EDB2 and EDB3

470

	Abundance of clones (%)		
Affiliation group	EDB1	EDB2	EDB3
Alphaproteobacteria	1.28	1.52	1.72
Deltaproteobacteria	38.46	28.79	31.03
Gammaproteobacteria	28.21	40.91	41.38
Betaproteobacteria	0	0	1.72
Epsilonproteobacteria	11.54	0	3.45
Bacteroidetes	2.56	4.55	0
Actinobacterium	0	1.52	1.72
Verrucomicrobia group	1.28	1.52	8.62
Spirochaeta	1.28	1.52	0
Cyanobacteria	1.28	0	0
Eukaryota	14.10	21.21	10.34

471

472 **FIGURES LEGENDS**

473 **Fig. 1. (A)** Water treatment unit of the effluent coming from the petrochemical factory (a)
474 comprising primary sedimentation tank (b), retention basin (c) where the eight sampling
475 stations are indicated. The ninth sampling station was localised out of the retention basin in
476 the lagoon (d). **(B)** Over-oxygenated cyanobacterial mat developing at the sediment surface of
477 the retention basin. O_2 bubbles can be observed at the surface of the mat. **(C)** Black anoxic
478 sediments and oil floating at the water surface revealed after perturbation of the sediments.

479

480 **Fig. 2.** Total petroleum hydrocarbons concentrations in water and sediment of the Berre
481 retention basin at the different stations.

482

483 **Fig. 3.** nC17 and Pristane concentrations in the water and sediment from the eight sediment
484 stations of the retention basin.

485

486 **Fig. 4.** Bacterial community structure assemblage of the different stations. nMDS map based
487 on the analysis of T-RFLP patterns of PCR-amplified 16S rRNA gene. Percent similarity
488 represents the similarity between T-RFLP profiles, based on the presence of common T-RFs
489 within the profiles (Bray Curtis similarity). Plain and dashed lines represent respectively 66 %
490 and 71 % of similarity. The stations with similarities up to 66% are grouped in three clusters,
491 A, B, and C.

492

493 **Fig. 5.** CCA between the bacterial communities of each station (A) or the variables (B) and
494 THC in sediment and water. The bacterial community structure is represented using T-RFLP
495 profiles.

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497 **Fig. 6.** Phylogenetic tree showing the affiliation of clones to (A) *Gammaproteobacteria* and
498 (B) *Deltaproteobacteria* sequences. The distances were calculated by the Kimura 2-parameter
499 algorithm. Percentages of 1000 bootstrap resampling that supported the branching orders in
500 each analysis are shown above or near the relevant nodes (only values >50% are shown).
501 Phylogenetic trees were constructed using free Mega3 software after alignment with ClutalX
502 and ProSeq2 correction. o : oil degrading bacteria

503 Fig 1.

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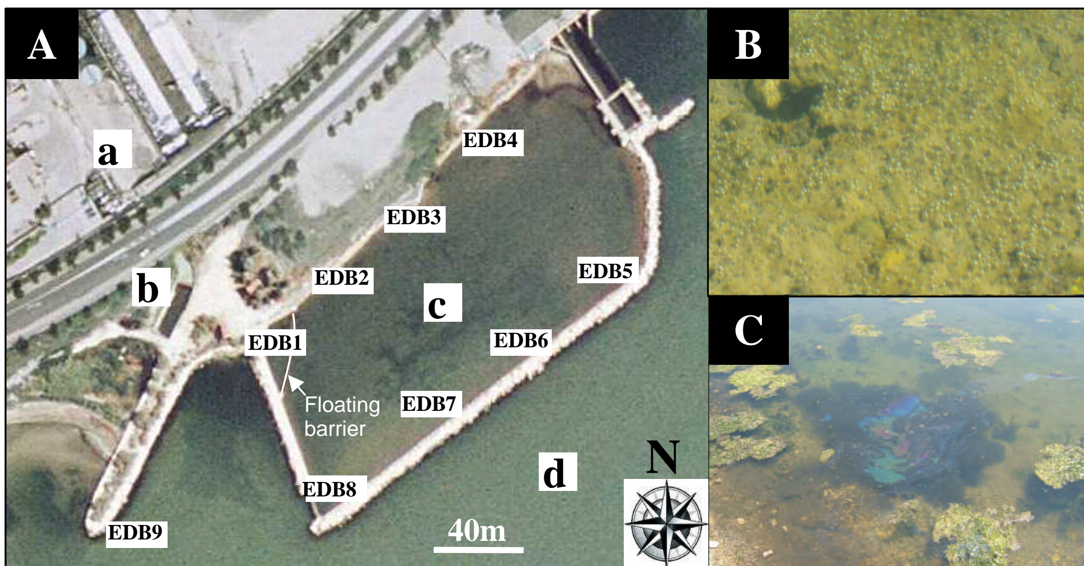

543 Fig 2.

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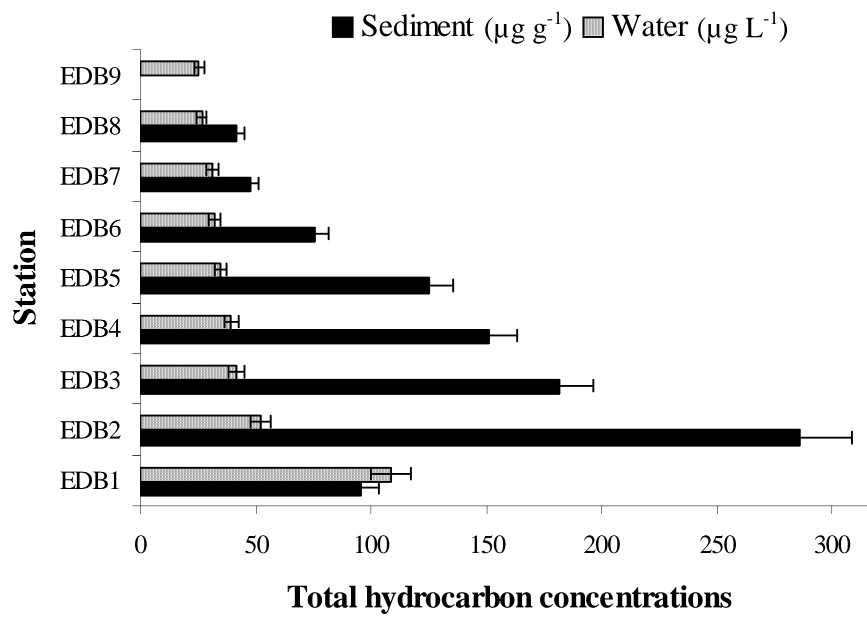

550 Fig 3.

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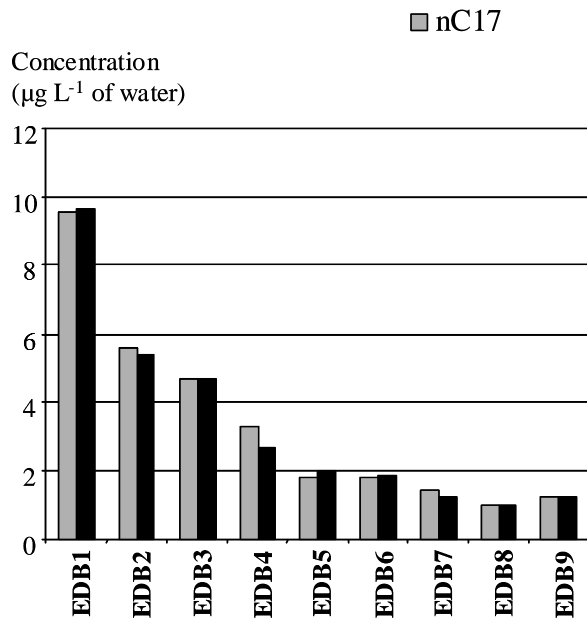

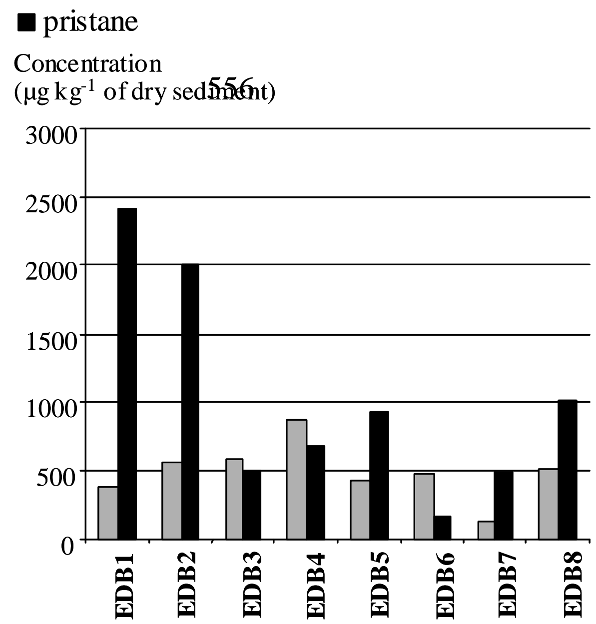

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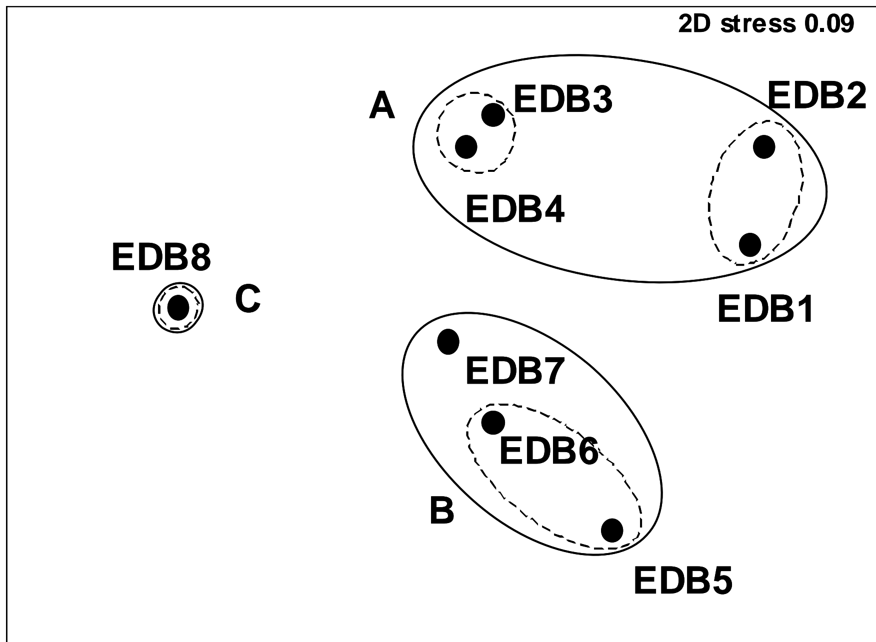
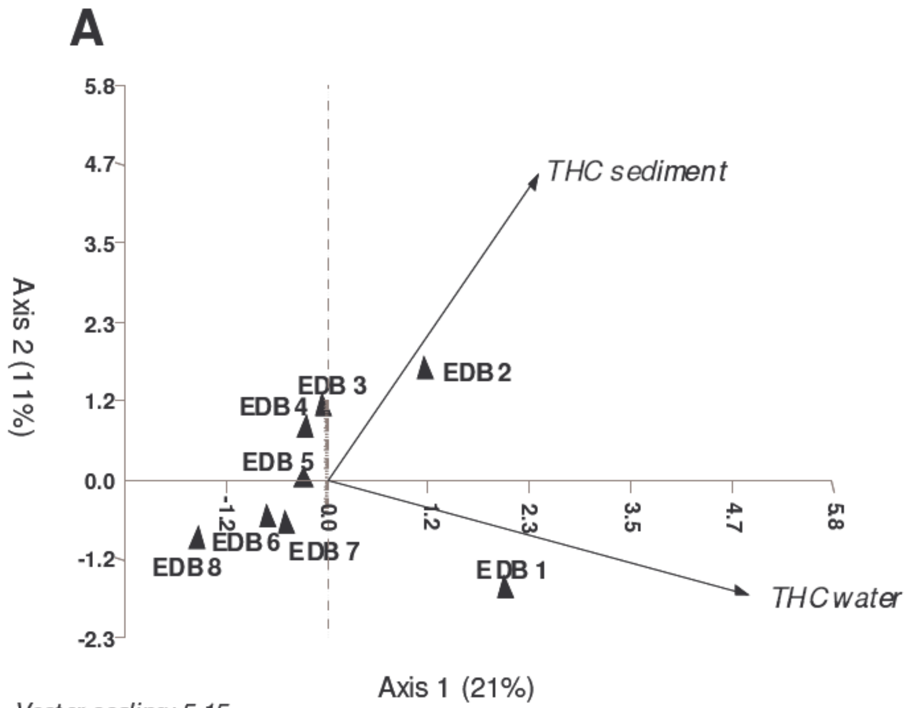


558 Fig 5.



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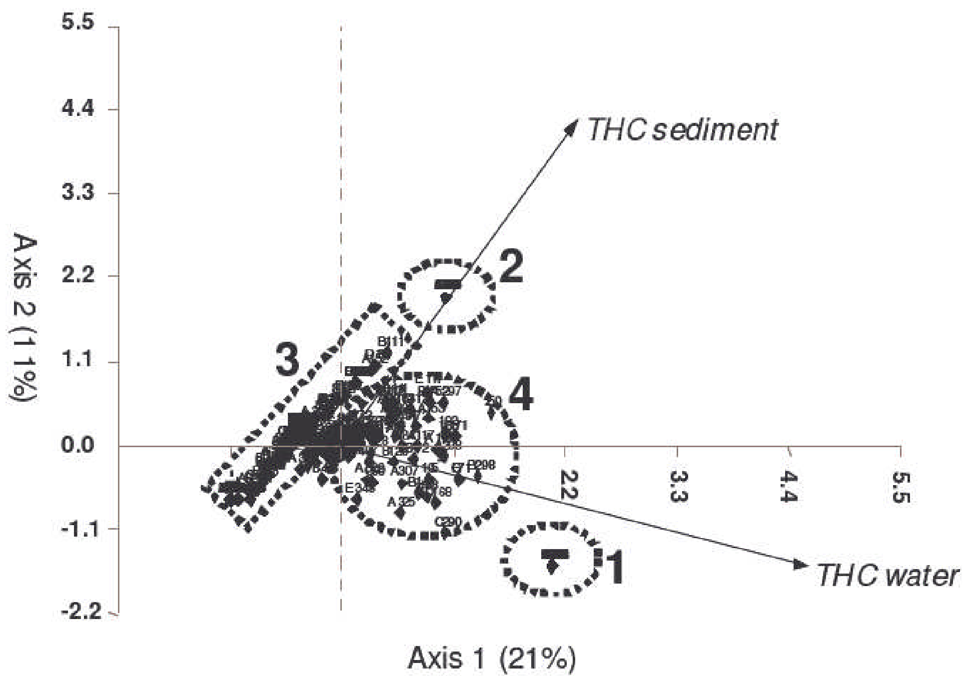

563 Fig 6.

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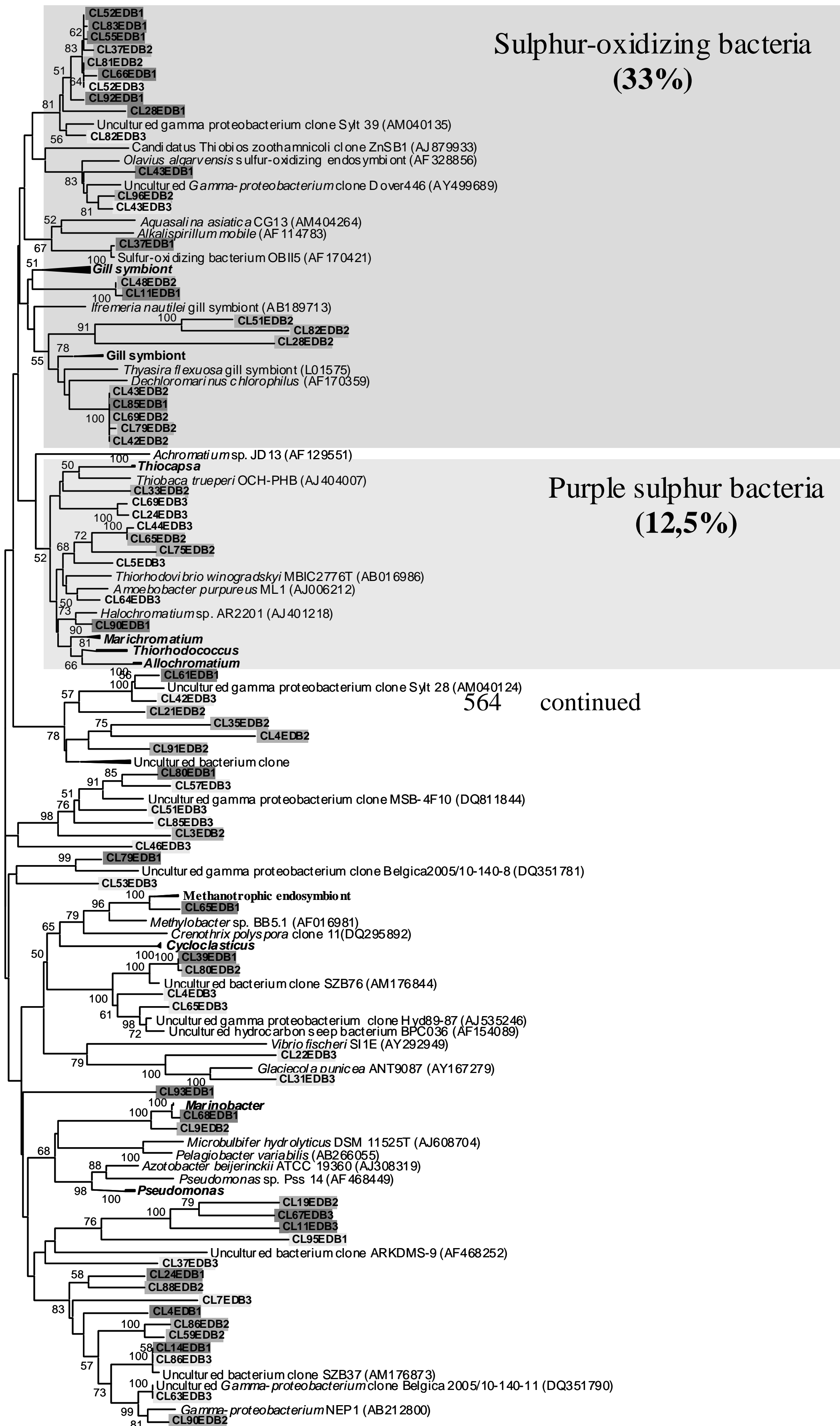

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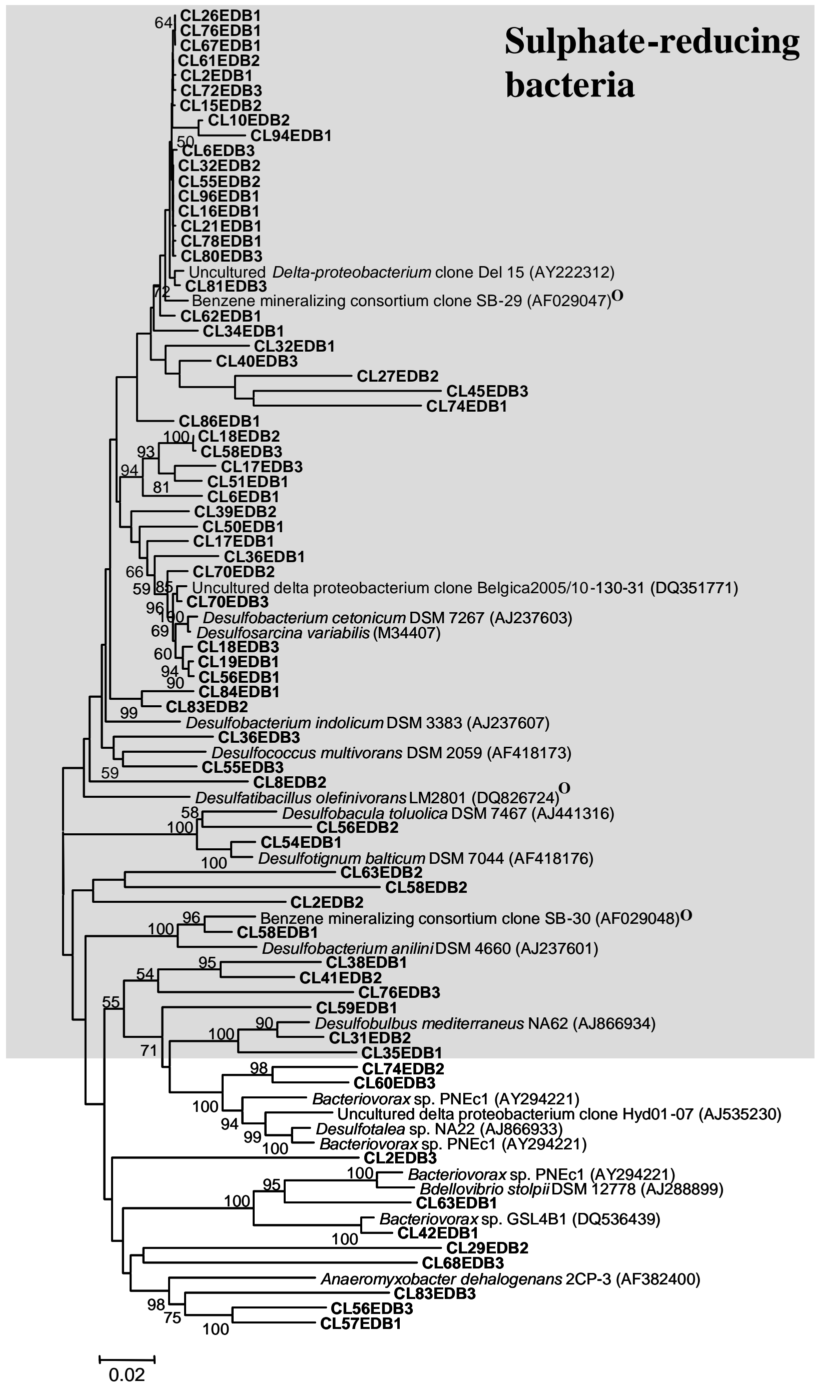

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