

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

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ABSTRACT

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

INTRODUCTION

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

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

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

reducing bacteria is frequently observed (Van Gernerden, 1993). We have described the structure of pristine hypersaline microbial mat communities of Salins-de-Giraud (Fourçans *et al.*, 2004), their dynamics (Fourçans *et al.*, 2006) and their capacity to degrade crude oil when maintained in microcosms (Bordenave *et al.*, 2004). Recently, we started to characterize microbial mats developed in restricted oil input area suggesting the possible effects of petroleum on microbial community composition (Hernández-Raquet *et al.*, 2006). To further investigate the potential impact of petroleum hydrocarbons on microbial community structure and diversity and hence provide a better understanding of natural attenuation process in coastal environment, chemical, molecular and multivariate analyses were combined together.

MATERIALS AND METHODS

Site and Sampling procedure. The Berre lagoon (France) has a long history of oil pollution, the north eastern area being described as the most contaminated of the lagoon, particularly in the ten upper centimetres of the sediments (Jacquot *et al.*, 1999). Located in this area (43°29'05''N; 5°11'17''E), the retention basin studied collected hydrocarbon charged water from a petrochemical industry since several decades (Fig 1). Highly used up to the 70's, it remains chronically supplied with contaminated rainfall water that flow from the industry to the primary sedimentation tank and from the sedimentation tank to the retention basin by overflowing system.

Samples were collected in May 2006 from nine stations screening the waste input (EDB1), the basin periphery (EDB2 to EDB8), and water out of the basin (EDB9) (Fig. 1). The station EDB1 is located in a restricted area limited by a floating barrier that retains the floating hydrocarbons. Water samples were first collected at the nine stations for hydrocarbon analysis. A thin beige mat of few millimetres cover the subsurface of black anoxic sediments. This mat and sub-surface sediments were sampled together, quickly homogenized and frozen

in liquid nitrogen for both hydrocarbon and molecular analysis. Samples were stored at -80°C until analysis.

Chemical analysis. Sediment samples (2 g) were chemically dried with 2 g of anhydrous sodium sulphate (Na_2SO_4). Dried samples were extracted with 6 mL of hexane:dichloromethane (1:1) by horizontal shaking at 150 oscillations per min over 16 h and finally sonicated for 30 min at 20°C . After centrifugation (5897 g for 20 minutes), extracts were cleaned on Supelclean™ solid phase extraction (SPE) tubes as recommended by the manufacturer (SPE Supelclean Envi™-18, Supelco Bellefonte, USA). Extracts were diluted 10 times for gas chromatography analysis.

Seawater samples (40 mL), collected in glass vials washed in acid solution and rinsed with hexane, were extracted using SPE tubes as recommended by the manufacturer (SPE Supelclean Envi™-18, Supelco Bellefonte, USA). After transfer of the water samples into the SPE tubes, extracts were eluted with 5 mL of hexane: dichloromethane (1:1) and then evaporated to 0.7 mL over an ice bath to minimize loss of semi-volatile low molecular weight polycyclic aromatic hydrocarbons (PAH).

Deuterated alkanes ($\text{C}_{10}^{\text{d}22}$, $\text{C}_{19}^{\text{d}40}$ and $\text{C}_{30}^{\text{d}62}$) and PAH (naphthalene^{d8}, anthracene^{d10}, chrysene^{d12} and perylene^{d12}) internal standards were added to the extracts at $0.5\text{ }\mu\text{g mL}^{-1}$ and $0.4\text{ }\mu\text{g mL}^{-1}$, respectively. Aliphatic hydrocarbons and PAH were identified and quantified by GC-MS using a Thermo Trace GC gas chromatograph coupled to a Thermo Trace DSQ® mass spectrometer as described previously (Coulon *et al.*, 2007).

For quality control, a $2\text{ ng }\mu\text{L}^{-1}$ diesel standard solution (ASTM $\text{C}_{12}\text{-C}_{60}$ quantitative, Supelco) and a $1\text{ ng }\mu\text{L}^{-1}$ PAH Mix Standard solution (Supelco) were analyzed every 15 samples. The recovery percentages of the extraction method used for sediment and seawater samples were 85% and 89%, respectively. The variation of the reproducibility of extraction and

quantification of samples were determined by successive extractions and injections (n = 6) of the same sample and estimated to +/- 8% in both cases.

DNA extraction. Total genomic DNA extractions from environmental samples were performed in triplicate using the alternative lysis method of the UltraClean™ Soil DNA isolation kit (MOBIO Laboratories Inc., USA). The manufacturer's instructions were followed except for the initial step of lysis where 500 µL of each sediment sample were vortexed horizontally during 20 minutes. Then, purified DNA was suspended in 50 µL of sterile water and examined by agarose gel electrophoresis. All extracted genomic DNA samples were stored at -20 °C until further processing.

PCR and T-RFLP analysis. The fluorescently labelled primers used for PCR amplification of bacterial 16S rRNA gene were TET 8F (5'-tetrachlorofluorescein phosphoramidite-AGAGTTTGATCCTGGCTCAG-3') (Lane, 1991) and HEX 1489R (5'-hexachlorofluorescein phosphoramidite-TACCTTGTTACGACTTCA-3') (Weisburg, 1991). PCR and T-RFLP analysis were carried out as described previously (Bruneel *et al.*, 2006) using the Taq DNA polymerase (Eurobio). The fluorescent PCR products were viewed on 1% w/v agarose gels, cleaned with PCR purification kit (GE Healthcare) and 10 µl of purified product digested separately with 3 U of enzyme *Hae*III, *Hinf*I or *Hpa*II for 3 h at 37°C (New England Biolabs). 1 µl of restriction digests were then mixed with 20 µl of deionized formamide and 0,5 µl of a TAMRA labelled Genescan 500 bp internal size standard (Applied Biosystems), denatured for 5 min at 95°C, and immediately transferred to ice. Triplicate samples were loaded onto an ABI PRISM 310 automated genetic analyser (Applied Biosystems). T-RFLP profiles were aligned by identifying and grouping homologous fragments, and normalized by calculating relative abundances of each T-RFs from height fluorescence intensity. Combining data from each restriction enzyme, we compared normalized T-RFLP profiles. For statistical analysis,

the averages of height of terminal restriction fragments (T-RFs) detected in triplicates with each endonuclease and each primer were used. In order to identify similarities between the bacterial communities at the different sites, two-dimensional non-metric multidimensional scaling ordination (2D-nMDS) based on presence/absence transformation and Bray Curtis similarity of all treated T-RFLP data were carried out with Primer6 (Plymouth Routines In Multivariate Ecological Research, version 6.1.6). Canonical Correspondence Analysis (CCA) was performed to examine the influence of environmental variables (salinity, pH and conductivity of water, hydrocarbon concentrations and distributions in water and sediment) on the structure of bacterial communities using MVSP software (Multi-Variate Statistical Package 3.12d, Kovach Computing Services, 1985-2001, UK).

Clone library. To characterize the bacterial populations inhabiting three stations of the retention basin (EDB1, EDB2, EDB3), 16S rRNA genes were amplified and cloned using unlabeled 8F and 1489R primers. The PCR products were cloned in *Escherichia coli* TOP10F' (Invitrogen) using the pCR2.1 Topo TA cloning kit (Invitrogen, Inc.). Cloned 16S rRNA gene fragments were amplified using M13 primers surrounding the cloning site. Inserts were sequenced using the Big Dye[®] Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequences (about 1400 bp) were first analysed using the CHECK CHIMERA program on the RDP Database Project website (<http://rdp8.cme.msu.edu/html/>) (Cole *et al.*, 2003). Then the sequences were compared with the GenBank nucleotide database library by BLAST on-line searches (Altschul *et al.*, 1997). Multiple sequence alignment of clones was performed by using CLUSTALX (Thompson *et al.*, 1997) and PROcessor of SEquences v2.91 (Filatov, 2002). Phylogenies were constructed with the Molecular Evolutionary Genetics Analysis v3.0 program (Kumar *et al.*, 2004) using Kimura two parameters model and Neighbour-joining algorithm. Significance of branching order was determined by

bootstrap analysis with 1000 resampled data sets. PAST (PAleontological Statistics v1.60) software from <http://folk.uio.no/ohammer/past/> website was used to perform rarefaction analysis and calculate diversity indices for each clone library with clone phenotype similarity defined at 97% 16S rRNA sequence similarity. In order to determine the significance of differences between the clone libraries, LIBSHUFF method was applied (Singleton *et al.*, 2001).

Nucleotide sequence accession numbers. The sequences determined in this study have been submitted to the EMBL database and assigned Accession Nos. **AM882511** to **AM882649**.

RESULTS

Hydrocarbons distribution in the retention basin. Total extractable hydrocarbon content (THC) in water and sediment samples ranged between 25 and 109 $\mu\text{g L}^{-1}$ and between 42 and 286 mg kg^{-1} , respectively (Fig. 2). About 70 compounds were found within the sub-surface sediment of the different stations sampled including 29 alkanes ranging from C_{10} to C_{36} , 38 PAHs and 3 conservative biomarkers, e.g. C_{30} -hopane. Except EDB1 station where THC concentrations in sediment were lower than expected, the chemical analyses showed a clear gradient of petroleum hydrocarbons contamination in water and sediment following the basin periphery from EDB2 to EDB8 (Fig. 2). THC in water from EDB8 were similar to those found in EDB9 showing the efficiency of this basin in hydrocarbon trapping, as well as the possible influence of the lagoon water in the basin. For all stations, oil concentrations in sediment were at least 1000 times higher than in water and hydrocarbon concentration sediment/water ratios were five-fold higher at EDB2 than at EDB7 or EDB8.

The hydrocarbon composition in the different stations showed that alkanes represented 78 to 92% of the total hydrocarbon in water against 10 to 38% in sediment (data not shown). Conversely, PAHs represented 8 to 22% of total hydrocarbon concentration in water against 61 to 89% in sediment (data not shown). Alkyl-homologues of phenanthrene and pyrene were representing together more 80% of the PAHs within the sediment samples. Overall, the hydrocarbon fingerprint analysis showed that THC concentration within the lagoon correlated with the distance from the inlet where sedimentation and accumulation of the hydrocarbons were clearly observed between the stations EDB2 and 8. Except to the station 8, the diagnostic weathering ratio $\sum\text{chrysenes}/\sum\text{phenanthrenes}$ was unchanged at all stations indicating that no weathering process was occurring within the sediment samples. However, significant losses occurred in low molecular weight n-alkanes, as shown by the distribution change of nC17 and pristane concentration in station EDB1 and EDB2 (Fig. 3).

Bacterial community analysis by 16S rRNA gene T-RFLP fingerprinting. T-RFLP patterns analysis (Fig. 4) showed eight different bacterial community structure forming three clusters with similarity up than 66%: A grouping EDB1 to EDB4, B grouping EDB5 to EDB7, and C including only EDB8 community. Canonical correspondence analysis (CCA) combining T-RFLP data and different environmental factors such as salinity, pH, conductivity of water revealed no clear influence of these parameters in the bacterial community structure of the samples (data not shown). Nevertheless, CCA analysis realised with T-RFLP data and hydrocarbon concentrations in water and sediment indicated that water and sediment hydrocarbon contents are the main measured variables that explain 32% of the bacterial community distribution (Fig. 5A). The pattern of bacterial communities structure from EDB2 to EDB8 followed the oil sediment concentration axis while the bacterial community structure of EDB1 seemed to be influenced by others environmental factors as oil content in water. The

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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TABLE LEGEND

Table 1: Phylogenetic sequences other than *Gamma*- and *Deltaproteobacteria* identified in 16S rRNA gene libraries.

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

^O : isolated from hydrocarbon polluted system ^{An} : anaerobic bacteria

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

470

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

471

FIGURES LEGENDS

Fig. 1. (A) Water treatment unit of the effluent coming from the petrochemical factory (a) comprising primary sedimentation tank (b), retention basin (c) where the eight sampling stations are indicated. The ninth sampling station was localised out of the retention basin in the lagoon (d). **(B)** Over-oxygenated cyanobacterial mat developing at the sediment surface of the retention basin. O₂ bubbles can be observed at the surface of the mat. **(C)** Black anoxic sediments and oil floating at the water surface revealed after perturbation of the sediments.

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

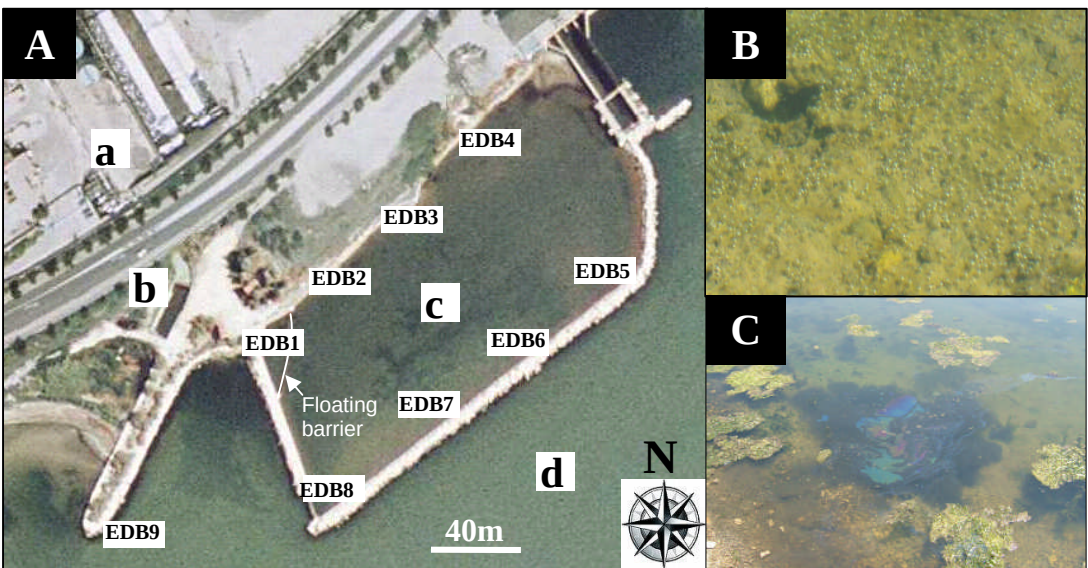
Fig. 3. nC₁₇ and Pristane concentrations in the water and sediment from the eight sediment stations of the retention basin.

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

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

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

Fig 1.



543 Fig 2.

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547

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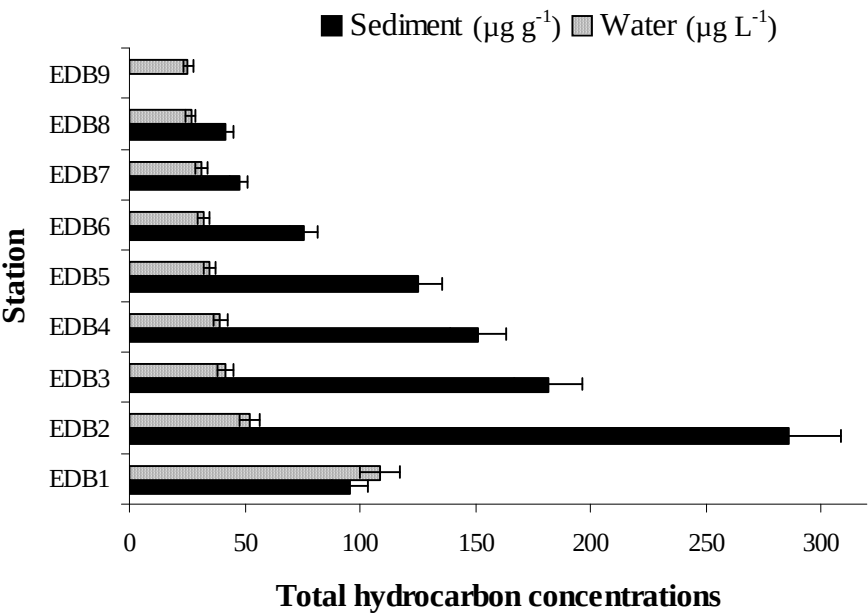
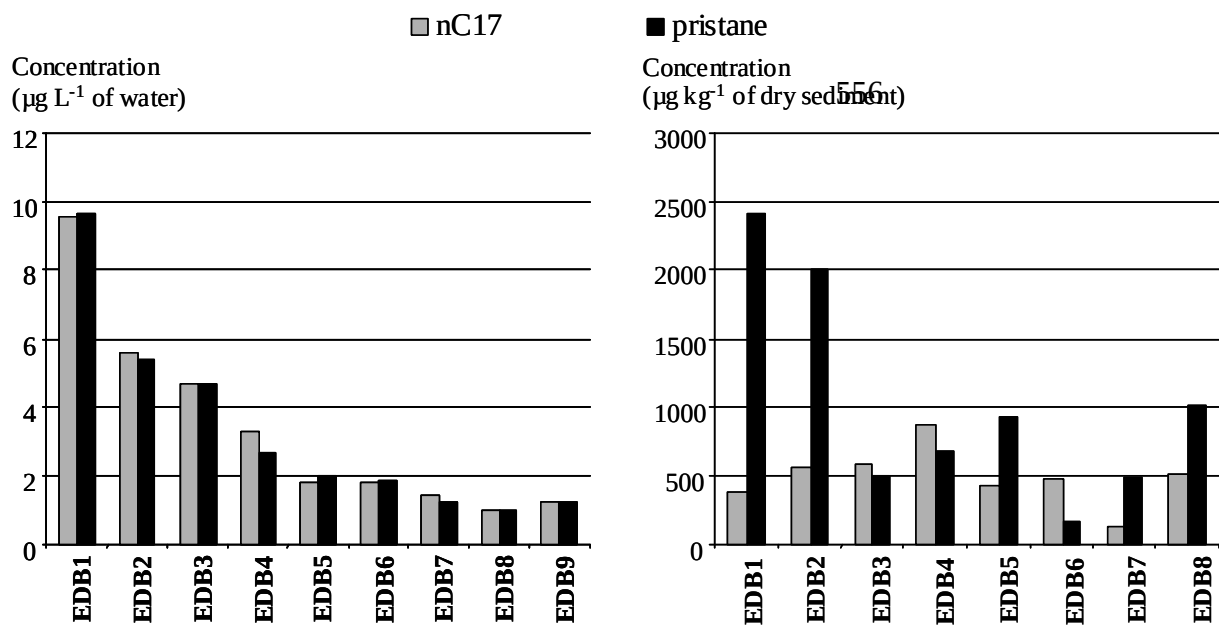
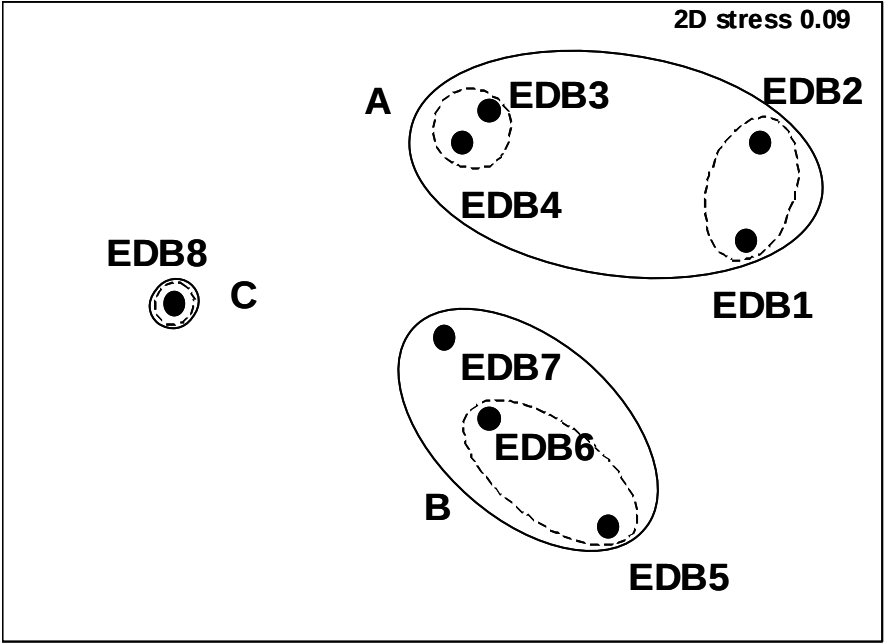
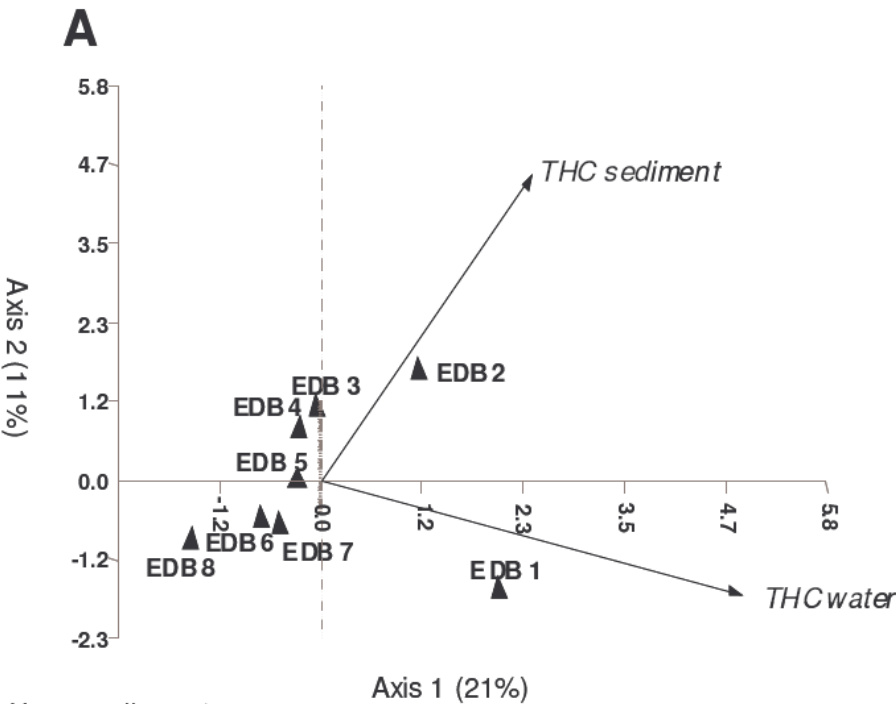


Fig 3.



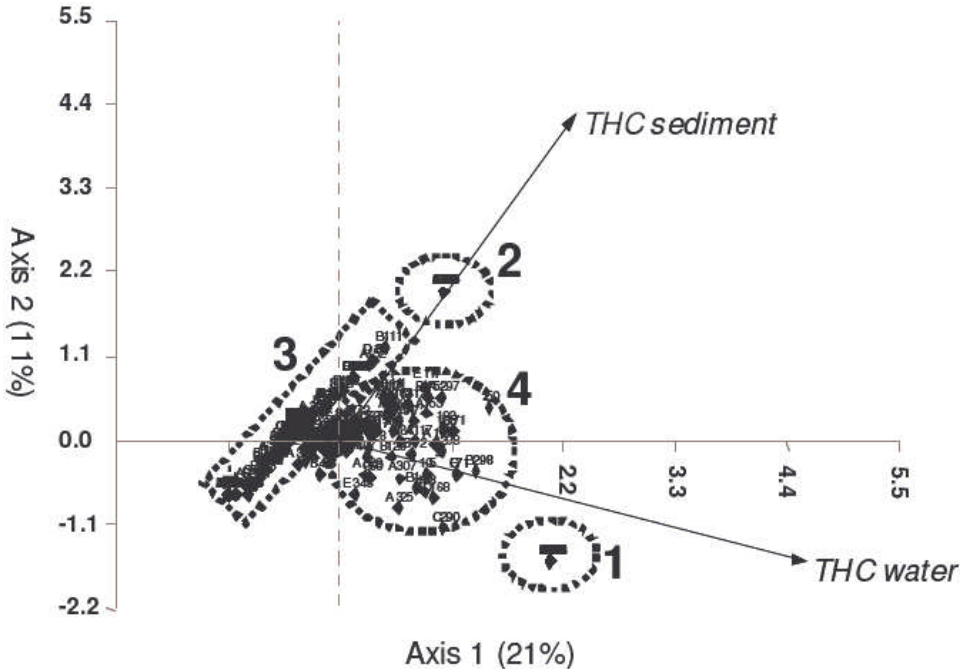


558 Fig 5.



560 Vector scaling: 5, 15

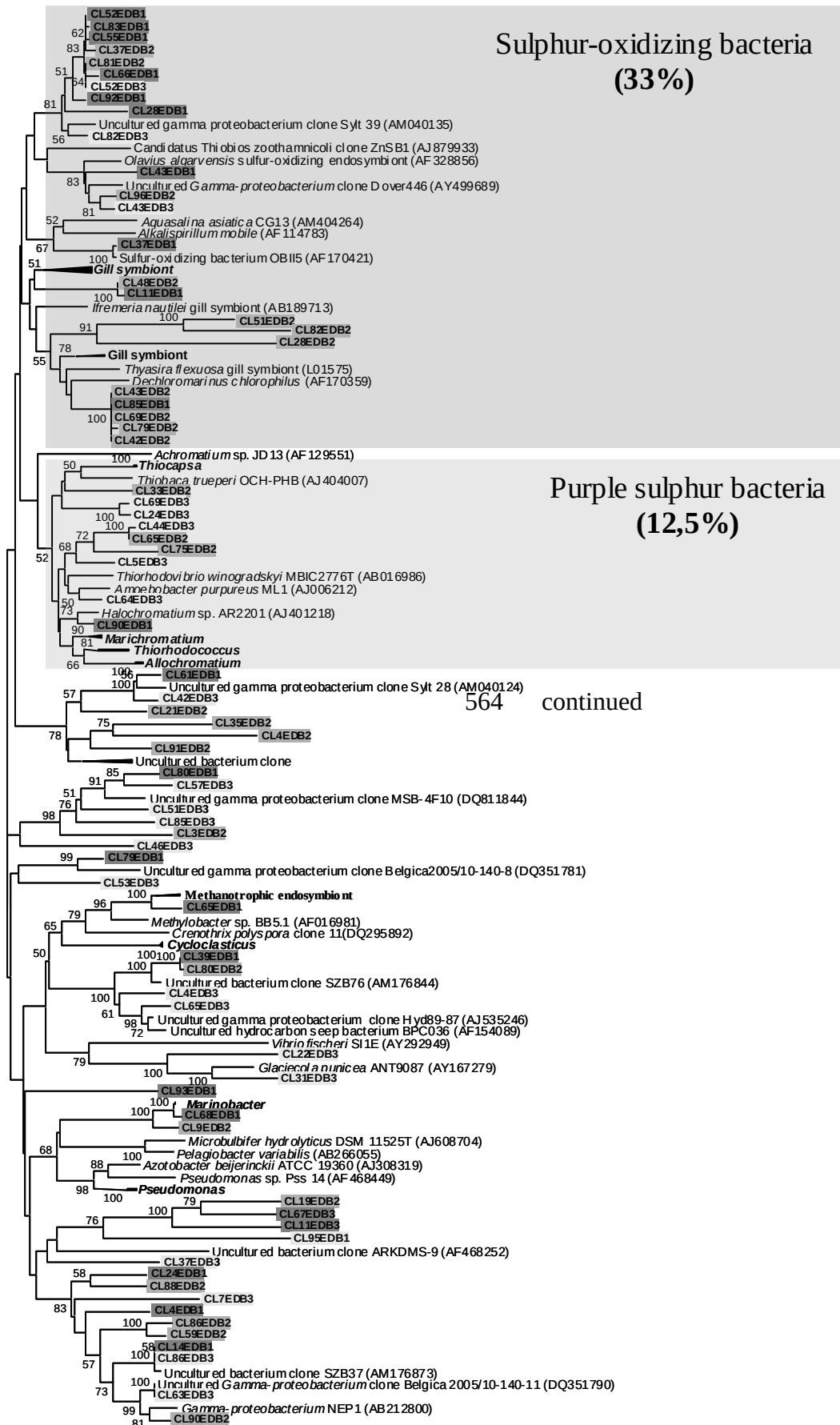
562 **B**



Vector scaling: 4, 86

Fig 6.

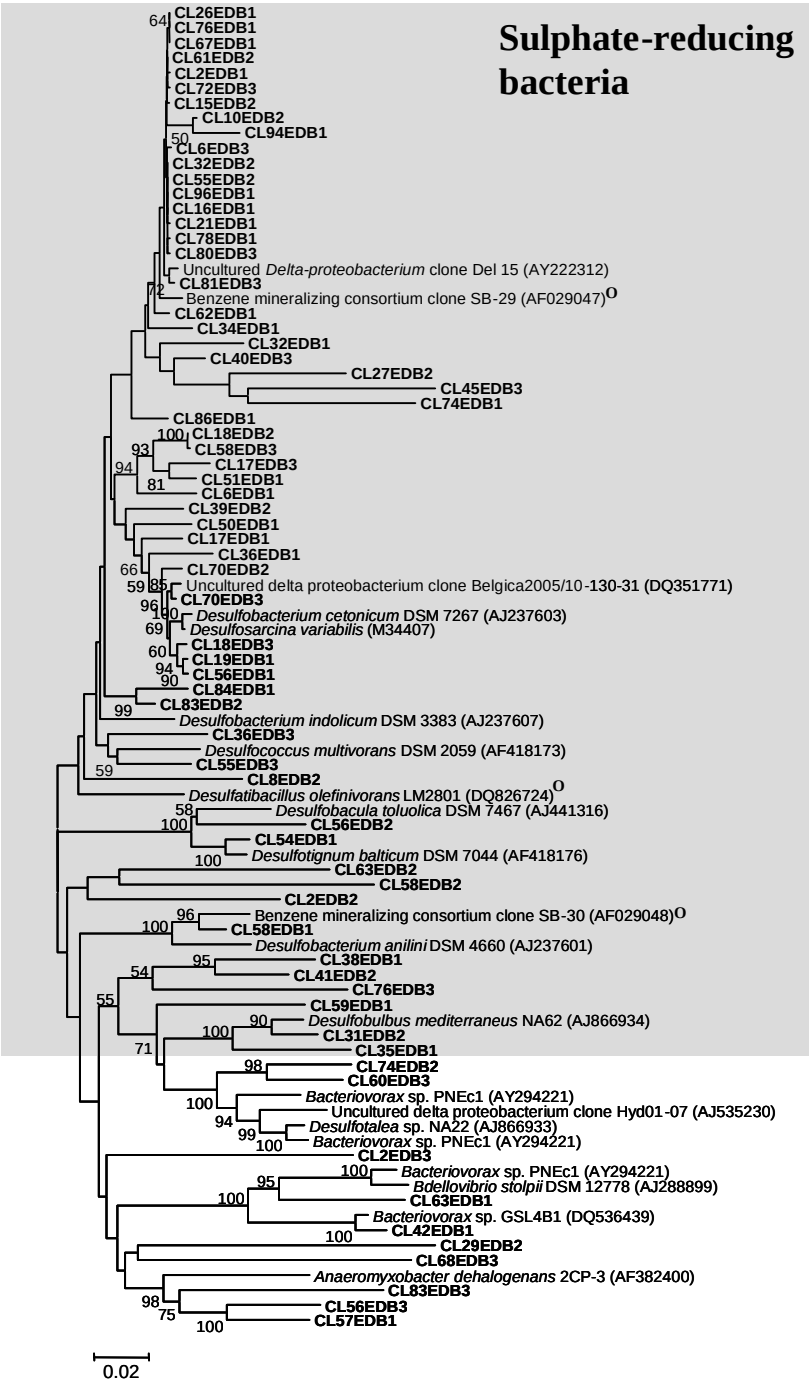
A



continued

0.02

B





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Structure of sediment-associated bacterial communities along a hydrocarbon contamination gradient in coastal sediment

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