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The impact of long-term hydrocarbon exposure on the structure, activity, and biogeochemical functioning of microbial mats

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ABSTRACT

Photosynthetic microbial mats are metabolically structured systems driven by solar light. They are ubiquitous and can grow in hydrocarbon-polluted sites. Our aim is to determine the impact of chronic hydrocarbon contamination on the structure, activity, and functioning of a microbial mat. We compared it to an uncontaminated mat harboring similar geochemical characteristics. The mats were sampled in spring and fall for 2 years. Seasonal variations were observed for the reference mat: sulfur cycle-related bacteria dominated spring samples, while *Cyanobacteria* dominated in autumn. The contaminated mat showed minor seasonal variation; a progressive increase of *Cyanobacteria* was noticed, indicating a perturbation of the classical seasonal behavior. Hydrocarbon content was the main factor explaining the differences in the microbial community structure; however, hydrocarbonoclastic bacteria were among rare or transient Operational Taxonomic Units (OTUs) in the contaminated mat. We suggest that in long-term contaminated systems, hydrocarbonoclastic bacteria cannot be considered a sentinel of contamination.

1. Introduction

Microbial mats dominated by *Cyanobacteria* are considered to be the oldest structured ecosystem on Earth (Margulis et al., 1980). They are nearly auto-sufficient systems in terms of nutrient cycling, containing high taxonomic and metabolic diversity within few-millimeter scales (Bolhuis and Stal, 2011). Microbial populations within a mat are stratified by steep light, oxygen, sulfide, and pH gradients; in turn, their metabolisms participate in maintaining these gradients. *Cyanobacteria* play key roles within a photosynthetic mat. They are precursors of microbial mats, since they fix dinitrogen and carbon dioxide, supporting the community growth (Severin and Stal, 2010). Other functional groups in photosynthetic microbial mats are aerobic/anaerobic heterotrophs, fermenters, sulfide oxidizers, and methanogens (van Gemerden, 1993). The structure of the mats changes depending on season, as purple sulfur bacteria increase at the end of winter and spring and cyanobacteria at the end of summer and fall (Pinckney et al., 1996). Microbial mats can be found in a diverse range of environments around the world, including but not limited to hot springs (Coman et al., 2013; Roeselers et al., 2007), polar ponds (Vincent and Whitton, 2002), and hypersaline waters (Allen et al., 2009; Dillon et al., 2009; Schneider et al., 2013). They also have been observed in petroleum-contaminated areas (Barth, 2003; Paissé et al., 2008).

Previous studies on hydrocarbon-contaminated microbial mats showed a reduction of microbial diversity in the short term associated with the disappearance of certain groups of microorganisms and a strong selection for specialist hydrocarbon-degrading marine bacteria (*i.e.*, *Alcanivorax* and *Marinobacter*) (Abed et al., 2007; Bordenave et al., 2007). *Alcanivorax* belong to the obligate hydrocarbonoclastic bacteria, which are known to feed exclusively on hydrocarbons (Yakimov et al., 2007). Other obligate hydrocarbonoclastic bacteria are *Cycloclasticus* spp., *Oleiphilus* spp., *Oleispira* spp., *Thalassolituus* spp., and some members of the genus *Planomicrobium*. *Alcanivorax* and related alkane degraders have been detected rapidly after an oil input (Head et al.,

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2006). *Cycloclasticus* spp., an aromatic hydrocarbon degrader, was shown to appear at later stages, when the alkanes have been degraded (Head et al., 2006). Microbial diversity in chronically or long-term hydrocarbon-polluted microbial mats is less documented. High diversity has been observed in chronically hydrocarbon-polluted microbial mats even though 16S rRNA gene sequences related to well-known hydrocarbon degraders (*i.e.*, *Alcanivorax* and *Cycloclasticus*) were not (or rarely) detected (Paissé et al., 2008). Finally, microbial mats show a high resilience capacity after petroleum contamination (Bordenave et al., 2007), suggesting that the petroleum impact is only transient and that the mat structure and functioning are robust enough to recover.

The archaeal community has been tackled rarely in hydrocarbon-polluted environments. In oiled sediments, the *Euryarchaeota* dominated, with particular contributions of *Methanococcoides*, *Methanosarcina*, and *Methanolobus* sequences (Miralles et al., 2010). The *Euryarchaeota* have been also found to be dominant in an uncontaminated microbial mat developing on the Dutch barrier island Schiermonnikoog, mainly by the *Methanobacteria* and the *Methanomicrobia* (Bolhuis and Stal, 2011). As far as we know, the archaeal community never has been investigated in hydrocarbon-polluted microbial mats.

The goal of this study was to determine the impact of chronic hydrocarbon contamination on the biogeochemical functioning, structure, and activity of the prokaryotic community in natural microbial mats. In order to determine to which extent hydrocarbon contamination is able to drive the structure and activity of microbial mats, we compared a highly hydrocarbon-contaminated mat, whose *in vitro* hydrocarbon degradation capacity has been proven (Paissé et al., 2010), with a reference mat. The second mat is located in close proximity to the same lagoon and shows similar physical and chemical parameters but without a hydrocarbon contamination history. We performed an extensive biogeochemical characterization of both mats and deep phylogenetic and transcriptomic analyses using high-throughput sequencing of the bacterial and archaeal community 16S rRNA genes and transcripts. Hydrocarbonoclastic bacteria were specifically targeted, since previously they were poorly observed in the contaminated mat (Paissé et al., 2008). Because microbial mat functioning is influenced by seasonal variation, the analyses were performed in spring and autumn during 2 consecutive years in order to assess the seasonal variation of mat structure and activity and to determine the impact of hydrocarbon contamination.

2. Materials and methods

2.1. Sampling sites and procedure

The Berre lagoon is located on the French coast of the Mediterranean Sea near Marseille. Microbial mat samples were collected from two sites within the lagoon at a distance of 4.9 km: a contaminated site located in a retention basin receiving hydrocarbon wastewaters from a petrochemical industry (EDB1) and a non-hydrocarbon-contaminated site located within the “Les salins du Lion” bird reserve (SL) considered as a reference. The EDB1 retention basin had been collecting hydrocarbon-charged waters from the petrochemical industry for decades, and it currently is continuously supplied with hydrocarbon-contaminated rainfall waters (Paissé et al., 2008). The waters flow from industrial facilities to a sedimentation tank and then to the retention basin by an overflow system. In September 2009, an accidental oil spill occurred due to the overflow of a hydrocarbon-polluted water retention pond (Beau-Monvoisin, 2009). Four sampling campaigns were carried out: two in spring (April 2011 and 2012) and two in autumn (September 2011 and 2012), named Apr11 and Apr12 for April sampling and Sept11 and Sept12 for September sampling, respectively. The first 2 cm of sediment were collected in triplicate for molecular analysis and stored at −80 °C until analyses. Sediments for hydrocarbon analyses were sampled with a glass container and, for metal and other chemical analyses, with a polypropylene container.

2.2. Physical and chemical analyses

Complete physical and chemical characterization was performed for Sept11, Apr12, and Sept12 campaigns. Metals and transition metals (Al, As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Sn, and Zn) were quantified in sediments *via* inductively coupled plasma mass spectrometry by Ultra Trace Analyses Aquitaine (Pau, France). Sediments were freeze-dried and passed through a 2-mm sieve. Total sediment mineralization was performed in a microwave oven using 0.2 g of sediment, 2 mL of concentrated HNO_3, and 2 mL of concentrated HF. The solution was then diluted with MilliQ water. Metal concentrations were measured with an internal standard of rhodium (Rh). Water samples were quantified without pre-treatment with an Rh internal standard. Hydrocarbon analyses (alkanes and polyaromatic hydrocarbons [PAH]) in sediments were performed by Cedre (Brest, France) using gas chromatography coupled to mass spectrometry, as described by Stauffert et al. (2013). Sulfates and sulfides were quantified in water with the method described by Kolmert et al. (2000) and with the methylene blue method (Cline, 1969), respectively. Total carbon (TC) and total sulfur (TS) contents were measured in freeze-dried sediments by infrared spectroscopy using a LECO C-S 125. Organic carbon content also was measured in freeze-dried material after removal of carbonates with 2 M HCl from 50 mg of powdered sample (Etcheber et al., 1999; Pastor et al., 2011). Total nitrogen (TN) content in the sediment was measured in freeze-dried material by combustion using an automatic Thermo Finnigan EA 1112 Series Flash Elemental Analyzer. The C/N ratios were expressed as atomic ratios. Redox potential, pH, salinity, and dissolved oxygen were measured *in situ* (*via* a WTW pH/mV 340 meter, an Atago S10 refractometer, and a CellOx 325 electrode, respectively). Net photosynthesis, areal rates of gross photosynthesis, and dark and light respiration were measured in microbial mats as described by Pringault et al. (2015). The pore water concentration of NH_4^+, NO_2^-, and NO_3^-, the total denitrification, and the nitrogen fixation were measured as described by Bonin and Michotey (2006). The sulfate reduction rates were determined using the single-step chromium reduction method (Fossing and Jørgensen, 1989) by adding 1 mL of $35SO_4^{2-}$ (37 kBeq) in 5 mL of homogenized sediment and incubated for 6 h at 21 °C. Specific activities of $35S^{2-}$ and $35SO_4^{2-}$ were determined with a Beckman LS6500 liquid scintillation counter.

2.3. DNA/RNA extraction and cDNA synthesis

DNA and RNA co-extractions were performed using an RNA PowerSoil Total RNA Isolation Kit (MO BIO Laboratories, Inc.) coupled with an AllprepDNA/RNA Mini Kit and an RNase-Free DNase set (QIAGEN). RNA qualities were verified using an RNA nanochip on a Bioanalyzer 2100 (Agilent); only RNA extracts with RNA quality higher than 7 RIN (RNA Integrity Numbers) were used for following experiments. Absence of DNA contamination within RNA samples was determined by PCR amplification of RNA without reverse transcription. cDNA synthesis was achieved on RNA using M-MLV RT and RNase OUT (Invitrogen) with 1 μL of RNA. Reverse transcription was performed with random primers (1 μL at 5 U/mL) according to manufacturer's instructions.

2.4. Quantification of genes and transcripts

16S rRNA genes and transcripts of archaea and bacteria were quantified using the DyNAmo Flash SYBR Green qPCR Kit (ThermoFisher Scientific) in an Mx 3005PTM (Stratagene) as described by Paissé et al. (2012) using bac1055YF and bac1392R primers (Ritalahti et al., 2006) for bacteria and arch349F and arch806R primers (Takai and Horikoshi, 2000) for archaea. PCR products were cloned in the pCR2.1 Topo TA Cloning Kit (Invitrogen) to generate the standard gene count curve. The standard curve for the transcript quantification was performed with the cDNA obtained after *in vitro* transcription. The cloned PCR

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products were transcribed *in vitro* using the Megascript T7 Kit (Ambion) and reverse transcribed using the protocol described above. Reactions were cycled as described by Paissé et al. (2012) with 30 s of annealing at 50 °C or 58 °C for bacteria or archaea, respectively. Analysis was performed as described by Paissé et al. (2012).

2.5. Gene and transcript sequencing

In order to determine the bacteria and archaeal community compositions, the 16S rDNA bacterial genes and cDNA were amplified with primers 104F and 530R (Jami and Mizrahi, 2012), whereas the 16S rRNA archaeal genes and cDNA were amplified with the above defined primers for qPCR. Forward and reverse primers contained, respectively, the adaptors CTTTCCCTACACGACGCTCTTCCGATCT and GGAGTTCAGACGTGTGCTCTTCCGATCT. Thermal cycling for 16S rDNA was carried out as follows: 5 min at 94 °C; 40 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 30 s; and a final extension for 5 min at 72 °C. The PCR conditions for archaeal genes and transcripts were the same as for qPCR, with the exception of the initial denaturation and the final extension, which were made for 5 min at 94 °C and 72 °C, respectively. Amplicons were sequenced using MiSeq 250-paired technology (Illumina). Since MiSeq produces paired 250-bp reads, their ends overlap and can be concatenated in order to generate high-quality, full-length sequences of the entire region in a single run. Single multiplexing was performed using custom 6-bp index sequences, which were added to the reverse primers during a second PCR with 12 cycles using a forward primer (AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC) and a reverse primer (CAAGCAGAAGACGGCATACGAGAT-index-GTGACTGGAGTTCAGACGTGT). The resulting PCR products were purified and loaded into the Illumina MiSeq cartridge according to the manufacturer's instructions. After the run quality check using PhiX, each paired-end sequence was assigned to its samples using the integrated index. Raw sequences were submitted to the National Center for Biotechnology Information Sequence Read Archive under the accession number PRJNA295127.

2.6. 16S rRNA bioinformatic analysis

Paired reads were first concatenated with Flash (Magoč and Salzberg, 2011) to retain only those comprising complete amplicon sequences. Concatenated sequences were processed using USEARCH version 7.0.1090_i86linux32 (Edgar, 2013), as detailed in the Supplementary information.

To extract putative obligate oil-degrading bacteria from mat communities, reference sequences from the bacterial OTUs were aligned with Blast (Altschul et al., 1990) against 16S rDNA sequences of the obligate oil-degrading bacteria described by Yakimov et al. (2007). Sequences having >95% identity were retrieved, and a phylogenetic tree was constructed using the maximum likelihood method using MEGA software version 6 (Tamura et al., 2013). The gene and transcript relative abundances per site (mean of the four campaigns) were added using the Interactive Tree of Life web tool (Letunic and Bork, 2011, 2007). Visualization of dominant families was conducted using Circos (Krzywinski et al., 2009) on the six dominant families for genes and transcripts.

2.7. Statistical analysis

All analyses were performed in triplicate. For molecular analysis, in order to assess the replicate homogeneity, we first performed a cluster analysis using Bray–Curtis distances (Vegan package, R) (Oksanen et al., 2013; R Core Team, 2015) with the OTU tables. For all analyses, the variability among replicates was much lower than between samples. Replicates were then combined, and 100 successive random community samplings with a size corresponding to the minimum number of sequences per replicate were carried out in order to maximize sample diversity (Ardilly, 2006; Lejeune, 2006). The following describe the analyses performed using these normalized data. Taxonomic richness and diversity estimators were calculated using the Mothur software package (Schloss et al., 2009). Site descriptors were analyzed by principal component analysis (PCA) using the FactoMineR package (Lê et al., 2008); variables with cos2 below 0.5 on each factorial plane are not shown on the corresponding correlation circles of the PCA. Canonical Correspondence Analysis (CCA) (Vegan package) was used to find the environmental parameters (temperature, pH, salinity, TC, TN, TS, total hydrocarbons, and total heavy metals) that explain the diversity of the present and active bacterial and archaeal community; only uncorrelated variables were considered. The Wilcoxon signed-rank test was applied to test the effects of the sites or the seasons on the microbial abundance, activity, and richness and diversity estimators (Vegan package, R) (Oksanen et al., 2013; R Core Team, 2015). Taxonomical comparisons between sites and seasons were performed with STAMP (Parks et al., 2014) using the White's non-parametric *t*-test with an effect size filter for the difference between proportion >1%.

3. Results

3.1. Biogeochemical description of the mats

Hydrocarbon contents at EDB1 were 4–7-fold higher for alkanes and 28–330-fold higher for PAHs than those at SL (Table S1). As such, Cr and Cu contents were also higher at EDB1, with concentrations above the low effect range (ERL) and above the medium effect range (ERM) for Pb, Zn, and Ni (USA Environmental Protection Agency guidelines defined by Long et al. (1995)). The Hg was very abundant at both sites, with concentrations over the ERM; SL showed Cd, Zn, and Ni concentrations above the ERL for some campaigns (Table S1).

PCA was performed with the complete dataset to describe the sites (data not shown, percentages of variance dim.1 = 45.81% and dim.2 = 24.33%). Concentrations of PAHs (indeno[1,2,3,-cd]pyrene, fluorene, chrysene, anthracene, and phenanthrene) and Zn were the main physical chemical parameters explaining the separation of the sites (PCA analysis, correlation > 0.96 and p-value $< 5.83 \times 10^{-7}$). Different PCA analyses were performed with the data separated into three categories: (a) primary environmental parameters, (b) metal and metalloids contents, and (c) hydrocarbon contents (Fig. 1).

When considering the primary environmental parameters (Fig. 1a), the sites were separated by axis 2, mainly explained by the C/N ratio and the carbon contents. Both sites exhibited high metal concentration levels, but a clear separation can be observed (Fig. 1b) along axis 1 with high concentrations of Cu, Zn, Ni, As, Pb, Fe, Cr, and Sn at EDB1, whereas SL exhibited high Cd concentration. Both sites contained high Al concentrations during the Apr12 campaign, showing temporal variation in metal content. However, the main difference resulted from the extremely high hydrocarbon contents in the retention basin (EDB1). A shift of the hydrocarbon composition in the contaminated mat was visible over time (Fig. 1c); the mat sampled in Sept11 was characterized by high levels of short-chain alkanes ($<C_{28}$), the mat of Apr12 was characterized by significant levels of medium-weight PAHs, and the mat from the last campaign by heavy PAHs. Indeed, PAHs accounted for 61.5% of the total hydrocarbon content in the mat of Sept11 and 89.9% in the last campaign.

3.2. Diversity and activity of prokaryotic communities

Gene abundances (16S rRNA gene quantification) ranged from 1.7×10^{10}–7.1×10^{10} and 1.5×10^{8}–5×10^{8} copies/g of dry sediment for bacteria and archaea, respectively. No significant differences were observed between sites for neither prokaryotic gene abundances nor for archaeal transcripts; only bacterial transcripts were more abundant at the reference site relative to the contaminated one (Wilcoxon test p-value = 0.0003062). Prokaryotic richness and diversity indexes also

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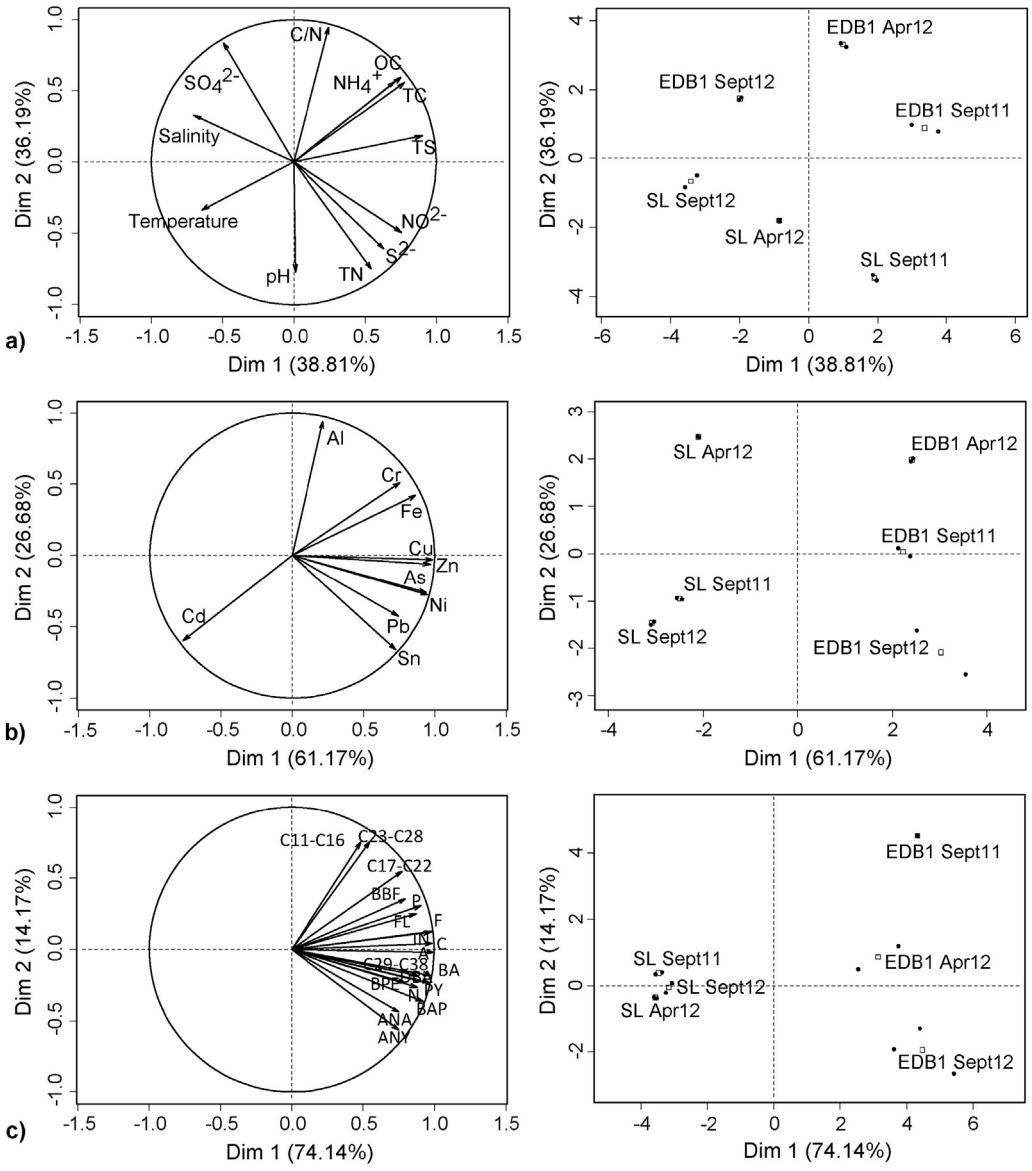

Fig. 1. Principal component analysis of parameters describing EDB1 and SL sites based on physical-chemical parameters (a), metal contents (b), and alkanes and EPA's 16 priority PAH contents (c). Each dot represents one replicate, the averages of replicates are enclosed. Abbreviations are: (a) C/N, carbon/nitrogen; OC, organic carbon; TC, total carbon; TN, total nitrogen, TS, total sulfur; (c) A, anthracene; ANA, acenaphtene; ANY, acenaphtylene; BA, benzo[a]anthracene; BBF, benzo[b + k]fluoranthene; BAP, benzo[a]pyrene; BPE, benzo(g,h,i)perylene; C, chrysene; DBA, dibenz(a,h)anthracene; F, fluorene; FL, fluoranthene; IN, indeno(1,2,3-cd)pyrene; N, naphtalene; P, phenanthrene; PY, pyrene. The parameters with a cos2 below 0.5 are not shown (Inorganic carbon; NO_3^-; Hg).

were similar (Wilcoxon test p-value > 0.05) at both sites (Table S2). Both bacterial and archaeal communities clustered based on the sampling site (clustering based on the Bray–Curtis distance; Fig. 2), with the exception of the last sampling of EDB1. A lower clustering level was observed that separates September from April samples. A second clustering level based on season could be detected. The dominant phyla at both sites were the *Cyanobacteria*, *Proteobacteria*, *Bacteriodetes*, and *Chloroflexi* for the bacteria (Fig. 3a) and the *Parvarchaeota*, *Euryarchaeota*, the *Crenarchaeota* for the archaea (Fig. 3b).

A total of 24,191 bacterial OTUs were found in the mats, and, excluding the EDB1_Sept12 sample, which diverged from other samples, 722 OTUs (2.9%) were found common to all samples, representing between 30.5% and 52.11% of the relative abundance (data not shown). A total of 1826 OTUs were shared between SL samples; they accounted for 64.40–75.59% of the relative abundance (Fig. S2), depending on samples. SL mats were dominated by the *Oscillatoriales* (*Oscillatoriophycideae*), *Desulfobacterales* (*Deltaproteobacteria*), *Chromatiales* (*Gammaproteobacteria*), and *Anaerolineales* (*Anaerolineae*) families (Fig. 3). These four families accounted for 33.2–38.1% of the sequences. The EDB1 mat community showed important differences between the Sept12 sample and the others (Fig. 2a). Only 313 OTUs were shared among all the EDB1 samples, accounting for 26.41–45.18% of the EDB1 communities (Fig. S1a). Excluding the Sept12 sample, 2524 OTUs were common to EDB1 samples, representing 71.67–77.39% of the EDB1 bacterial community. As for reference site, phototrophic bacteria (*Oscillatoriales* and *Chromatiales*) and sulfate-reducing bacteria (*Desulfobacterales*) were the dominant functional groups in these mats (Fig. 3a).

The active part of the bacterial community was largely dominated by the *Cyanobacteria*, mainly the *Oscillatoriales* (Fig. 3a). Cyanobacteria-related OTUs increased through all the investigated campaigns at the EDB1 site, accounting for 6.01% of the transcripts for the Apr11 campaign, 55.21% for the Sept11 campaign, and 70.65% for the Apr12 campaign, and dominated completely the last campaign with 93.95% of the transcripts. Besides the *Oscillatoriales*, the *Stigonematales* (*Nostocophycideae*) appeared dominant in the last campaign. The

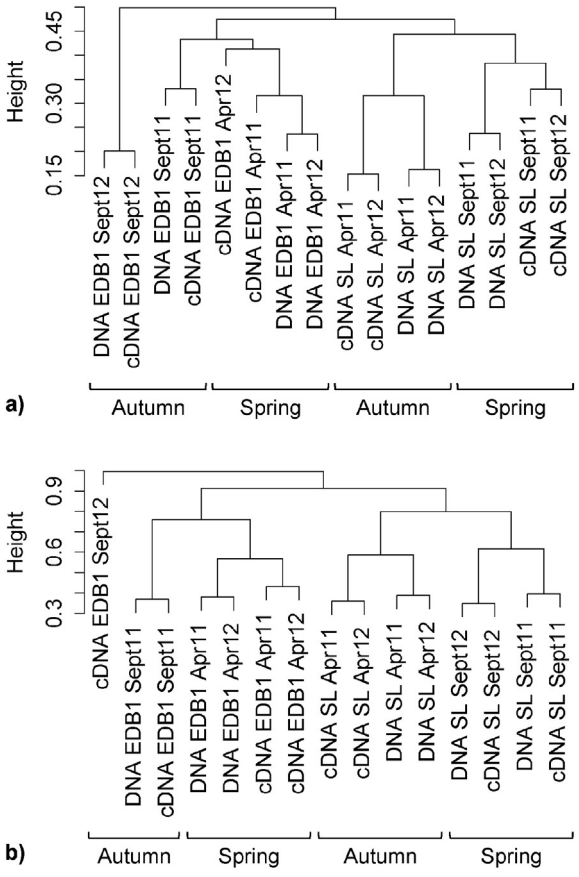

Fig. 2. Clustering of *Bacteria* (a) and *Archaea* (b) communities present (DNA) and active (cDNA) based on Bray Curtis distances.

Proteobacteria also dominated the active part of the community, with the sulfate reducers affiliated with the *Desulfobacterales*.

General clustering based on biogeochemical activities grouped samples according to seasons only for SL (Fig. 4). Photosynthesis was found to be different between sites (Fig. 4); the reference site showed higher net production (Sept11) or higher gross photosynthesis and respiration (Sept12) in autumn samples, showing pronounced seasonal variation. No such seasonal variation could be detected at the contaminated site.

A total of 24 bacterial orders was found differentially abundant, with 10 differently active between sites. At the genus level, 51 bacterial genera showed significantly different abundances (p-value < 0.05). Among them, 40 were more abundant at SL and 11 more abundant at EDB1. Most of these differences, although significant, were very low (< 1%). The genera with differences >1% (Fig. 5a) were related to the sulfur cycle, including sulfate-reducing (*Desulfotignum*) and sulfur-oxidizing bacteria (*Thiocapsa* and *Thiobacillus*). Similar trends were observed for the active community (transcriptomic based); 37 genera were differentially expressed, most of them were more active at SL (32 genera), and only one genus exhibited a difference >1%.

Around 50% of the archaeal OTUs were common between sites in all campaigns (1577 OTUs for EDB1, 1200 OTUs for SL), represented from 81.34–90.51% of the relative abundance for EDB1 and from 71.47–83.64% for SL. Three main phyla were detected: the *Parvarchaeota*, *Euryarchaeota*, and *Crenarchaeota*; the whole community and the active part were similar (Fig. 2b). The *Euryarchaeota* were primarily affiliated with the *Methanomicrobia* methanogens. Finally, OTUs 4 and 9, highly active at SL (up to 6.26% of the relative abundance of the transcripts), could not be affiliated below the kingdom level. Three archaeal classes were differentially abundant between sites, including two with a difference >1% (Fig. 5b). They were related to the Marine Benthic Group B (MBGB) (more abundant at EDB1) and to the Miscellaneous Crenarchaeotal Group (MCG) (more abundant at SL). This latter class also was more active at SL and was the only one observed to be significantly different among transcripts.

3.3. Seasonal modifications of bacterial diversity

Neither gene nor transcript abundances showed differences between seasons (Fig. S1). Similarly, no significant differences (Wilcoxon test) were observed for the richness and diversity estimators when comparing between seasons.

Although the seasonal clustering was lower than the site clustering, the SL community showed important seasonal variation (difference up to 10%). A total of 26 orders was differentially abundant in spring or fall, and 24 were differentially active. For comparison, the contaminated site showed only 10 orders differentially abundant and six differentially expressed. Most of these differences, although significant (p-value < 0.05), were very low (<1%). Among differences >1%, a higher abundance and activity of the *Gamma-* and *Deltaproteobacteria* in spring campaigns were observed at SL (Fig. 6a). Among the *Gammaproteobacteria*, the *Chromatiales* (Fig. 6a), and, particularly, the OTUs related to the *Thiocapsa* genus, were more abundant in spring. For the *Deltaproteobacteria*, the sulfate-reducing bacteria affiliated with the *Desulfobacterales* were significantly more active in spring. In autumn, the *Cyanobacteria* within the *Oscillatoriales* were more abundant. Differences in *Cyanobacteria* transcripts were not significant at the order level but were observed at the phylum level (p-value = 0.013, difference of 29.83%), as increases of *Cyanobacteria* were related to different groups. Seasonal variations in EDB1 mats, while less pronounced (differences <4%), were observed for the sulfurogenic *Desulfuromonadales*, which were more abundant in spring. In fall, the phototrophic *Pseudoanabaenales* and *Nostocales* were more abundant or more active.

Seasonal variation also was observed for the archaeal community. The YLA114 order within the *Parvarchaeota* highly dominated fall samples. The *Euryarcheota* were more abundant and more active in spring (Fig. 2b). They were primarily affiliated with the *Thermoplasmata* group E2 and with the *Methanosarcinales* and *Methanomicrobiales* methanogens (Fig. 6b).

3.4. Influence of environmental parameters in bacterial and archaeal communities

Sediment hydrocarbon contents were the main variables that significantly explained the structure of the bacterial community distribution (Fig. 7a) and the active part of the bacterial and archaeal community, as well (Fig. 7c and d). The hydrocarbon contents were also correlated with the heavy metal contents. The EDB1 site was associated with high total hydrocarbon contents. Besides hydrocarbon contents, TC content significantly influenced the EDB1 archaeal community structure (Fig. 7b).

3.5. Hydrocarbonoclastic bacteria in microbial mats

Eight OTUs within the *Gammaproteobacteria* class were related to obligate oil-degrading bacteria (Fig. 8). Altogether, they represented <2% of the overall bacterial community in the contaminated mat. OTU41, affiliated with *Cycloclasticus oligotrophus* (97.7% identity), represented up to 2.84% of EDB1 Apr11 transcripts and was the most active OTU among the *Gammaproteobacteria* for this campaign. However, the relative abundance and activity of this OTU varied over time, reaching <0.001% for April campaigns. At SL, this OTU accounted for 0.001% in April 2012 transcripts. *Alcanivorax*-related OTUs (OTUs 3283, 3442, and 4729) were rare at EDB1, representing <0.0052% of the bacterial community, and were only found to be active at Apr11 EDB1. OTUs related to this genus also were observed at the reference site but only for the Sept11 SL sample. Other hydrocarbonoclastic bacteria related

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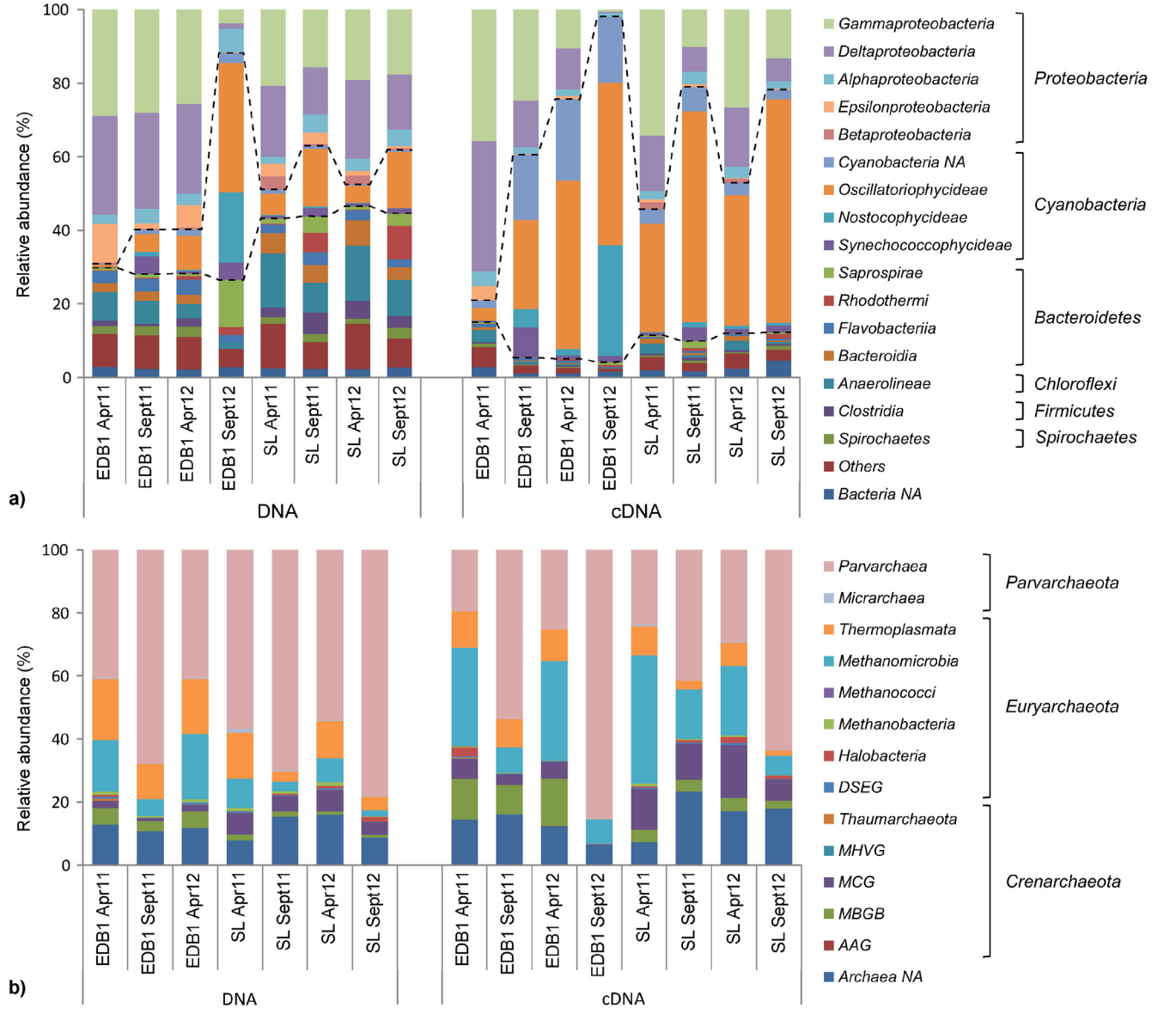

Fig. 3. Taxonomic affiliation of the 16SrRNA gene and transcripts of *Bacteria* (a) and *Archaea* (b) at the class level. *Proteobacteria* and *Cyanobacteria* phyla are separated by dotted lines.

to *Marinobacter hydrocarbonoclasticus* were observed to be more active at the reference site, accounting for up to 0.008% of the transcripts. Among dominant *Deltaproteobacteria*, no oil-degrading-related OTUs could be observed. With the exception of the known obligate oil-degrading bacteria, it is difficult to determine the potential degradation capacities of bacterial OTUs, because, in general, they are strain specific.

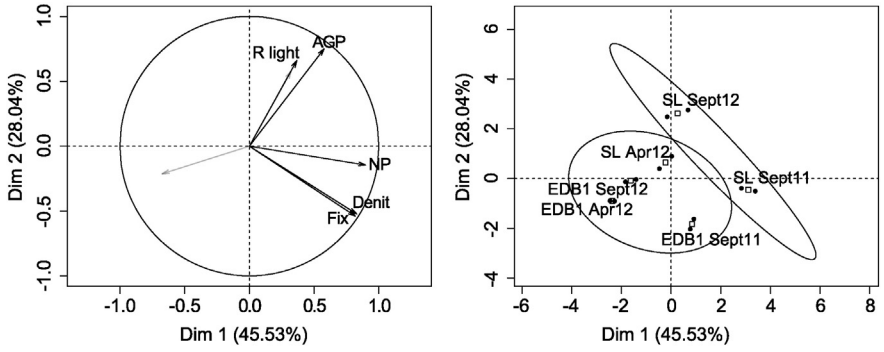

Fig. 4. Principal component analysis of parameters describing EDB1 and SL sites based on biological activities. Each dot represents one replicate, the averages of replicates are enclosed. Ellipses represented the groups defined by k-means clustering method using Euclidean distances. Abbreviations are: AGP, areal rates of gross photosynthesis; Denit, total denitrification; Fix, nitrogen fixation; NP, net photosynthesis; R respiration. The parameters with a cos2 below 0.5 are not shown (sulfate reduction rate; respiration dark).

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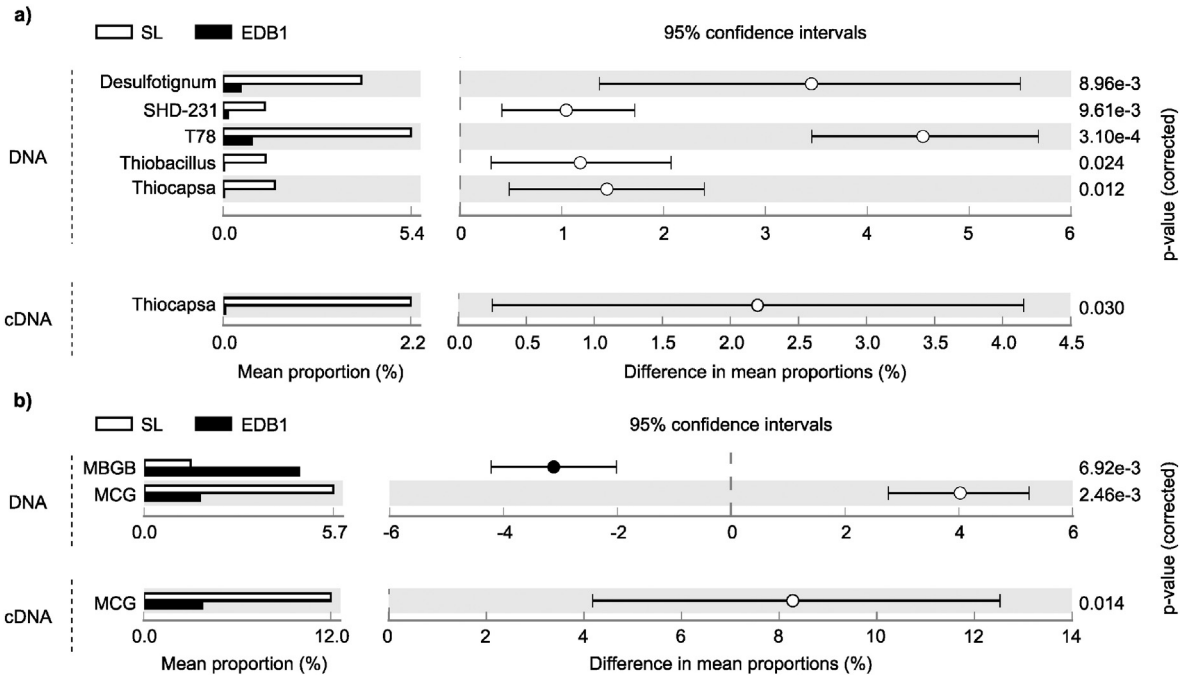

Fig. 5. Extended error bar plot showing the bacterial genera (a) and archaeal classes (b) differentially abundant or active between sites (p-value < 0.05; White's non-parametric *t*-test). Only groups with mean differences between sites larger than 1% are shown.

4. Discussion

Most of the studies related to polluted environments mainly have focused on the capacity of biodegradation of contaminated sediments through the isolation of hydrocarbonoclastic bacteria or the detection of hydrocarbonoclastic bacteria or genes involved in biodegradation (Bordenave et al., 2008; Paissé et al., 2012; Todorova et al., 2014). Microcosm studies have demonstrated that hydrocarbon contamination induces an increase of hydrocarbonoclastic bacterial community abundance (Coulon et al., 2007; Paissé et al., 2010), and, thus, a

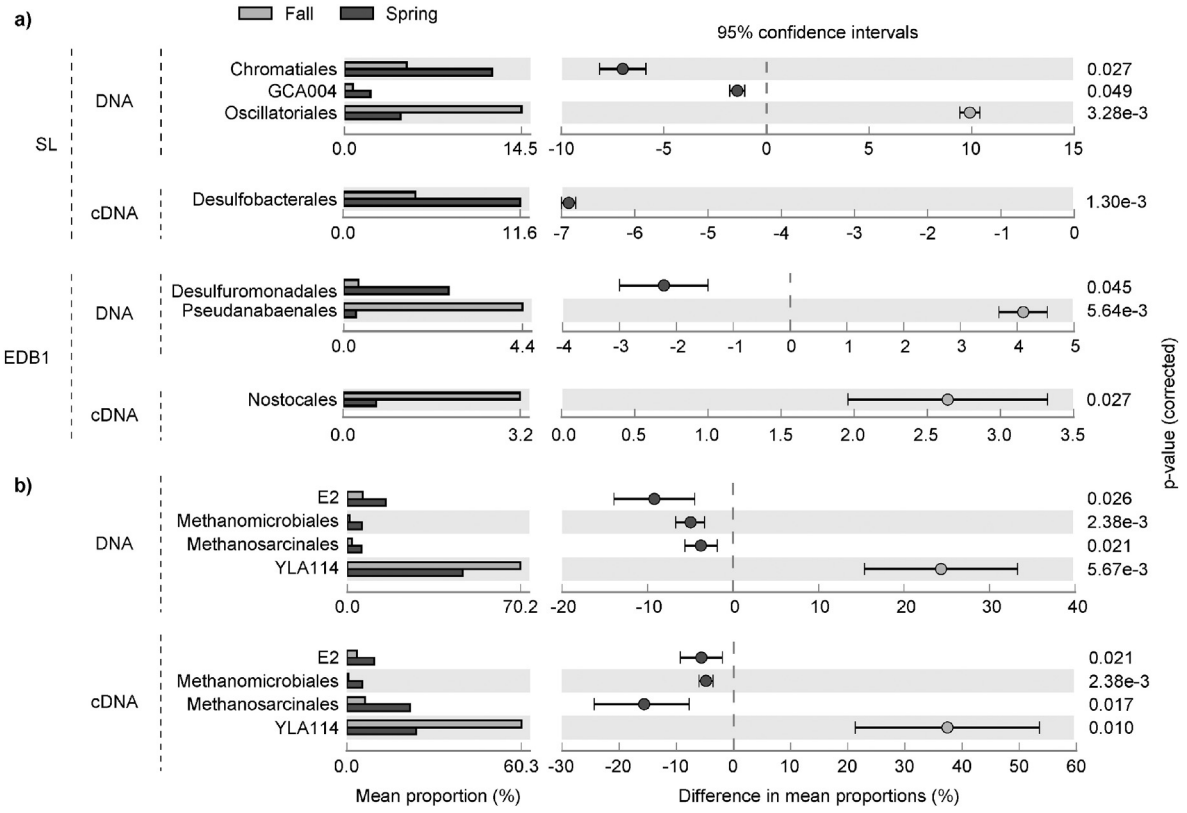

Fig. 6. Extended error bar plot showing the bacterial (a) and archaeal (b) orders differentially abundant or active between seasons (p-value < 0.05; White's non-parametric *t*-test). Only orders with mean differences between seasons larger than 1% are shown.

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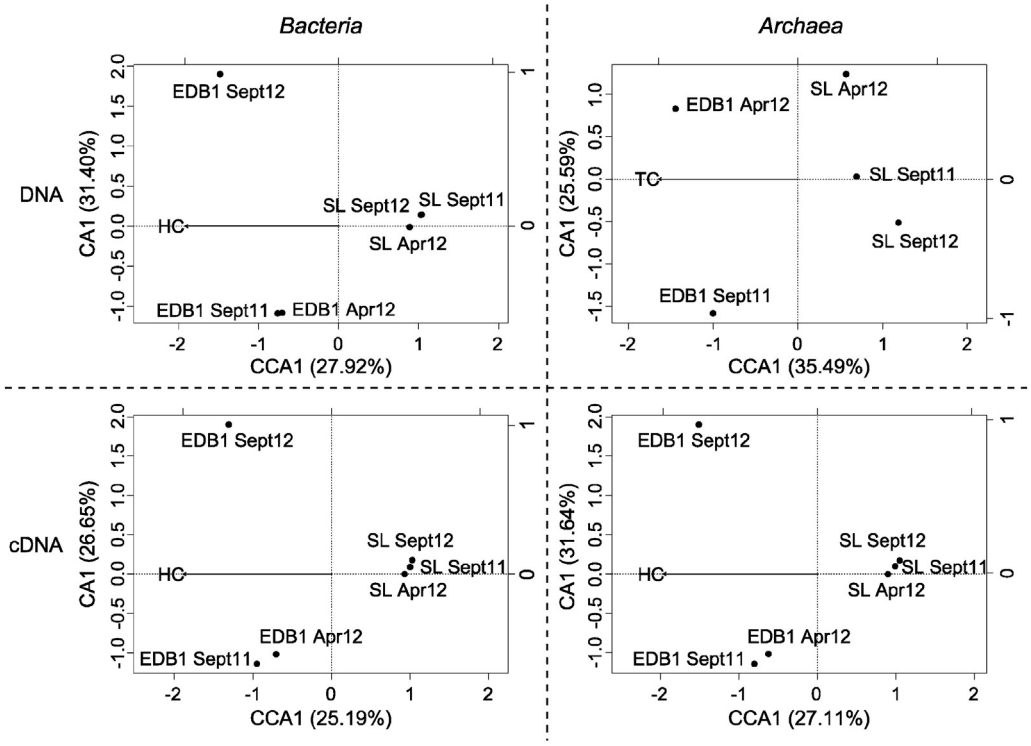

Fig. 7. CCA based on the 16SrRNA bacterial and archaeal gene and transcripts of each community and the biogeochemical parameters. Only parameters having a significant effect (p-value < 0.05, Anova) are shown. Abbreviations are: HC, total hydrocarbon and TC, total carbon.

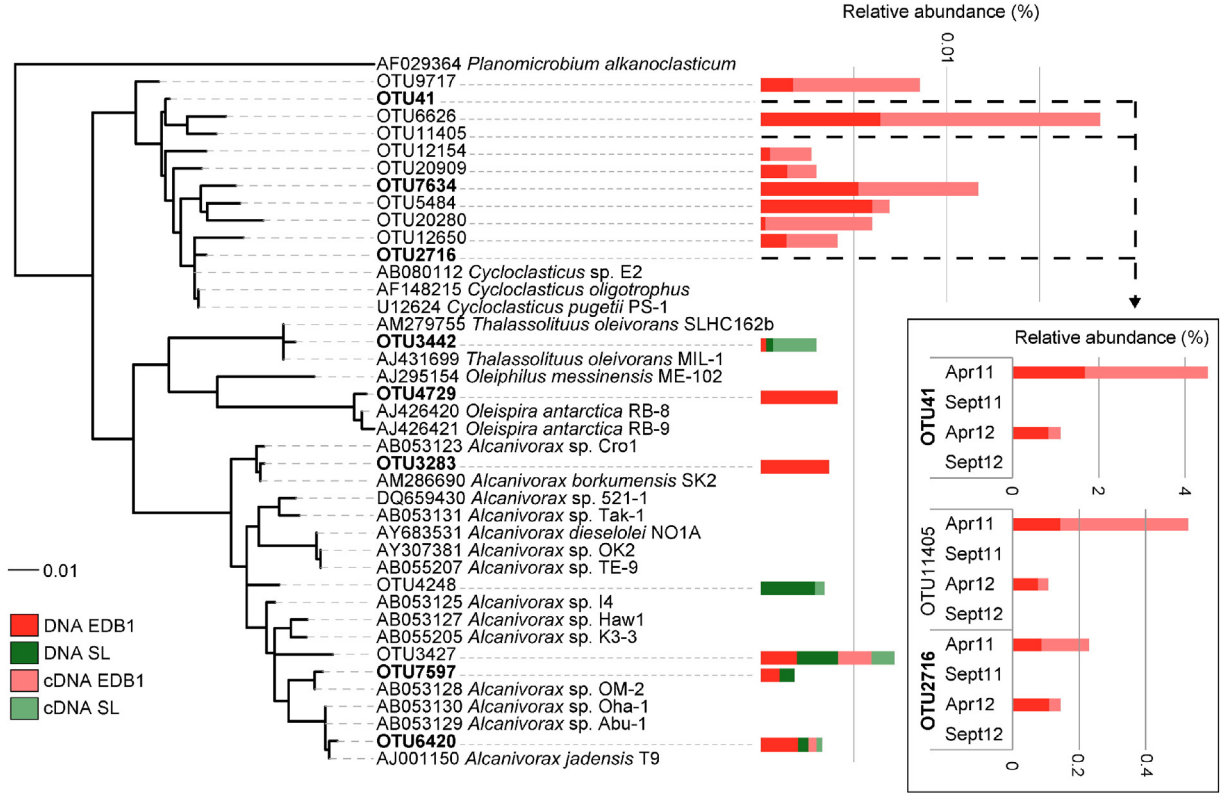

Fig. 8. Phylogenetic affiliations of obligate hydrocarbonoclastic bacteria according to Yakimov et al., (2007). The tree, based on 439 nucleotide positions, was constructed using the maximum likelihood. OTUs sequences sharing >95% of identity with obligate hydrocarbonoclastic bacteria 16S gene are shown; bold OTUs shared >97% of identity. The relative abundances of genes (DNA) and transcripts (cDNA) for selected OTUs (mean of the four campaigns) are shown on the bar chart. OTU 41, 11405 and 2716 relative abundances are shown for the four campaigns.

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modification in the bacterial community structure. However, the extent of this phenomenon in a natural system remains unknown. Bordenave et al. (2008) demonstrated that gene diversity involved in PAH degradation (*i.e.*, dioxygenases) was higher in a chronically contaminated microbial mat than in a pristine mat after *in vitro* contamination. The effect of hydrocarbon contamination in a closed controlled system may be more important than in an *in situ* one, where many external factors affect the system. In natural ecosystems, most of the studies have focused on the response of the microbial community to accidental oil spills (Acosta-González et al., 2013; Kasai et al., 2001; Kostka et al., 2011; Mason et al., 2012), but almost nothing is known about the structure (Abed et al., 2014; Paissé et al., 2008) and the activity of chronically oil-contaminated environments.

4.1. Comparison of the structure, activities, and seasonality of microbial mat communities

The bacterial community of both (contaminated and reference) mats was dominated by the *Proteobacteria*, *Cyanobacteria*, and *Bacteroidetes*, as previously observed (Bolhuis and Stal, 2011; Severin et al., 2010). Community structure of samples was first discriminated by sites and at second level by seasons. The active part of the community revealed the *Cyanobacteria* as being the main contributors to the microbial mat functioning. Sulfate cycle-related bacteria were found to be dominant in both mats. At the reference site, organisms related to the sulfur cycle were more abundant and more active in spring, especially the sulfate-reducing bacteria affiliated with the *Desulfobacterales* and the sulfur-oxidizing bacteria affiliated with the *Chromatiales*. No seasonal variation of these functional groups could be observed at the contaminated site. Nevertheless, higher abundances of sulfurogenic-related bacteria were observed in spring campaigns. The archaeal *Crenarchaeota* and the methanogens related to the *Euryarchaeota* also were found to be highly abundant and active in both mats, as described elsewhere (Bolhuis and Stal, 2011; Robertson et al., 2009), showing that methane cycling has occurred in Berre lagoon microbial mats.

The differences in the microbial mat structure concerned rare or minor OTUs. Thus, their biogeochemical functioning was quite similar. The main differences were related to the photosynthesis and the net production, whereas no impact of sulfate reduction rates was observed. The reference mat showed marked seasonal dynamics with higher photosynthetic activities in autumn, as already suggested by yearlong monitoring of microbial mats (Bolhuis and Stal, 2011; Pinckney et al., 1996). The main driver of this photosynthesis was *Oscillatoriales*-related bacteria, which were found to be abundant and active in fall. The increase of *Oscillatoriales* and other filamentous *Cyanobacteria* has been described previously in microbial mats and intertidal flats for the summer–fall period (Bowlin et al., 2012; Scholz and Liebezeit, 2012). Seasonal changes in pristine intertidal flats have been explained mainly by light and temperature (Barranguet et al., 1998; Blanchard et al., 1996). Such seasonality could not be observed in the contaminated mat. Minor seasonal differences could be attributed to the increase of *Pseudanabaenales*-related 16S genes and *Nostococales* 16S transcripts. At the phylum level, *Cyanobacteria*-related transcripts increased all throughout the campaigns, showing a possible constant evolution of the community related to hydrocarbon content. The *Cyanobacteria* are the precursors and the main drivers of microbial mats (Wieland et al., 2005), it already has been demonstrated that hydrocarbon contamination could induce the photosynthetic activities (Benthien et al., 2004). The overall results suggest a reinstallation process of contaminated mats that recover from accidental input (see below) and point to a possible resilience of the mat.

4.2. Hydrocarbon drives microbial mat structure

The main difference in environmental parameters among mats was the hydrocarbon content, which affected the bacterial and archaeal communities, as demonstrated by CCA. Among other environmental parameters, the impact of petroleum contamination on microbial mat structure has been reported previously (Abed et al., 2014). An accidental oil spill occurred in September 2009 in the contaminated mat, after the overflow of the hydrocarbon-polluted water retention pond (Beau-Monvoisin, 2009), and introduced hydrocarbon molecules massively into EDB1. The hydrocarbon composition in the contaminated site changed over time, with higher concentrations of branched PAHs and heavyweight hydrocarbon molecules in the last campaign, suggesting the progressive degradation of the more easily degradable hydrocarbons (n-alkane and PAHs with low numbers of rings [Atlas, 1981]). The hydrocarbon values reached in 2012 were close to those observed in 2006 by Paissé et al. (2008). These changes also could induce a modification in the microbial community structure, explaining the lower seasonal variations at this site. Petroleum has been shown to modify the microbial mat community structure *in vitro* (Abed et al., 2002; Bordenave et al., 2007) and to increase the photosynthesis and the abundance of *Cyanobacteria* in petroleum-contaminated mats (Abed et al., 2014; Benthien et al., 2004), as observed in this study.

4.3. Hydrocarbons do not select hydrocarbonoclastic bacteria

Most of the hydrocarbonoclastic-related OTUs did not shown any difference in abundance and activity. Those hydrocarbonoclastic bacteria related to *Alcanivorax*, *Oleispira*, or *Thalassolituus* represented a minor part of the active community. Nevertheless, some PAH-degrading bacteria, such as *Cycloclasticus* spp., were more abundant and active at the contaminated site but were transient in the EDB1 community. Neither overabundance nor overexpression were noticed for known hydrocarbonoclastic bacteria at the contaminated site at the genus level. Since hydrocarbons seem not to select specifically the hydrocarbonoclastic population, two questions can be raised: (1) to what extent can those bacteria be considered sentinels of the hydrocarbon pollution and (2) what is their role in hydrocarbon degradation in chronically contaminated systems? Although *in vitro* studies (Yakimov et al., 2005) and punctual oil pollution (Kostka et al., 2011; Mason et al., 2012; Newton et al., 2013) demonstrated the selection of these specialized bacteria (for a review, see Head et al., 2006), these selections were observed only in the first stage of pollution. Other studies demonstrated that there was a rapid recovery (*i.e.*, resilience) to the original bacterial community structure after punctual oil pollution (Bordenave et al., 2007; Röling et al., 2002). In the investigated contaminated EDB1, previous studies with microcosms and artificial contamination showed a significant increase of hydrocarbon-degrading related bacteria belonging to *Alcanivorax* and *Cycloclasticus* genera (Paissé et al., 2010). However, few known obligate hydrocarbonoclastic bacteria were observed *in situ*. These low abundances could be attributed to the low bioavailability of the high molecular weight PAHs present *in situ*, in contrast to the alkane and low molecular weight PAHs added in experimental studies (Dandie et al., 2010). The aged hydrocarbons found in hydrocarbon-contaminated sites are generally considered less available than freshly added material (Alexander, 2000; Macrae and Hall, 1998). Finally, the sediment of EDB1 is rich in organic matter, partially produced by the photosynthetic organisms, which could bypass the use of heavy hydrocarbon molecules. More than selecting specialized hydrocarbonoclastic bacteria, hydrocarbons could inhibit some sensitive microorganisms. Some genera that appeared significantly more abundant or active in the reference mat should be inhibited by the toxicity of hydrocarbon compounds, as observed in other studies (Bachoon et al., 2001; Zhou et al., 2009). Thus, the selectiveness of oil in rich and chronic contaminated sites should be the consequence of toxicity more than the effect of carbon source input.

5. Conclusion

This comparative *in situ* study showed that chronic hydrocarbon contamination in natural conditions induces only minor perturbations

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in the biogeochemical functioning and the bacterial composition of microbial mats. Although the classical seasonal variation was observed in the reference mat (phototrophic community dominant in autumn and sulfur cycle-related bacteria and methanogens dominant in spring), the contaminated site showed a progressive increase of the cyanobacterial component and no marked seasonal changes in the other functional groups. Thus, we suggest that the mat in the contaminated site is evolving to acquire a stable structure. This pattern corresponds to an evolution of the hydrocarbon composition characterized by the progressive loss of the easily degradable hydrocarbons. Surprisingly, the known hydrocarbonoclastic bacteria were not specific to the contaminated mat; moreover, some hydrocarbonoclastic bacteria were more abundant and active in the reference site. We conclude that the hydrocarbon contamination did not select a specialized hydrocarbonoclastic community in the chronic contaminated Berre lagoon mat.

Author contributions

MG, RG and OP conceived the study, JA, OP, PB and MG collected samples, JA, OP, BD, OB and PB conducted lab work, JA, NB, EB, PS and CK analyzed data, and JA wrote the manuscript with contributions from MG, RG, OP, PB, CK and PS. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marpolbul.2016.07.023.

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