Biodegradation (2009) 20:467–486
DOI 10.1007/s10532-008-9235-x

ORIGINAL PAPER

Molecular diversity analysis and bacterial population dynamics of an adapted seawater microbiota during the degradation of Tunisian zarzatine oil

Ines Zrafi-Nouira · Sonda Guermazi · Rakia Chouari · Nimer M. D. Safi · Eric Pelletier · Amina Bakhrouf · Dalila Saidane-Mosbahi · Abdelghani Sghir

Received: 6 March 2008 / Accepted: 10 November 2008 / Published online: 4 December 2008

Abstract The indigenous microbiota of polluted coastal seawater in Tunisia was enriched by increasing the concentration of zarzatine crude oil. The resulting adapted microbiota was incubated with zarzatine crude oil as the only carbon and energy source. Crude oil biodegradation capacity and bacterial population dynamics of the microbiota were evaluated every week for 28 days (day 7, day 14, day 21, and day 28). Results show that the percentage of petroleum degradation was 23.9, 32.1, 65.3, and 77.8%, respectively. At day 28, non-aromatic and aromatic hydrocarbon degradation rates reached 92.6 and 68.7%, respectively. Bacterial composition of the adapted microflora was analysed by 16S rRNA gene cloning and sequencing, using total genomic DNA extracted from the adapted microflora at days 0, 7, 14, 21, and 28. Five clone libraries were constructed and a total of 430 sequences were generated and grouped into OTUs using the ARB software package. Phylogenetic analysis of the adapted microbiota shows the presence of four phylogenetic groups: *Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes*. Diversity indices show a clear decrease in bacterial diversity of the adapted microflora according to the incubation time. The *Proteobacteria* are the most predominant (>80%) at day 7, day 14 and day 21 but not at day 28 for which the microbiota was reduced to only one OTU affiliated with the genus *Kocuria* of the *Actinobacteria*. This study shows that the degradation of zarzatine crude oil components depends on the activity of a specialized and dynamic seawater consortium composed of different phylogenetic taxa depending on the substrate complexity.

Keywords 16S rRNA gene · Biodegradation · Seawater microbiota · Zarzatine crude oil

Abbreviations

AH	Aromatic hydrocarbon
AM	Adapted Microbiota

I. Zrafi-Nouira (✉) · A. Bakhrouf · D. Saidane-Mosbahi
Laboratoire d'Analyse, Traitement et Valorisation des Polluants de l'Environnement et des Produits, Faculté de Pharmacie de Monastir, Rue Avicenne, 5019 Monastir, Tunisia
e-mail: zrafi_ines@yahoo.fr

S. Guermazi · E. Pelletier · A. Sghir
CNRS-UMR 8030, Evry, France

S. Guermazi · E. Pelletier · A. Sghir
CEA-Genoscope, Evry, France

S. Guermazi · A. Sghir
Université d'Evry Val d'Essonne, 2 rue Gaston Crémieux, 91057 Evry, France

R. Chouari
Faculté des Sciences de Bizerte, Université 7 Novembre à Carthage, 7021 Jarzouna Bizerte, Tunisia

N. M. D. Safi
Institute of Chemistry and Biology of the Marine Environment (ICBM), Carl von Ossietzky University of Oldenburg, 26111 Oldenburg, Germany

NAH Non-aromatic hydrocarbon
TPH Total Petroleum Hydrocarbon
UCM Unresolved complex mixture
ZCO Zarzatine crude oil

Introduction

Oil pollution and concern about it became important at the beginning of the nineteenth century. Microbial degradation is the major natural route for cleaning up oil spills (Kasai et al. 2001). Pollutants are subject to spontaneous decomposition by oxidation, hydrolysis, or ultraviolet rays, and attack by the natural microbiota. Total decontamination is extremely variable, especially when the pollutant is as complex as crude oil. The latter is composed of hundreds of compounds (Bertrand and Mille 1989). The major fractions are the non aromatic hydrocarbons (NAH) and the aromatic hydrocarbons (AH). The NAH fraction includes aliphatic hydrocarbons, branched isoalkanes such as pristane and phytane and cycloalkanes which are represented by an unresolved complex mixture (UCM). While each compound represents potentially a different carbon and energy source for growth, in some cases, hydrocarbons can have an inhibitory effect on microbial growth. Most hydrocarbon-degrading bacteria will typically degrade a small range of either aliphatic or aromatic compounds (McKew et al. 2007). Microorganisms able to use hydrocarbons are very diverse. The literature cites 79 bacterial, 103 fungal and 14 algal genera that can use hydrocarbons as a sole source of carbon and energy (Prince 2005). In 1946, Zobell (Zobell 1946) noted the hydrocarbonoclast property of bacteria and many studies have confirmed his observations (Reisfeld et al. 1972; Walker and Colwell 1974; Bartha 1977). Within the last two decades, molecular techniques based on comparative analysis of 16S rRNA genes have contributed to improve our knowledge of microbial diversity. Characterizing microbial diversity and identifying microorganisms which play a key role in the degradation of pollutants could be useful in defining new strategies for bioremediation. Many studies examined the impact of oil pollution on the bacterial community structure, or the presence of oil-degrading microorganisms (Hernandez-Raquet et al. 2006; Winderl et al. 2007; Alekhina et al. 2007). In the present paper we report the analysis of the diversity and dynamics of an adapted microbiota of crude oil polluted seawater under laboratory conditions. We elucidate relationships between the major hydrocarbon classes of crude oil, microbial composition and dynamics during oil degradation.

Materials and methods

Sampling

Seawater was collected from the rejection zone of the STIR refinery (Tunisian Company of Industrial Refining) in Jarzouna, Bizerte, Tunisia (37°17′ 57.10″ N, 9°51′ 53.61″ E). The sampling site was located in the vicinity of the rejection area of seawater used in the treatment of the petroleum effluent, the refining process, and the cleaning of the oil reservoirs. The treatment consists of successive decantation basins to recover the oil, with the used seawater rejected into the sampling area. The area of interest is a high industrialized zone. Industrial activity includes metallurgic industry, naval construction and tire production (Trabelsi and Driss 2005). In addition, there is a base of the Tunisian Navy Marine, many local wastewater discharge sites, intensive traffic of boats and harbour activities and solid landfill. Our previous results demonstrated chronic petroleum pollution at this same sampling site (Zrafi-Nouira et al. 2008). The average levels of petroleum hydrocarbons, pH, temperature, salinity and dissolved oxygen of the seawater at the sampling site were 98 mg/L, 8.3, 24°C, 37‰ and 2.5 mg/L respectively.

Crude oil

Zarzatine crude oil (ZCO) is a light crude oil, which has a density of 810 and a viscosity of 3.4 at 20°C. It was provided by the STIR refinery. In the biodegradation experiment, ZCO was used as the sole carbon and energy source. For biodegradation assays, the oil was placed in an air-tight closed vial and sterilized at 120°C for 20 min.

Microbial consortia

To separate free-living microbes from other organisms and particles which are in the seawater, samples were filtered through 2 μm filters (Millipore). Indigenous microbiota was obtained by centrifugation of 1 L of filtered seawater at 14,000*g* for 15 min. The supernatant was then eliminated and the pellet containing the bacterial fraction was used for direct enrichment procedures. The enrichment procedure was carried out by inoculating the totality of the pellet (approximately 10^9 CFU/ml) after centrifugation, in 1 L of sterilized non-polluted seawater. The first step of enrichment consists of adding progressively 1 mg of ZCO at a rate of 250 μg/week for one month. The consortium was then centrifuged and directly subjected to a second step of enrichment using different concentrations of ZCO from 1 to 50 mg/L with an increment of 5 mg, over 91 days. Then 50 ml of seawater from each enrichment experiment (1, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mg/l) were removed under sterile conditions, after thorough agitation for 5 min. The final microflora of the ten mixed samples was obtained after centrifugation. This consortium represents the adapted microflora. The purpose of performing this pre-treatment experiment was to obtain a microflora adapted to a large range of ZCO concentrations. Fresh aliquots of the adapted microflora were used to inoculate the degradation microcosm (see biodegradation assays). Cultures were transferred weekly to fresh seawater containing 1 mg/L of ZCO for 1 year. Aliquots of the adapted microflora were conserved at −80°C until DNA extraction.

Biodegradation assays

Biodegradation assays were carried out in 500-ml flasks containing 200 μg of ZCO and 200 ml of sterilized non-polluted seawater inoculated directly with 5 ml of fresh culture of 10^9 CFU/ml of adapted microflora. The flasks were incubated at 25°C without agitation. Microcosms were incubated in triplicate for 1, 2, 3, and 4 weeks. For the quantification of abiotic loss, a parallel experiment was performed and used as negative control, differing from the latter by adding 0.5 g of $HgCl_2$ growth inhibitor. Non- inoculated flasks were included. At the end of the incubation period, a subsample (50 ml) was taken from each microcosm after a thorough mixing at predetermined intervals and used for hydrocarbon extraction from both biodegradation and control flasks. Subsamples of 150 ml were used to obtain microflora from day 7, day 14, day 21, and day 28. Microbial consortia was prepared and stored at −80°C until DNA extraction.

Analysis of residual crude oil

Residual ZCO from inoculated and control cultures were subjected to an organic extraction using chloroform. The analytical procedure used for total petroleum hydrocarbon (TPH) extraction was a modification of the method described by Saliot (1981). Briefly, hydrocarbons in water samples (50 ml) were liquid–liquid extracted three times, for 1 h with 150 ml of pure chloroform, using a separatory funnel. The recovery of this extraction method ranged between 88.4 and 89.2% ($n = 4$) for zarzatine crude oil. The extracts were then concentrated to 2 ml using rotary evaporation (BÜCHI). Following chloroform evaporation, the extract was fractionated by adsorption liquid chromatography (LC) using a column of alumina and silica-gel, and gradient solvents as eluent: n-hexane and 2:1 n-hexane/chloroform for saturated and unsaturated non-aromatic hydrocarbons (NAH), and aromatic hydrocarbons (AH), respectively. Fractionation was performed in a silica microcolumn of 0.5 cm × 15 cm (silica gel 60 of 63–200 μm). Prior to use, the silica was cleaned with chloroform, subjected to a 1-h wash at 50°C under magnetic agitation, and filtered through a glass fiber filter (type GF/A, 47 mm diam., 1.6 μm retention) (Whatman International, Kent, UK). The silica was then conditioned overnight at 110°C. Following solvent evaporation, TPH, NAH and AH concentration were determined.

GC/FID analysis

The NAHs were analyzed with a Hewlett-Packard 5890 gas chromatograph equipped with a temperature controlled injector, a flame ionization detector (GC/FID), and a capillary column HP5: 5% diphenyl, 95% dimethylpolisiloxane (25 m × 0.32 mm × 0.52 μm). The oven temperature program was as follows: 1 min at 80°C, from 80 to 280°C at 4°C/min and 10 min at 280°C. Injector and detector temperatures were 250°C and 280°C, respectively. The samples were solubilised in 1 ml cyclohexane and an aliquot of 1 μl of each

extract was injected following the addition of external standard (n-eicosene) at a rate of 20 μg/ml.

Determination of biodegradation efficiency

The rate of recovery, which corresponds to the ratio of the quantity of oil in the control flask at the end of the incubation to the initial amount of oil, ranges from 91.1% to 93.4% ($n = 6$). The efficiency of biodegradation corresponding to each incubation period was calculated for the various fractions of TPH, NAH or AH at various incubation periods proportionally to the fractions obtained from the negative control, using the following formula: (% of biodegradation) = [(amount of residual hydrocarbon in the control microcosm) − (amount of residual hydrocarbon in the inoculated microcosms)]/(amount of residual hydrocarbon in the control microcosm).

DNA extraction, PCR amplification, cloning and sequencing of 16S rRNA gene

Direct extraction of total microbial DNA from the adapted microflora (day 0) and from the other microflora obtained at days 7, 14, 21 and 28 was performed as described by Chouari et al. (2003). Amplification of 16S rRNA genes was performed using *Bacteria* forward primer 0008F (Hicks et al. 1992) and universal reverse primer 1390R (Zheng et al. 1996). The PCR thermal profile, cloning, sequencing, chimera check and phylogenetic rRNA gene sequence analysis were performed as described in the study of Chouari et al. (2003, 2005). All sequences having more than 1200 nucleotides were imported into the ARB database and compared to the EMBL database using BLAST.

Sequences from EMBL with the best BLAST score were imported into the ARB database when necessary (http://www.arb-home.de). They were aligned using the "Fast Aligner" tool of the ARB program and then manually checked. A distance matrix was generated and used by the DOTUR computer program to define Operational Taxonomic Units (OTUs). A 97% 16S rRNA sequence similarity threshold was used to define an OTU. Rarefaction curves for each clone library as well as the estimation of the Simpson, Shannon and Chao diversity indices were generated using DOTUR software (Schloss and Handelsman 2006). Diversity coverage was also calculated as described by Good (1953).

Nucleotide sequence accession numbers

16S rRNA gene sequences determined in this study were deposited in the EMBL database under accession numbers CU914816 to CU915245.

Results

Analysis of ZCO degradation by AM

Degradation of ZCO by the adapted microflora was studied by monitoring the disappearance of TPH, NAH and AH through the determination of their biodegradation percentages, and confirmed by chromatographic analysis of the residual crude oil at the beginning and the end of the incubation period (day 28). The amount of TPH and AH decreased after 3 days. NAH biodegradation started at day 3 and reached the highest value (92.6%) at the end of the incubation period (Fig. 1) whereas the AH biodegradation started only at day 7 and continued to increase to reach a maximum of 68.7% at day 28. A slight decrease in NAH degradation was detected at day 21; this might be related to the accumulation of metabolites following hydrocarbon decomposition. Detailed chromatogram analysis showed visible degradation of *n*-alkanes ranging from *n*-C14 to *n*-C24 during the first week (Fig. 2). Branched alkanes such as 2, 6, 10, 14-tetramethylpentadecane (pristane) and 2, 6, 10, 14,-tetramethylhexadecane were degraded during the second week of incubation. At the end of the third week, the majority of *n*-alkanes such as *n*-decane, *n*-dodecane and *n*-tetradecane were totally degraded, except for some iso-alkanes and *n*-alkanes which were totally degraded after a month of incubation. The signal intensity of the UCM (unresolved complex mixture) whose major composites are the *cyclo*-alkanes and the highly substituted *n*-alkanes, increases from day 0 to day 14 (Fig. 2). This can be explained by the selective biodegradation of *n*-alkanes causing a relative high concentration of *cyclo*-alkanes at the expense of *n*-alkanes and *iso*-alkanes. The UCM degradation signal starts decreasing from day 14 to day 28. Hence at the end of 4th week, the main residual ZCO compounds were *cyclo*-alkanes.

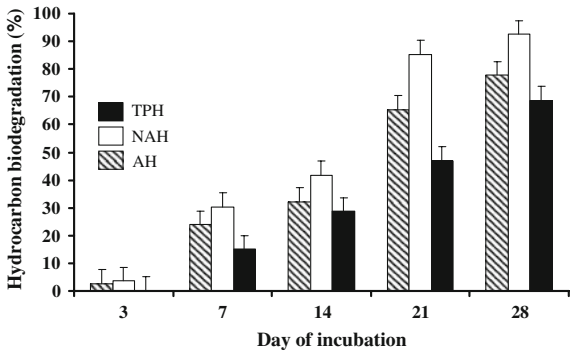

Fig. 1 Histogram showing the evolution of the biodegradation of TPH, NAH and AH fractions of ZCO by the adapted microflora at various incubation periods. TPH: Total Petroleum Hydrocarbons; NAH: Non Aromatic Hydrocarbons; AH: Aromatic Hydrocarbons

Analysis of AH degradation shows that this class of hydrocarbon compounds is less degraded than NAH and presents a lag time ranging from 0 to 7 days (Fig. 1). Our study shows a high AH degradation rate at the end of the incubation period, which reached 68.7% at day 28. We concluded that the adapted consortium exhibits a high level of degradation of both NAH and AH components of ZCO.

Molecular analysis of the adapted microbiota

The adapted microflora represents the natural bacterial population of seawater from Jarzouna Bizerte, Tunisia (Mediterranean Sea), adjacent to an oil refinery. Our results are the first report on the composition of bacterial communities of this region.

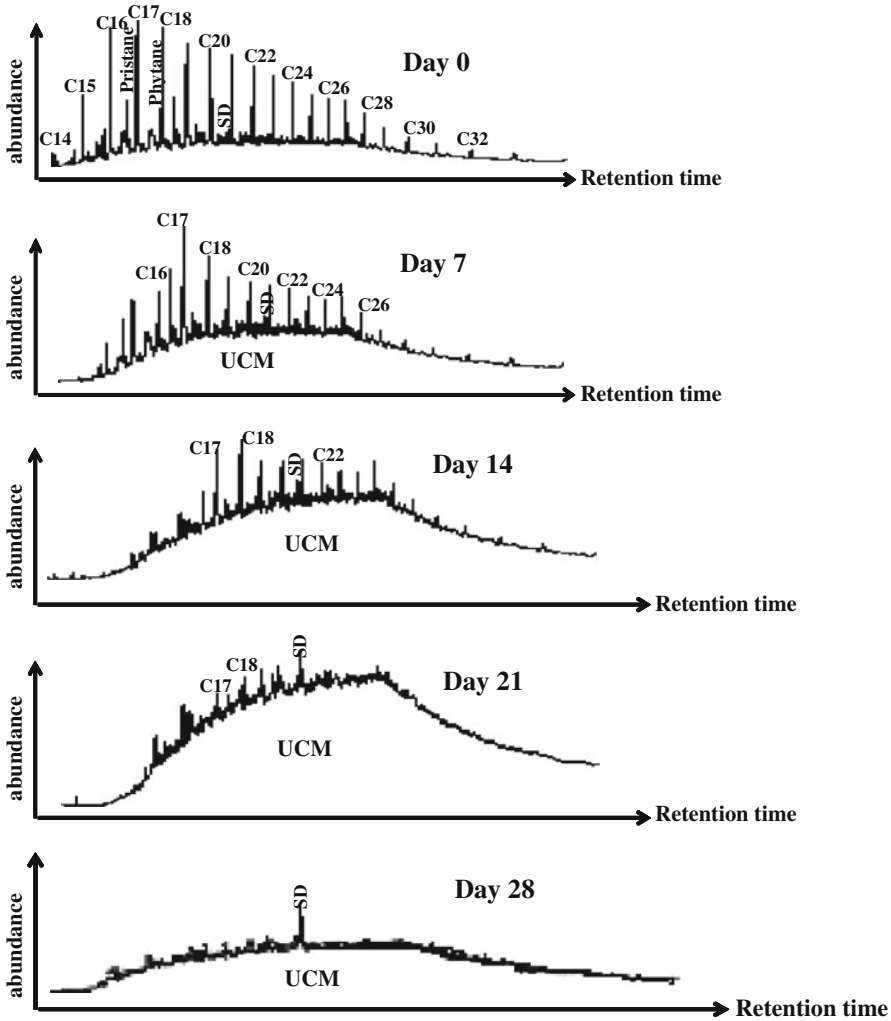

Fig. 2 Chromatograms showing NAH fractions, at different incubation periods during ZCO degradation, by the adapted microflora. GC traces were obtained following NAH fractionation in a capillary column. Individual hydrocarbons are shown with their carbon number. The distribution of peaks with retention time demonstrates individual hydrocarbon degradation in each incubation time. UCM: Unresolved complex mixture; SD: standard

A total of 94 clone sequences were obtained for the adapted microflora clone library (Table 1). They showed a percentage of sequence similarity to the closely related sequence organism found in public databases; pourcentages of sequence similarity ranged between 91 and 100%. The 16S rRNA gene sequences were grouped into 22 OTUs from which 16 OTUs are affiliated with cultivated microorganisms and only one OTU is affiliated with a noncultivated microorganism with ≥97% sequence similarity. A total of five OTUs represent novel OTUs sharing less than 97% sequence identity with their closest relatives. The distribution and affiliation of the adapted microflora is shown in the phylogenetic tree (Fig. 3). Four bacterial divisions are represented: *Proteobacteria* represent the predominant group, composed of *Alpha-, Beta-, and Gamma-subclasses* representing, respectively 36.4, 13.6, and 27.3% of total OTUs. *Firmicutes*, *Bacteroidetes,* and *Actinobacteria* represent 13.6, 4.55, and 4.55% of the total, respectively (Table 2).

The *Alphaproteobacteria* are represented by sequences affiliated with four different genera within the *Rhizobiales* order (*Pseudaminobacter, Nitratireductor*, *Ochrobactrum* and *Novosphingobium)*. The *Pseudaminobacter* genus is dominant (23 clones) in the AM consortium and is represented by OTUs affiliated with *Pseudaminobacter* sp. *W11-4* (DQ65 9452), described by Wang et al. (unplished) as a member of an efficient pyrene-degrading bacterial consortium from the Pacific Open Sea Sediment. *Nitratireductor* is represented by one OTU affiliated with *Nitratireductor aquibiodomus* (AF534573) which was isolated by Labbé et al. (2002) from a marine methanol-fed denitrifying reactor at the Montreal Biodome in Canada. *Ochrobactrum* and *Novosphingobium* genera were represented by unique OTU affiliated with *Ochrobactrum anthropi* (AJ276036) and uncultured bacterium DQ129610, respectively. The *Betaproteobacteria* subclass is represented by 24 clones affiliated with *Alcaligenes faecalis* (AY548384), two clones affiliated with *Alcaligenes faecalis* (EF427887) and *Alcaligenes* sp. *CO14* (DQ643040) and 10 clones affiliated with not yet cultured species (four clones are related to the uncultured beta proteobacterium DQ366010 while the six others are affiliated with the uncultured soil bacterium AF423224). *Alcaligenes faecalis* (AY548384) was recently isolated by Wilson and Metcalf (2005) and characterized as being an active isolate involved in the oxidation of reduced phosphorus compounds, *Alcaligenes faecalis* (EF427887) is a strain isolated by Kazy et al. (unpublished) and described as having a role in phenol biodegradation, whereas *Alcaligenes* sp. *CO14* (*DQ643040*) was described by Tian et al. (unpublished) as a heavy metal-resistant bacterium from deep-sea sediments. It has been reported that cultures of *Alcaligenes* play a major role in AH degradation (Weissenfels et al. 1990). The uncultured beta Proteobacterium DQ366010 was detected by Aislabiea et al.

Fig. 3 Evolutionary distance dendogram constructed using the Neighbor-Joining method showing the affiliation of the environmental bacterial 16S rRNA gene sequences recovered from the enriched polluted seawater from the Jarzouna coast of Tunisia during ZCO degradation at various times of incubation. The phylogenetic tree was calculated using the ARB software package, as described in experimental procedures. The scale bar corresponds to a 10% estimated difference in nucleotide sequence positions. *Plasmodiophora brassicae* is used as an outgroup. The identified phylogenetic groups are indicated by vertical bars on the right of the tree. Only one representative of each OTU is represented in the tree. The number of clones forming each OTU is indicated in brackets

Table 1 Analyses of bacterial clone libraries using DOTUR software

Library	Number of clones	Number of OTUs[b]	Coverage[a]	Diversity indices		
				Chao	Shannon (H)	Simpson (I/D)
Adapted microflora	94	22	71.3	44.75	2.16	0.18
Day 7	99	27	72.7	35.25	2.51	0.14
Day 14	87	12	85	27	1.56	0.27
Day 21	56	9	85.2	14	1.17	0.49
Day 28	94	1	94.7	1	0	0.65

[a] Operational taxonomic unit, the number of OTUs was calculated with a threshold value of 97% 16S rRNA sequence similarity

[b] $[1 - (n/N)] \times 100$ (Good 1953)

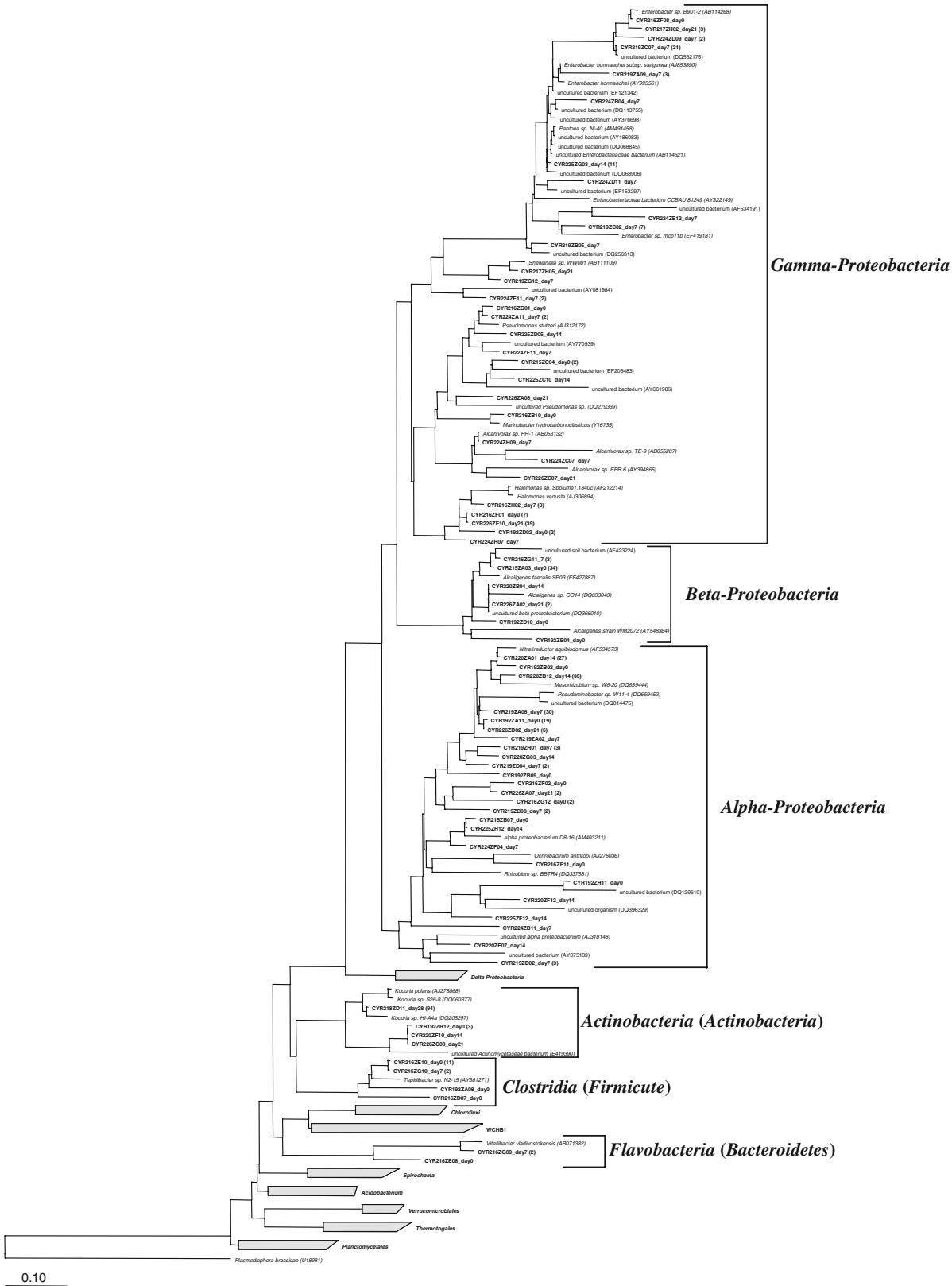
Gamma-Proteobacteria
Beta-Proteobacteria
Alpha-Proteobacteria
Actinobacteria (Actinobacteria)
Clostridia (Firmicute)
Flavobacteria (Bacteroidetes)
Delta Proteobacteria
Chloroflexi
WCHB1
Spirochaeta
Acidobacterium
Verrucomicrobiales
Thermotogales
Planctomycetales
Plasmodiophora brassicae (U18981)
0.10

Table 2 Distribution of clone sequences and OTUs analyzed during ZCO biodegradation

Library	Group affiliation	Sub-group affiliation	Number of clones	Number of OTUs	% OTUs	% of cultivated[a] OTUs (≥97%)	% of not yet cultivated OTUs (≥97%)	% of novel OTUs
Adapted microflora	Proteobacteria	Alpha	27	8	36.4	87.5	12.5	0
		Beta	36	3	13.6	66.7	0	33.3
		Gamma	14	6	27.3	83.3	0	16.7
	Firmicute	Clostridia	13	3	13.6	33.3	0	66.7
	Actinobacteria	Actinobacteria	3	1	4.55	0	0	100
	Bacteroidetes	Flavobacteria	1	1	4.55	100	0	1
Day 7	Proteobacteria	Alpha	43	8	29.6	50	0	50
		Beta	3	1	3.7	100	0	0
		Gamma	49	16	59.3	62.5	0	37.5
	Firmicutes	Clostridia	2	1	3.7	100	0	0
	Bacteroidetes	Flavobacteria	2	1	3.7	100	0	0
Day 14	Proteobacteria	Alpha	68	7	58.4	42.8	0	57.2
		Beta	5	1	8.3	0	100	0
		Gamma	13	3	25	0	33.3	66.7
	Actinobacteria	Actinobacteria	1	1	8.3	0	0	100
Day 21	Proteobacteria	Alpha	8	2	22.2	100	0	0
		Beta	2	1	11.1	0	100	0
		Gamma	45	5	55.6	80	0	20
Day 28	Actinobacteria	Actinobacteria	1	1	11.1	0	100	0

[a] The number of OTUs was calculated with a threshold value of 97% 16S rRNA sequence similarity

(2006) from soil samples from Marble Point and Wright Valley, Victoria Land, Antarctica. The *Gammaproteobacteria* sequences of the adapted microflora are affiliated with cultured species: *Pseudomonas stutzeri* (AJ312172), *Marinobacter hydrocarbonoclasticus* (Y16735), *Halomonas venusta* (AJ306894), *Halomonas* sp. *18bAG* (AJ640133), *Halomonas* sp. *JL1044* (DQ985041), *Enterobacter* sp. *B901-2* and with only one uncultured species: uncultured bacterium EF205483 with <95% sequence similarity. This bacterium was detected by Lau and Pointing (unpublished) in a comparative study of bacterial diversity in geographically separated geothermal regions in central Tibet. *Pseudomonas stutzeri* was isolated by Sikorski et al. (2002) from marine sediments and soils. The contribution of pure cultures of *Pseudomonads* to hydrocarbon degradation has been well documented (Ahn et al. 1998; Syutsubo et al. 2001; Hara et al. 2003; Röling et al. 2002). They are often considered as model organisms for pollution degradation (Popp et al. 2006). *Enterobacter* sp. *B901-2* was detected by Minamisawa et al. (2004) in an anaerobic nitrogen-fixing consortium. Finally, *Firmicutes* and *Bacteroidetes* phyla are represented by 13 clones closely related to *Tepidibacter* sp. *N2-15* (AY581271) and one clone affiliated with *Vitellibacter vladivostokensis* (AB071382) respectively.

Microbial population dynamics during ZCO biodegradation

The overall microbiota modifications at different incubation periods are represented in Tables 1 and 2. In the light of this analysis we observed an important bacterial shift during each period of biodegradation. Phylogenetic diversity is highest during day 7 of incubation and decreases progressively with the incubation time. Community composition changes began during the first week of incubation and remained significant during the whole incubation period, especially during the two last weeks of incubation. Hence at day 21 and day 28, we observed a major decrease in bacterial diversity, confirmed by diversity indices such as the Shannon index, and Simpson's dominance index as shown in Table 1. Calculated clone libraries-coverage shows that >71%

of the bacterial diversity could be accounted for (Table 1). Figure 3 summarizes the abundance of phylum in the clone libraries and their closest relatives in public databases.

At day 7, the *Gammaproteobacteria* are more abundant than in the adapted microflora library, reaching 59.3% of the total bacterial population. The *Alphaproteobacteria* make up 29.6% of OTUs and exhibited major changes in taxonomic representation compared with day 0. The only taxon that was still detected, after day 7 of incubation, within the *Alphaproteobacteria subclass* is the *Pseudaminobacter* sp., with a considerable change in its ratio. The number of clones affiliated with this genus (*Pseudaminobacter* sp.) increased from 23 to 36 clones that are grouped into three OTUs. The emerging new taxa within the *Alphaproteobacteria subclass* during the first week of incubation were closely related to: *Paracoccus thiocyanatus* (AJ864463) described as involved in sulfur-oxidizing chemolithoautotrophs from the rhizosphere soil of an Indian tropical leguminous plant (Ghosh and Roy 2006), and D8-16 (AM403211) described by Krieger et al. (unpublished) as a member of nitrate-reducing and denitrifying bacteria in a marine aquaculture biofilter. One novel OTU sharing 92.1% similarity with the *Rhizobium* sp. *BBTR4* (DQ337581) was found. It has been isolated by Zeng et al. from swine effluent environments, and showed tetracycline and tylosin resistance genes (unpublished). Finally, two uncultured bacteria within the *Alphaproteobacteria* subclass were detected in this period and are affiliated with AY375139 and DQ814475. About 50% of the OTUs affiliated with the *Alphaproteobacteria* subclass, and identified during the first week, are considered as novel OTUs.

After one week, the *Betaproteobacteria* are represented by only 3.7% of the total number of OTUs and are grouped into a single OTU affiliated with *Alcaligenes faecalis* subsp. *faecalis* (AY548384). However, during this incubation period the *Gammaproteobacteria* become the predominant and most diverse subgroup. The clones forming this subclass were affiliated with different taxa and were grouped into 16 OTUs (Table 2). It is important to notice the emergence in this period of clones affiliated with different uncultured bacteria. The most numerous of these were: uncultured bacterium EF121342 (nine clones), uncultured bacterium DQ532176 (eight clones), uncultured bacterium AY375139 (three clones) showing only 89% similarity to the most closely related sequence in the database. Twelve novel sequences, which have not been detected before in other environments, were detected during this period and were grouped into six OTUs representing 37.5% of the total OTUs. They share 91–96% of identity with their closest relatives. The emergence of these uncultured clones suggests that they contribute to the biodegradation of ZCO during the first week of incubation. Many of these novel OTUs were represented by a unique clone. We also noticed the presence of *Alcanivorax* genera, which is well known in petroleum degradation, and also *Pseudomonas, Shewanella, Halomonas a*nd *Enterobacter genera.*

At the end of the second week, the phylogenetic diversity seems to be reduced. We found 32 clones affiliated with *Pseudoaminobacter* while 25 others were affiliated with *Nitratiductor aquibiodomus*. This subclass represents 58.4% of the total OTUs, whereas the *Gamma-* and the *Betaproteobacteria* subclasses enclose respectively 25% and 8.3% of the recovered OTUs, during the second week of ZCO degradation. The results show a continual dynamic in the relative abundance of the different *Proteobacteria* subclasses during oil degradation. In fact at the second week of incubation the percentage of the *Alpha-* and *Betaproteobacteria* OTUs increases whereas those of *Gammaproteobacteria* OTUs decreases. On the other hand, the *Bacteroidetes* and the *Firmicutes* groups, which are represented by one clone each, affiliated, respectively with *Vitellibacter* (AB071382) and *Tepidibacter* sp. *N2-15* (AY581271) during the first week, were not detected during the second week. The number of uncultured bacterial species was also significantly reduced and other uncultured species were detected such as the uncultured *Pseudomonas* sp. DQ2796339, the uncultured alpha proteobacteruim AJ318148, the uncultured bacterium AY661986, the uncultured beta proteobacteruim DQ366010 and the uncultured organism DQ396339. Four clones were affiliated with *Mezorhizobium* sp. *W6-20* (DQ659444) described by Wang et al. as a member of an efficient pyrene-degrading bacterial consortium from the Pacific open Sea sediment (unpublished). Moreover, 57.2 and 66.7% of the recovered OTUs for the *Alpha-* and *Gamma-* subclass are novel.

Comparison of the microflora composition obtained at day 21 and day 28, showed that there were several

changes in bacterial composition and a reduction in bacterial diversity. At the third week, the remaining clones were distributed among the *Actinobacteria* and the *Proteobacteria* groups. The *Gamma-* subclass makes up 55.6% of the total OTUs while the *Alphaproteobacteria* decreases to 22.2% of the total. Only one OTU is affiliated with the *Betaproteobacteria*. The *Gammaproteobacteria* were represented by *Pseudaminobacter, Alcanivorax, Enterobacter, Halomonas and Shewanella. The major phylogenetic bacterium is Halomonas venusta* (AJ306894) and it is represented by 36 clones. We noticed the emergence of unique clones such as *Alcanivorax* sp. *EPR 6* (AY394865) which is involved in petroleum hydrocarbon degradation and *Halomoas* sp. (AF212214). *Actinobacteria* are represented on day 0, day 7 and day 21 by one novel OTU sharing 96% similarity with an uncultured *Actinomycetaceae bacterium*.

At 28 days of incubation, a total of 94 clones were recovered. All of these clones are grouped into one cultivable OTU, affiliated with the *Actinobacteria* group and especially with: *Kocuria polaris* (AJ278868). This bacterium was isolated from an Antarctic *Cyanobacterial* mat sample by Reddy et al. (2003) and described as an orange-pigmented psychrophilic bacterium. It is known that the *Actinobacteria* are one of the most predominant phylogenetic group retrieved in marine ecosystems and that some taxa of this groups may play an important role in bioremediation associated with anaerobic TPH degradation (Rehmann et al. 2001; Pineda-Flores et al. 2004). We suggested that these taxa may have an important role in cyloalkane hydrocarbons and aromatic hydrocarbons of ZCO oil degradation at the 4th week of incubation. The description of the nearest relatives given in public database can provide an idea about the taxa which are detected (Table 3), but no conclusions relative to their direct role or metabolic function can be inferred. Nerveless, this phylogenetic description is important for the exploration of the bacterial composition of the consortium.

Discussion

Efficiency of ZCO degradation by seawater adapted microflora

The results of the present study showed that the heavy zarzatine crude oil (ZCO) was significantly biodegraded after 28 days of incubation with adapted microbiota from polluted coastal seawater in Tunisia. Hydrocarbon degradation was carried out by the indigenous microbiota of the Jarzouna coast, wich is known to be considerably polluted by petroleum compounds. The adapted microflora, resulting from enriching native microbiota at various petroleum concentrations, showed considerable efficiency in the biodegradation rate of both non-aromatic and aromatic fractions. This suggests that the native microflora could have a positive effect on hydrocarbon degradation.

The aliphatic hydrocarbon degradation rate was not constant over the 4 weeks of incubation. Oudot et al. (1998) showed that oil biodegradation is not regular and that it becomes more intense about the fourth and the eighth week of incubation. At the end of the incubation period, both *n*-alkanes and *iso*-alkanes were totally removed, including short and long hydrocarbon chains. It is well documented that shorter-chain *n*-alkanes are first degraded in crude oil followed by the longer-chain-length fractions (Wang et al. 1995a, b; Harayama et al. 1999); McKew et al., (2007) reported that bacterial degradation starts respectively at days 5, 7, 10, and 25, for *n*-alkanes ranging from (C_{10}–C_{14}), (C_{16}–C_{20}), pristane and (C_{22}–C_{32}). In, 2006, Ozaki et al. showed that 56% of the saturated hydrocarbons are degraded by a K-3 consortium from petroleum contaminated soil. Brakstad and Lødeng (2004) used seawater for biodegradation studies of fresh Statfjord oil and noticed a degradation percentage of *n*-alkanes (C_{12}–C_{36}) ranging from 86 to 91% after 2 months of incubation and that the *n*-alkane half-life varied between 14 and 27 days using a seawater consortium.

For *iso*-alkanes, it was reported that an increase in branching decreases hydrocarbon degradation (Mckenna and Kallio 1971; Pirnik et al. 1974). This may explain the low rate of degradation of branched compound such as pristane and phytane (Mckenna and Kallio 1971; Pirnik et al. 1974; Rontani and Giusti 1986; Ko and Lebeault 1999; Alvarez et al. 2001). However branching at the α position does not prevent β-oxidation (Schaeffer et al. 1979; Rontani and Giusti 1986). Moreover, co-oxidations involving microbial species with complementary enzymatic activities cannot be excluded (Bertrand et al. 1983; Leahy and Colwell 1990). Hence, we can suggest that the adapted microflora may contain specialized microorganisms

Table 3 16S rRNA gene sequence similarity to the closest sequences in GenBank

Taxon	Accession no. in Genbank	Similarity (%)
Pseudaminobacter sp. *W11-4*	DQ659452	95.65–100
Uncultured *Actinomycetaceae bacterium*	EF419390	96.86–96.9
Enterobacter sp. *B901-2*	AB114268	99.05–99.86
Halomonas venusta	AJ306894	92.84–98.99
Alcaligenes faecalis subsp. *faecalis*	AY548384	91.12–99.86
Nitratireductor aquibiodomus	AF534573	98.58–99.41
Alcaligenes faecalis subsp. *faecalis*	EF427887	99.29
Alcaligenes sp. *CO14*	DQ643040	99.28
Alcanivorax sp. *EPR 6*	AY394865	99.12
Alcanivorax sp. *PR-1*	AB053132	99.86
Alcanivorax sp. *TE-9*	AB055207	95.98
Alpha proteobacterium D8-16	AM403211	98.33
Enterobacter hormaechei	AY995561	99.86
Enterobacter hormaechei subsp. *steigerwaltii*	AJ853890	98.8–99.39
Enterobacter sp. *mcp11b*	EF419181	99.4
Enterobacteriaceae bacterium CCBAU 81249	AY322149	99.4
Halomonas sp. *18bAG*	AJ640133	99.23–99.56
Halomonas sp. *JL1044*	DQ985041	97.78–98.72
Halomonas sp. *Sbplume1.1840c*	AF212214	98.7–98.92
Halomonas variabilis	AY204638	95.95
Kocuria polaris	AJ278868	97.9–98.99
Kocuria sp. *HI-A4a*	DQ205297	98.39–99.27
Kocuria sp. *S26-8*	DQ060377	98.48–99.13
Marinobacter hydrocarbonoclasticus	Y16735	99.84
Mesorhizobium sp. *W6-20*	DQ659444	98.70–99.4
Ochrobactrum anthropi	AJ276036	99.92
Pantoea sp. *Nj-40*	AM491458	99.57
Paracoccus thiocyanatus	AJ864463	99.02
Pseudomonas stutzeri	AJ312172	97.64–99.78
Rhizobium sp. *BBTR4*	DQ337581	92.1
Shewanella putrefaciens	DQ307729	97.42
Shewanella sp. *WW001*	AB111109	99.19
Tepidibacter sp. *N2-15*	AY581271	95.3–99.7
Uncultured alpha proteobacterium	AJ318148	91.37
Uncultured bacterium	AF534191	95.14
Uncultured bacterium	AF534213	99.19
Uncultured bacterium	AY081984	96.68–97.04
Uncultured bacterium	AY186083	99.78
Uncultured bacterium	AY375139	89.53–89.68
Uncultured bacterium	AY376698	98.61–98.96
Uncultured bacterium	AY661986	93.81
Uncultured bacterium	AY770939	98.78
Uncultured bacterium	DQ068845	99.35
Uncultured bacterium	DQ068848	99.57–99.64
Uncultured bacterium	DQ068906	96.05–96.13

Table 3 continued

Taxon	Accession no. in Genbank	Similarity (%)
Uncultured bacterium	DQ113755	97.5
Uncultured bacterium	DQ129610	96.94–97.99
Uncultured bacterium	DQ256313	97.57
Uncultured bacterium	DQ532176	99.06–99.93
Uncultured bacterium	DQ814475	98.55–99.02
Uncultured bacterium	DQ814842	96.27
Uncultured bacterium	EF121342	99.93–100
Uncultured bacterium	EF153297	94.45
Uncultured bacterium	EF205483	95.11–95.44
Uncultured beta proteobacterium	DQ366010	99.78–100
Uncultured *Enterobacteriaceae* bacterium	AB114621	99.63
Uncultured organism	DQ396339	93.44
Uncultured *Pseudomonas* sp.	DQ279339	95.01
Uncultured soil bacterium	AF423224	99.56–99.85
Vitellibacter vladivostokensis	AB071382	98.25–99.93

with complementary roles in degradation of branched alkanes. Cycloalkanes were also considerably degraded by adapted microflora but not completely removed at the end of incubation period. Atlas (1981) showed that degradation of *cycloalkanes* is very limited. Penet et al. (2004) reported similar results in their study of gasoline and diesel oil degradation.

Aromatic degradation by the adapted microflora was lower than the degradation of the aliphatic fraction. These results confirm the relative recalcitrant degradation of AH fraction as described by several authors (Harayama et al. 1999; Reid et al. 2002). However Ozaki and Fujita (2006) described bacterial consortia which are able to degrade AH at higher rates. Table 4 provides bibliographic information on the main hydrocarbon degradation studies. However, it is difficult to compare our results with other reported studies because of the great differences in experimental conditions (crude oil type, crude oil concentration, type of microbiota inoculation, the amount of the bacterial amendment, physico-chemical parameters, etc.).

Bacterial characterization of seawater adapted microflora

Detailed analyses of the adapted microflora clone library indicated that the abundant phylotypes in the seawater microflora enriched by zarzatine crude oil are distributed amoung the *Alpha-, Beta-*, and *Gammaproteobacteria*. Minor phylotypes are distributed within the *Actinobacteria, Firmicutes,* and *Bacteroidetes* groups. The dominance of the *Proteobacteria* has been extensively documented in the literature in hydrocarbon-contaminated environments (seawater, soil, ice, estuary, and river). The predominance of the *Proteobacteria* in polluted seawater has been demonstrated in several studies (Brakstad and Lødeng 2004; Brakstad et al. 2004; Kasai et al. 2005; Marc et al. 2005). We noticed that many molecular diversity studies have reported similar results regarding the contribution of the *Proteobacteria* as the dominant phylogenetic group in hydrocarbon degradation. MacNaughton et al. (1999), in their experiments conducted using oil contaminated sand, demonstrated the dominance of the *Alphaproteobacteria* subclass at the expense of *Gammaproteobacteria group*. They also reported that the *Gammaproteobacteria* are first detected at week 8 after oil contamination. Popp et al. (2006) concluded that the dominance of the *Gammaproteobacteria* may result from the degradation of high levels of contaminants. The involvement of the *Actinobacteria, Firmicutes,* and *Bacteroidetes* in hydrocarbon biodegradation is also extensively documented (Chang et al. 2000; Popp et al. 2006). Although the *Proteobacteria* represent the predominant group in most of the studies conducted on hydrocarbon polluted environments, the taxonomic

Table 4 Hydrocarbon biodegradation efficiency of AM, and microfloras recovered at day 7, 14, 21, and 28 in this study

Carbon sources	Microbiota origin	Spices and/or, dominant microorganisms	Incubation period	%B[a]	Reference
Zarzatine Crude oil	Seawater adjacent to oil refinery (Tunisia)	Adapted microflora			This study
TPH		Day 7	7 days	23.9	
Saturated fraction			7 days	30	
Aromatic fraction			7 days	15	
TPH		Day 14	14 days	32.1	
Saturated fraction			14 days	42	
Aromatic fraction			14 days	28.6	
TPH		Day 21	21 days	65.3	
Saturated fraction			21 days	85.3	
Aromatic fraction			21 days	47	
TPH		Day 28	28 days	77.8	
Saturated fraction			28 days	92.6	
Aromatic fraction			28 days	68.7	
Oil refinery tank bottom sludge (OTBS)	Contaminated soil; groundwater; petrochemical waste sludge refinery-wastewater plants	Adapted microbial consortia: Consortium consisting of four microorganisms (three bacteria and one yeast): *Acinetobacter calcoaceticus Nocardiodes simplex; Pseudomonas alcaligenes Rhodotorula graminis*			Gallego et al. (2007)
n-Alkanes			10 days	100	
Branched alkanes			10 days	44	
Cyclo-alkane			10 days	85	
Aromatics			10 days	31–55	
Crude oil	Seawater from Stanford le Hope adjacent to a major oil refinery (UK)	Consortiums: *Thalassolituus oleivorans, Rosobacter*, *Arcobacter, Oceanospirillum.*			McKew et al. (2007)
Low-molecular-weight alkanes (C_{10}–C_{18})			10 weeks	99	
High-molecular-weight alkanes (C_{20}–C_{32}) and pristane			10 weeks	41–84	
Polycyclic aromatic hydrocarbons (PAHs).			10 weeks	32–88	

Table 4 continued

Carbon sources	Microbiota origin	Spices and/or, dominant microorganisms	Incubation period	%B[a]	Reference
Crude oil	Petroleum contaminated soils at oilfields prefectures (Japan)	K-3 consortium: *Pseudomonas aeruginesa, Ochrobactrum anthropi, Uncultred bacterium (AY218747) and (AY218737), Uncultred Alpha (AY218747) and (AY218737), Uncultred Alphabacterium (AY144193).*			Ozaki and Fujita (2006)
Saturated hydrocarbon of crude oil			7 days	56	
Aromatics + saturated			7 days	20	
Aromatics			7 days	3	
Aromatics of crude oil			7 days	46	
Crude petroleum	Heavily creosote-contaminated soil from wood treatment plant (near Barcelone)	Eubacterial community: *Sphingomonas, Azospirillum Xanthomonas, Alcaligenes, Achromobacter*			Viñas et al. (2005)
TPH			200 days	72–79	
PAHs			200 days	83–87	
Fresh Statfjord oil	Seawater from North Sea (German) near oil platform	Consortiums: *Sphingobacteria, Flavobacteria, Pseudoalteromonas, Alteromonas, Vibrio, and Rosobacter*			Brakstad et al. (2004)
n-Alkanes (C_{12}–C_{36})			2 months	86–91	
Crude petroleum	Hydrocarbon-contaminated sites (near Barcelone, Spain)	Three microbial consortia with no composition detail:			Viñas et al. (2002)
Saturated fraction		TD consortia	7 days	48	
Polyaromatic fraction			7 days	11	
Saturated fraction		F1AA	15 days	60	
Polyaromatic fraction			15 days	7	
Saturated fraction		AM	7 days	34	
Polyaromatic fraction			7 days	19	

Table 4 continued

Carbon sources	Microbiota origin	Spices and/or, dominant microorganisms	Incubation period	%B[a]	Reference
Hydrocarbon model compounds	Heavily polluted site (Wadi Gaza)	Indigenous Bacteria and cyanobacteria mats: *G. fermenta, H. foetida, Anabaena cylindrica Phormidium faveolarum, Phormidium corium*			Abed et al. (2002)
Phenantrene and dibenzothophene			7 days	100	
n-Octadecene and pristane			7 days	25 and 34	
Assam crude oil	Oil-contaminated samples from coastal regions (near Mumbai, India)	*Rhodococcus* sp.			Sharma and Pant (2000)
Aliphatic fraction			72 h	50	
Pristane			72 h	30	
Crude oil	Several contaminated sites	Mixed biomass: bacteria and fungi: *Pseudomonas, Brevundimonas Sphingomonas Acinetobacter, Rhodococcus, Arthrobacter Corynebacterium, Aspergillus, Penicillium, Beauveria, Acremonium, Cladosporium*			Chaîneau et al. (1999)
TPH			3 months	40	
Saturated fraction			3 months	15–80	
Aromatic fraction			3 months	64	
Saturated fraction		*Ps. Chlorophis*	3 months	40	
Aromatic fraction			3 months	16	
Saturated fraction		*Sphingomonas paucimobilis*	3 months	56	
Aromatic fraction			3 months	34	
Saturated fraction		*Acinotobacter baumannii*	3 months	68	
Aromatic fraction			3 months	8	
Crude oil	Contaminated soil	Mixed biomass with no composition detail			Oudot et al. (1998)
TPH			35 weeks	40.2	
Aromatic fraction			35 weeks	24.3	

[a] Percentage of biodegradation

A report of the efficiency of microfloras recovered in other studies are included in this table

composition of the analyzed populations displayed many differences. This may indicate that every indigenous population is different. These differences may be attributed to environmental conditions (temperature, pH, salinity, O_2, etc.) and the nature of the substrate to be degraded (crude oil, refined product, asphalt, etc.). In fact different natural bacterial microflora have been described worldwide, and each microflora is specific to its environment. McKew et al. (2007) reported that the present microbiota from the Thames estuary in the UK wich has adapted to crude oil degradation is dominated by *Thalassolituus oleivorans* and composed of various other genera such as *Oceanospirillum, Roseobacter,* and *Arcobacter*. In petroleum-contaminated soils from the north of Canada (Juck et al. 2000), the bacterial population is composed mainly of *Arthrobater, Nocardioides,* and *Xanthomonas*. The German Bight in the North Sea (Brakstad and Lødeng 2004) is characterized by the presence of *Sphingobacteria* and *Flavobacteria, Pseudoalteromonas, Alteromonas, Vibrio, and Roseobacter*. Bacterial populations of soil samples from the polluted zone in Shizuoka, Japan (Kasai et al. 2005) contains *Variovorax, Acidovorax, Burkholderia, Thiobacillus, Alcaligens* and other microorganisms. Within the clone libraries obtained from natural asphalts of the Rancho La Brea Tar Pits in California (Kim and Crowley 2007), the predominant bacteria are affiliated with *Chromatiales, Xanthomonadaceae, Pseudomonadaceae,* and *Rhodobacteraceae*.

The adapted microflora library of the Jarzouna coastal taxonomic composition is relatively different from the communities described in the literature. Preliminary studies have revealed diverse microbial communities that are able to live on complex petroleum hydrocarbon mixtures. Nonetheless, very little is known about the changes in natural consortia after specific enrichment with crude oil. Sequence analysis of the dominant taxa within the 16S rRNA gene library of the adapted microflora revealed specific selection for bacteria highly resistant to high ZCO concentrations. Our results reveal a wide range of phylogenetic groups within the bacterial domain, in which the most abundant OTUs are closely related to *Pseudoaminobacter, Alcaligenes, Nitratireductor and Halomonas* but also to *Alcanivorax, Pseudomonas, Mesorizobuim, Shewanella and Marinobacter hydrocarbonoclasticus.*

It is important to notice that it is very difficult to infer functionality at the phylum level and it will be necessary to study functional genes. Molecular analysis of the composition of bacterial seawater represents an essential step for improving traditional isolation of hydrocarbon degraders.

Hydrocarbon composition changes and bacterial shift during oil biodegradation

Detailed analyses of different incubation stages during ZCO degradation revealed differences in both chemical composition and microbial community composition. To reveal bacterial changes, diversity indices, rarefaction and phylogenetic analysis were carried out for different periods of ZCO degradation. The use of diversity parameters in microbial ecology studies have been described previously by several authors (Hill et al. 2003; Hughes et al. 2001; Pedrós-Alió 2006). Our results are in agreement with those of Brakstad and Lødeng (2004) which indicate that the reduction of microbial diversity is observed early, from day 7 to day 21. In fact, the addition of oil can dramatically reduce bacterial diversity (Röling et al. 2002). Shannon diversity indexes shown in Table 1 seem to be low when compared to the results obtained in other studies (this index was reported to vary between 4 and 5 by Schloss and Handelsman 2006). This might be ascribed to the fact that seawater is in general less diverse than sediment or soil. Furthermore, lower values of the Shannon index were previously found in aged petroleum-contaminated samples as indicated by Saul et al. (2005); Kaplan and Kitts (2004). Our results are in agreement with those previously found by Popp et al. (2006).

In the present study we found that bacterial diversity is gradually reduced during the biodegradation process. At day 0 (adapted microflora), four divisions are represented while only three are represented on days 7, 14, and 21, and only one division by day 28. We found that on days 7, 14, and 21, 16S rRNA gene sequences recovered are affiliated with the same microorganisms detected in the adapted microflora library but with variation in their proportions and the emergence of species not detected before. The major dominant group is the *Proteobacteria,* especially *Alpha-*, *Beta-* and *Gammaproteobacteria*. Thus we noticed a change in the diversity and the relative

proportion of the clones composing these three subclasses during the different stages of ZCO biodegradation. We suggest that the balance between the *Alpha*- and the *Gammaproteobacteria* depends on the nature, levels and composition of the pollution source. The most abundant phyla retrieved in the adapted microflora on days 7, 14, 21 and 28 may be involved directly or indirectly in ZCO degradation. Meanwhile, we observed important shifts in the distribution of the bacterial phyla using different incubation times. At day 7 the microflora contains a greater breadth of OTU diversity. We noticed the emergence of novel OTUs with low sequence identity to their closest relatives described in public databases.

Predominant sequences in the clone libraries of the present study are affiliated with microorganisms which probably represent the main degraders of ZCO in the marine environment. Their presence in a polluted medium, their resistance during acclimatization to high amounts of oil and their proliferation during the biodegradation period, confirms the importance of these consortia in hydrocarbon degradation. The consortium obtained is under investigation in a pilot bioreactor for the treatment of the effluent from the refinery.

Conclusion

The aim of this study was to analyze the bacterial diversity during oil degradation in order to understand the bioremediation process in a marine environment. Molecular analysis of the microflora composition associated with hydrocarbon composition analysis provides evidence that microbial composition dynamics is related to changes in substrate composition, and there is a close relationship between the proliferation of specialized species and their function in the degradation of the different fractions of ZCO oil. Finally, to further elucidate the role of these bacterial groups in hydrocarbon degradation, catabolic gene expression profiling may be necessary.

Acknowledgments We thank STIR refinery employees of Bizerte for their help in seawater sampling. Chromatography analyses were performed in the laboratory of Biochemistry and Mass spectrometry at the Monastir Medical School Tunisia. We thank Sebastien Chaussonnerie for his help in sequence analyses and the CEA-Genoscope sequencing team for their technical assistance. We thank Suzan Cure for the reading of the manuscript. We are grateful to Rémy Marchal, Institut Français de Pétrole, France, for his help and his continuous encouragement. This study was partly supported by grants from the Ministry of Education, Scientific Research and Biotechnology of Tunisia and the University of Monastir.

References

Abed RM, Safi NM, Köster J, de Beer D, El-Nahhal Y, Rullkötter J, Garcia-Pichel F (2002) Microbial diversity of a heavily polluted microbial mat and its community changes following degradation of petroleum compounds. Appl Environ Microbiol 68:1674–1683. doi:10.1128/AEM.68.4.1674-1683.2002

Ahn IS, Ghiorse WC, Lion LW, Shuler ML (1998) Growth kinetics of Pseudomonas putida G7 on naphthalene and occurrence of naphthalene toxicity during nutrient deprivation. Biotechnol Bioeng 59(5):587–594. doi:10.1002/(SICI)1097-0290(19980905)59:5<587::AID-BIT9>3.0.CO;2-6

Aislabiea JM, Chhourb KL, Saulb DJ, Miyauchib S, Aytonb J, Paetzoldc RF, Balksd MR (2006) Dominant bacteria in soils of Marble Point and Wright Valley, Victoria Land, Antarctica. Soil Biol Biochem 38(10):3041–3056. doi:10.1016/j.soilbio.2006.02.018

Alekhina IA, Marie D, Petit JR, Lukin VV, Zubkov VM, Bulat SA (2007) Molecular analysis of bacterial diversity in kerosene-based drilling fluid from the deep ice borehole at Vostok, East Antarctica. FEMS Microbiol Ecol 59(2):289–299. doi:10.1111/j.1574-6941.2006.00271.x

Alvarez HM, Souto MF, Viale A, Pucci OH (2001) Biosynthesis of fatty acids and triacylglycerols by 2, 6, 10, 14-tetramethyl pentadecane-grown cells of Nocardia globerula 432. FEMS Microbiol Lett 200(2):195–200. doi:10.1111/j.1574-6968.2001.tb10715.x

Atlas RM (1981) Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol Rev 45(1):180–209

Bartha R (1977) The microbiology of aquatic oil spills. Adv Appl Microbiol 22:225–266. doi:10.1016/S0065-2164(08)70164-3

Bertrand JC, Mille G (1989) Devenir de la matière organique exogène. Un modèle: les hydrocarbures. In: Bianchi M, Marty D, Bertrand JC, Caumette P, etGauthier MJ (eds) Microorganismes dans les écosystèmes océaniques, Chapitre 13. Masson, Paris, pp 85–343

Bertrand JC, Rambeloarisoa JF, Rontani JF, Giusti G, Mattei G (1983) Microbial degradation of crude oil in sea water in continuous culture. Biotechnol Lett 5:567–572. doi:10.1007/BF01184950

Brakstad OG, Lødeng AGG (2004) Microbial diversity during biodegradation of crude oil in seawater from the North Sea. Microb Ecol 49(1):94–103. doi:10.1007/s00248-003-0225-6

Brakstad OG, Bonaunet K, Nordtug T, Johansen O (2004) Biotransformation and dissolution of petroleum hydrocarbons in natural flowing seawater at low temperature. Biodegradation 15(5):337–346. doi:10.1023/B:BIOD.0000042189.69946.07

Chaîneau CH, Morel J, Dupont J, Bury E, Oudot J (1999) Comparison of the fuel oil biodegradation potential of hydrocarbon-assimilating microorganisms isolated from a temperate agricultural soil. Sci Total Environ 227(2–3):237–247. doi:10.1016/S0048-9697(99)00033-9

Chang YJ, Stephen JR, Richter AP, Venosa AD, Brüggemann J, Macnaughton SJ, Kowalchuk GA, Haines JR, Kline E, White DC (2000) Phylogenetic analysis of aerobic freshwater and marine enrichment cultures efficient in hydrocarbon degradation: effect of profiling method. J Microbiol Methods 40(1):19–31. doi:10.1016/S0167-7012(99)00134-7

Chouari R, Le Paslier D, Daegelen P, Ginestet P, Weissenbach J, Sghir A (2003) Molecular evidence for novel planctomycete diversity in a municipal wastewater treatment plant. Appl Environ Microbiol 69(12):7354–7363. doi:10.1128/AEM.69.12.7354-7363.2003

Chouari R, Le Paslier D, Daegelen P, Ginestet P, Weissenbach J, Sghir A (2005) Novel predominant archaeal and bacterial groups revealed by molecular analysis of an anaerobic sludge digester. Environ Microbiol 7(8):1104–1115. doi:10.1111/j.1462-2920.2005.00795.x

Gallego JL, García-Martínez MJ, Llamas JF, Belloch C, Peláez AI, Sánchez J (2007) Biodegradation of oil tank bottom sludge using microbial consortia. Biodegradation 18(3):269–281. doi:10.1007/s10532-006-9061-y

Ghosh W, Roy P (2006) *Mesorhizobium thiogangeticum* sp. nov., a novel sulfur-oxidizing chemolithoautotroph from rhizosphere soil of an Indian tropical leguminous plant. Int J Syst Evol Microbiol 56:91–97. doi:10.1099/ijs.0.63967-0

Good IJ (1953) The population frequencies of species and the estimation of population parameters. Biometrika 40:237–264

Hara A, Syutsubo K, Harayama S (2003) *Alcanivorax* which prevails in oil-contaminated seawater exhibits broad substrate specificity for alkane degradation. Environ Microbiol 5(9):746–753. doi:10.1046/j.1468-2920.2003.00468.x

Harayama S, Kishira H, Kasai Y, Shutsubo K (1999) Petroleum biodegradation in marine environments. J Mol Microbiol Biotechnol 1(1):63–70

Hernandez-Raquet G, Budzinski H, Caumette P, Dabert P, Le Ménach K, Muyzer G, Duran R (2006) Molecular diversity studies of bacterial communities of oil polluted microbial mats from the Etang de Berre (France). FEMS Microbiol Ecol 58(3):550–562. doi:10.1111/j.1574-6941.2006.00187.x

Hicks RE, Amann RI, Stahl DA (1992) Dual staining of natural bacterioplankton with 4′, 6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl Environ Microbiol 58(7):2158–2163

Hill TCJ, Walsh KA, Harris JA, Moffett BF (2003) Using ecological diversity measures with bacterial communities. FEMS Microbiol Ecol 43:1–11. doi:10.1111/j.1574-6941.2003.tb01040.x

Hughes JB, Hellmann JJ, Ricketts TH, Bohannan BJ (2001) Counting the uncountable: statistical approaches to estimating microbial diversity. Appl Environ Microbiol 67(10):4399–4406. Erratum in: Appl Environ Microbiol. 68. 1: 448. doi:10.1128/AEM.67.10.4399-4406.2001

Juck D, Charles T, Whyte LG, Greer CW (2000) Polyphasic microbial community analysis of petroleum hydrocarbon-contaminated soils from two northern Canadian communities. FEMS Microbiol Ecol 33(3):241–249. doi:10.1111/j.1574-6941.2000.tb00746.x

Kaplan CW, Kitts CL (2004) Bacterial–bacterial succession in a petroleum land treatment unit. Appl Environ Microbiol 70(3):1777–1786. doi:10.1128/AEM.70.3.1777-1786.2004

Kasai Y, Kishira H, Syutsubo K, Harayama S (2001) Molecular detection of marine bacterial populations on beaches contaminated by the Nakhodka tanker oil-spill accident. Environ Microbiol 3(4):246–255. doi:10.1046/j.1462-2920.2001.00185.x

Kasai Y, Takahata Y, Hoaki T, Watanabe K (2005) Physiological and molecular characterization of a microbial community established in unsaturated petroleum-contaminated soil. Environ Microbiol 7(6):806–818. doi:10.1111/j.1462-2920.2005.00754.x

Kim JS, Crowley DE (2007) Microbial diversity in natural asphalts of the Rancho La Brea Tar Pits. Appl Environ Microbiol 73(14):4579–4591. doi:10.1128/AEM.01372-06

Ko SH, Lebeault JM (1999) Effect of mixed culture on co-oxidation during the degradation of saturated hydrocarbon mixture. J Appl Microbiol 87:72–79. doi:10.1046/j.1365-2672.1999.00797.x

Labbé N, Parent S, Villemur R (2002) *Nitratireductor aquibiodomus* gen. nov., sp., a novel α-protebacterium fom the marine denitrication system of the Montreal Biodome (Canada). Int J Syst Evol Microbiol 54(1):269–273. doi:10.1099/ijs.0.02793-0

Leahy JG, Colwell RR (1990) Microbial degradation of hydrocarbons in the environment. Microbiol Rev 54:305–315

MacNaughton SJ, Stephen JR, Venosa AD, Davis GA, Chang YJ, White DC (1999) Microbial population changes during bioremediation of an experimental oil spill. Appl Environ Microbiol 65:3566–3574

Marc V, Sabaté J, Espuny MJ, Solanas AM (2005) Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. Appl Environ Microbiol 71(11):7008–7018. doi:10.1128/AEM.71.11.7008-7018.2005

McKenna EJ, Kallio RE (1971) Microbial metabolism of the isoprenoid alkane pristine. Proc Natl Acad Sci USA 68:1552–1554. doi:10.1073/pnas.68.7.1552

McKew BA, Coulon F, Osborn AM, Timmis KN, McGenity TJ (2007) Determining the identity and roles of oil-metabolizing marine bacteria from the Thames estuary, UK. Environ Microbiol 9(1):165–176. doi:10.1111/j.1462-2920.2006.01125.x

Minamisawa K, Nishioka K, Miyaki T, Ye B, Miyamoto T, You M, Saito A, Saito M, Barraquio WL, Teaumroong N, Sein T, Sato T (2004) Anaerobic nitrogen-fixing consortia consisting of clostridia isolated from gramineous plants. Appl Environ Microbiol 70(5):3096–3102. doi:10.1128/AEM.70.5.3096-3102.2004

Oudot J, Merlin FX, Pinvidic P (1998) Weathering rate of oil components in a bioremediation experiment in Estuarine sediments. Mar Environ Res 45(2):113–125. doi:10.1016/S0141-1136(97)00024-X

Ozaki S, Fujita KishimotoT (2006) Isolation and phylogenetic characterization of microbial consortia able to degrade aromatic hydrocarbons at high rates. Microbes Environ 21(1):44–52. doi:10.1264/jsme2.21.44

Pedrós-Alió C (2006) Marine microbial diversity: can it be determined? Trends Microbiol 14(6):257–263. doi:10.1016/j.tim.2006.04.007

Penet S, Marchal R, Sghir A, Monot F (2004) Biodegradation of hydrocarbon cuts used for diesel oil formulation. Appl Microbiol Biotechnol 66(1):40–47. doi:10.1007/s00253-004-1660-0

Pineda-Flores G, Ball-Arguello G, Lira-Galeana C, Mesta-Howard MA (2004) A microbial consorptium isolated from a crude oil sample that uses asphaltenes as a carbon and energy source. Biodegradation 15:145–151. doi:10.1023/B:BIOD.0000026476.03744.bb

Pirnik MP, Atlas RM, Bartha R (1974) Metabolism by Brevibacterium erythrogenes: normal and branched alkanes. J Bacteriol 119(3):868–878

Popp N, Schlömann M, Mau M (2006) Bacterial diversity in the active stage of a bioremediation system for mineral oil hydrocarbon-contaminated soils. Microbiology 152(11):3291–3304. doi:10.1099/mic.0.29054-0

Prince RC (2005) Petroleum microbiology. In: Ollivier B, Magot M (eds) The microbiology of marine oil spill bioremediation. American Society for Microbiology Press, Washington DC, pp 317–336

Reddy GS, Prakash JS, Prabahar V, Matsumoto GI, Stackebrandt E, Shivaji S (2003) *Kocuria polaris* sp. nov., an orange-pigmented psychrophilic bacterium isolated from an Antarctic cyanobacterial mat sample. Int J Syst Evol Microbiol 53(1):183–187. doi:10.1099/ijs.0.02336-0

Rehmann K, Hertkorn N, Kettrup AA (2001) Fluoranthene metabolism in *Mycobacterium* sp. strain KR20: identity of pathway intermediates during degradation and growth. Microbiology 147(10):2783–2794

Reid BJ, Fermor TR, Semple KT (2002) Induction of PAH-catabolism in mushroom compost and its use in the biodegradation of soil-associated phenanthrene. Environ Pollut 118(1):65–73. doi:10.1016/S0269-7491(01)00239-1

Reisfeld A, Rosenberg E, Gutnick D (1972) Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. Appl Microbiol 24(3):363–368

Röling WF, Milner MG, Jones DM, Lee K, Daniel F, Swannell RJ, Head IM (2002) Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. Appl Environ Microbiol 68(11):5537–5548

Rontani JF, Giusti G (1986) Study of the biodegradation of polybranched alkanes by a marine bacterial community. Mar Chem 20:197–205. doi:10.1016/0304-4203(86)90039-3

Saliot A (1981) Natural hydrocarbons in sea water. In: Duursma EK, Dawson R (eds) Marine organic chemistry. Elsevier, Amsterdam, pp 327–374

Saul DJ, Aislabie JM, Brown CE, Harris L, Foght JM (2005) Hydrocarbon contamination changes the bacterial diversity of soil from around Scott Base, Antarctica. FEMS Microbiol Ecol 53(1):141–155. doi:10.1016/j.femsec.2004.11.007

Schaeffer TL, Cantwell SG, Brown JL, Watt DS, Fall RR (1979) Microbial growth on hydrocarbons: terminal branching inhibits biodegradation. Appl Environ Microbiol 38:742–746

Schloss PD, Handelsman J (2006) Introducing TreeClimber, a test to compare microbial community structures. Appl Environ Microbiol 72(4):2379–2384. doi:10.1128/AEM.72.4.2379-2384.2006

Sharma SL, Pant A (2000) Biodegrdation and conversion of alkanes and crude oil by marine *Rhododcoccus* sp. Biodegradation 11(5):289–294. doi:10.1023/A:1011185806974

Sikorski J, Mohle M, Wackernagel W (2002) Identification of complex composition, strong strain diversity and directional selection in local *Pseudomonas stutzeri* populations from marine sediment and soils. Environ Microbiol 4(8):465–476. doi:10.1046/j.1462-2920.2002.00325.x

Syutsubo K, Kishira H, Harayama S (2001) Development of specific oligonucleotide probes for the identification and in situ detection of hydrocarbon-degrading Alcanivorax strains. Environ Microbiol 3(6):371–379. doi:10.1046/j.1462-2920.2001.00204.x

Trabelsi S, Driss MR (2005) Polycyclic aromatic hydrocarbons in superficial coastal sediments from Bizerte Lagoon, Tunisia. Mar Pollut Bull 50:344–359. doi:10.1016/j.marpolbul.2004.11.031

Viñas M, Grifoll M, Sabaté J, Solanas AM (2002) Biodegradation of a crude oil by three microbial consortia of different origins and metabolic capabilities. J Ind Microbiol Biotechnol 28:252–260. doi:10.1038/sj.jim.7000236

Viñas M, Sabaté J, Espuny MJ, Solanas AM (2005) Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. Appl Environ Microbiol 71(11):7008–7018. doi:10.1128/AEM.71.11.7008-7018.2005

Walker JD, Colwell RR (1974) Microbial petroleum degradation: use of mixed hydrocarbon substrates. Appl Microbiol 27(6):1053–1060

Wang RF, Cao WW, Cerniglia CE (1995a) Phylogenetic analysis of polycyclic aromatic hydrocarbon degrading mycobacteria by 16S rRNA sequencing. FEMS Microbiol Lett 130(1):75–80

Wang ZD, Fingas M, Sergy G (1995b) Chemical characterization of crude oil residues from an arctic beach by GC/MS and GC/FID. Environ Sci Technol 29:2622–2631. doi:10.1021/es00010a025

Weissenfels WD, Beyer M, Klein J (1990) Degradation of phenanthrene, fluorene and fluoranthene by pure bacterial cultures. Appl Microbiol Biotechnol 32(4):479–484. doi:10.1007/BF00903787

Wilson MM, Metcalf WW (2005) Genetic diversity and horizontal transfer of genes involved in oxidation of reduced phosphorus compounds by *Alcaligenes faecalis WM2072*. Appl Environ Microbiol 71(1):290–296. doi:10.1128/AEM.71.1.290-296.2005

Winderl C, Schaefer S, Lueders T (2007) Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (bssA) genes as a functional marker. Environ Microbiol 9(4):1035–1046. doi:10.1111/j.1462-2920.2006.01230.x

Zheng D, Alm EW, Stahl DA, Raskin L (1996) Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. Appl Environ Microbiol 62:4504–4513

Zobell CE (1946) Action of microorganisms on hydrocarbons. Bacteriol Rev 10:1–49

Zrafi-Nouira I, Khedir-Ghenim Z, Zrafi F, Bahri R, Cheraeif I, Rouabhia M, Saidane-Mosbahi D (2008) Hydrocarbon pollution in the sediment from the Jarzouna-Bizerte coastal area of Tunisia (Mediterranean Sea). Bull Environ Contam Toxicol 80(6):566–572. doi:10.1007/s00128-008-9421-x