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Natural microbial diversity in superficial sediments of Milazzo Harbor (Sicily) and community successions during microcosm enrichment with various hydrocarbons

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Summary

Hydrocarbon-contaminated superficial sediments collected from the Harbor of Milazzo (Tirrenean Sea, northern Sicily), a zone strongly affected by anthropogenic activities, were examined for *in situ* biodegradative capacities. A culture-independent molecular phylogenetic approach was used to study the influence of hydrocarbon and nutrient addition on the activity and diversity of the indigenous microbiota during a microcosm evaluation. The autochthonous microbial community in non-polluted sediments was represented by eubacterial phylotypes grouped within *Proteobacteria*, *CFB* and *Firmicutes*. The archaeal domain was represented by members of Marine Group I of *Crenarchaeota*. The majority of recovered sequences was affiliated with heterotrophic genera *Clostridium* and *Vibrio*, typical members of eutrophic coastal environments. Amendments of hydrocarbons and mineral nutrients to microcosms dramatically changed the initial diversity of the microbial community. Only bacterial phylotypes affiliated with *Proteobacteria* and *CFB* division were detected. The decrease in diversity observed in several microcosms could be explained by the strong selection for microorganisms belonging to group of marine hydrocarbonoclastic γ-*Proteobacteria*, namely *Alcanivorax*, *Cycloclasticus*, *Marinobacter*, *Marinobacterium/Neptunomonas* and *Thalassolituus*. This study demonstrated that nutrient amendment to hydrocarbon-contaminated superficial sediments enhanced the indigenous microbial biodegradation activity and that highly specialized marine hydrocarbonoclastic bacteria, representing a minor fraction in the natural microbial community, play an important role in the biodegradation of petroleum hydrocarbons accidentally entering the coastal environment.

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Introduction

Hydrocarbon contamination of harbour sediments from marine oil transportation, pipe/tanker accidents, dumping of tanker ballast water, petroleum run-off from land, etc., is of great concern because of the toxicity of petroleum hydrocarbons. Many physical remediation strategies, such as sediment dredging and removal, are economically impractical and therefore inapplicable. Moreover, the usage of dredged material as reclaimed soil is very limited, especially in cases where the sediment contains a high concentration of toxic hydrocarbons (Myers and Bowman, 1999). Therefore, this suggests that bioremediation is the more pragmatic approach to the problem. This requires a better understanding of the activities of indigenous hydrocarbon degrading microorganisms to be used in biotreatments.

Bacteria are responsible for major environmental hydrocarbon degradation (Leahy and Colwell, 1990). Such processes occurring in marine ecosystems have been extensively studied (Atlas, 1981; Swannell *et al*., 1996; Head and Swannell, 1999; Harayama *et al*., 1999). Recently, a variety of marine hydrocarbon-degrading bacteria from several phyla (*Proteobacteria*, *CFB*, Gram-positive *Firmicutes*) have been isolated from sites all over the world (Gauthier *et al*., 1992; Dyksterhouse *et al*., 1995; Yakimov *et al*., 1998, 2003, 2004; Hedlund *et al*., 1999; Engelhardt *et al*., 2001; Golyshin *et al*., 2002). However, the growth of many of these hydrocarbon-degrading bacteria is limited by the low availability of nitrogen and phosphorus in seawater (Reisfeld *et al*., 1972; Atlas, 1981,

1988). Addition of N and P fertilizers has been shown to strongly enhance the biodegradation rates of microbial consortia *in situ* (Prince, 1993; Swannell *et al.*, 1996; Head and Swannell, 1999; Schäfer *et al.*, 2001). Rather than a single species, an assemblage of many different bacterial species is usually involved in the degradation of crude oil. Depending on the chemical nature of the contaminant, different biodegrading microorganisms coexisting in extant microbocenosis may became dominant as it was shown by the temporal fluctuation of population densities of hydrocarbonoclastic *Alcanivorax* and *Cycloclasticus* among the bacteria attached to the oil-polluted grains of gravel (Kasai *et al.*, 2002a; Röling *et al.*, 2002).

The goals of the present study are: (i) to characterize the organic contamination of surface sediments of Milazzo Harbor (Sicily, Italy); (ii) to perform a phylogenetic survey of the indigenous microbial community associated with this site and (iii) to monitor changes in the structure and composition of the bacterial community as a response to nutrient load and the addition of various hydrocarbons.

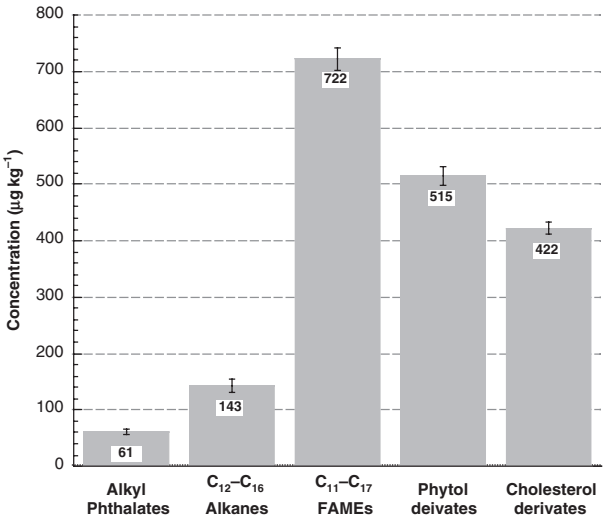

Fig. 1. Composition of major organic pollutants obtained from Milazzo Harbor sediment samples.

Results

Harbour sediment characterization

The topmost 5 cm of a sediment core (approximately 50 g) with a distinct colour stratification from brown-grey in the upper 4 cm to black in the lower 1 cm was obtained. The average total organic matter (TOM) content in the surface sediment of Milazzo Harbor was 3.53 ± 0.15%, whereas total nitrogen content was 0.32 ± 0.06%, yielding C/N ratio of 11.0. These values fell well within the observed ranges of other harbour sediments (approximately 1–4% and 9–13 for TOM and C/N respectively) (Sweeney *et al.*, 1980; Takada *et al.*, 1992; Eganhouse and Sherblom, 2001). Total extractable organic matter (TEO) content was 5.87 mg g^{-1}, whereas concentration of total unpolar organic compounds (TUC) was 2.58 mg g^{-1}, yielding the TUC/TEO ratio of 0.44, which is comparable to those reported from other coastal sites contaminated by municipal wastes (38–51% of TEO) (Eganhouse *et al.*, 1981; Eganhouse and Kaplan, 1982; Eganhouse *et al.*, 1983; Eganhouse and Sherblom, 2001). The hydrocarbon fraction was found to be complex and was characterized by a significant amount of unresolved complex mixture (UCM) 28% of TUC fraction. GC-MS analyses of UCM revealed the presence of molecular fragments typical for aliphatic hydrocarbons. Thus, UCM seemed to be comprised largely of a mixture of alkanes *n*-C16 to >*n*-C32 (branched and cyclic). Unresolved complex mixture with these characteristics could be caused by the presence of fossil fuels or crankcase oil and are not believed to originate from recent biogenic sources (Volkman and Maxwell, 1986; Volkman *et al.*, 1997). The composition of the major resolved fractions of organic compounds isolated from harbour sediments is summarized in Fig. 1 and contains normal C12-C_{16} alkanes (7.6%), C2-C_9 alkyl phthalates (3.3%), methylated C11-C_{17} fatty acids (38.7%), acyclic C18-C_{21} isoprenoids (27.7%) and sterols (epi- and coprostanol were dominant) (22.7%).

Clone library analysis of natural microbial community

We used a pair of universal primers (see *Experimental procedures*) to amplify bacterial 16S rRNA genes and, after clone library construction (hereinafter referred to as the 'NAT' library), 200 randomly picked clones containing 16S rDNA inserts have been de-replicated using restriction fragment length polymorphism (RFLP) analysis. Forty-five operative taxonomic units (OTU) were observed by RFLP profiling. Twenty-four and 11 OTUs made two large subgroups with matching RFLP patterns. RFLP patterns of 12 clones occurred only once in the clone library. Based on these data, the calculated diversity coverage for NAT library was relatively high (96%), indicating that relatively good coverage of clone diversity was achieved. Sixty-three out of a total 200 clones representing all OTUs were sequenced. Forty-one and 14 of these clones represented two large subgroups detected by RFLP analysis. Thirty phylotypes were discerned with each phylotype consisting of either a unique clone or a group of clones that clustered over 97%. This value of 16S rRNA sequence similarity, as proposed by Rossello-Mora and Amann (2001), could be used as an absolute boundary for species circumscription. An archaeal-specific primer set was also employed and amplification products were obtained. Three RFLP patterns were obtained for 30

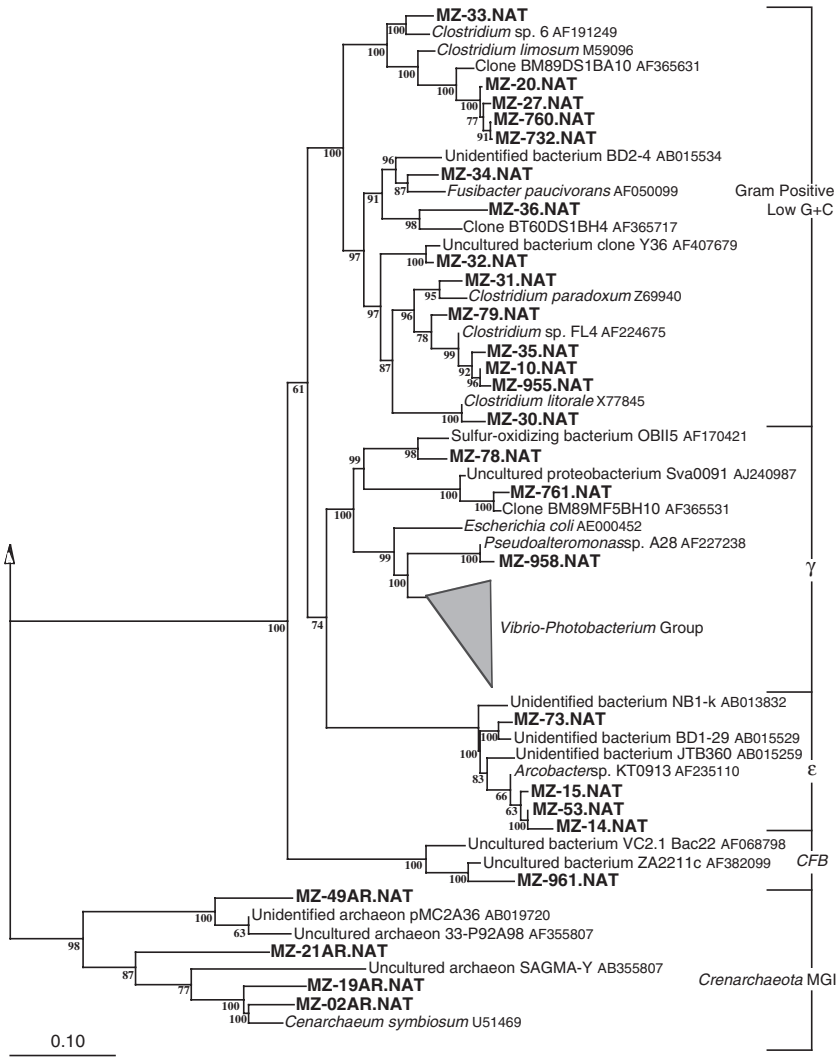

Fig. 2. Rooted phylogenetic tree of NAT clones clustered by Neighbour-joining of maximum likelihood values showing affiliation of partial bacterial 16S rRNA gene sequences to closest related sequences from either cultivated or cloned members of different microbial clusters. Clones obtained in this work are indicated in bold-type. Percentages of 1000 bootstrap resampling that supported the branching orders in each analysis are shown above or near the relevant nodes (only values ≥ 50% are shown). The tree was rooted with 16S rRNA gene sequences of *Methanococcus jannaschii* (M59126). The scale bar indicates 10% estimated sequence divergence.

clones and after sequencing, three sequence types were recovered. All these clones belong to Marine Group I of *Crenarchaeota***,** a relatively common group of archea in seawater (Massana *et al*., 2000; Lopez-Garcia *et al*., 2001).

Using the 530R sequencing primer we obtained 450–480 nucleotide sequences (mean size) corresponding to the positions approximately 20–500 of the 16S rRNA gene (*Escherichia coli* numbering). Several clones were almost identical to known 16S rRNA sequences from cultured organisms or environmental clones. Figures 2 and 3 summarize the phylogenetic positions of NAT clones sequenced.

Clostridia and related organisms were present in the NAT library with nine phylotypes and 14 sequenced clones, which made 24% of the total riboclone population. Phylogenetic analysis showed that MZ-10.NAT, MZ-35.NAT, MZ-79.NAT, MZ-955.NAT and MZ-30.NAT were very closely related to *Clostridium* sp. FL6 and *Clostridium litorale* (97.7–99.8%, respectively, of sequence identity). Three other clones, MZ-31.NAT, MZ-32.NAT and MZ-33.NAT revealed the highest values of similarity to *Clostridium paradoxum,* the riboclone Y36, retrieved from a hot artesian basin, and *Clostridium* sp. 6 (95.1%, 97.7% and 95.3% respectively). Four clones, MZ-20.NAT, MZ-27.NAT, MZ-732.NAT and MZ-760.NAT, formed a group affiliated with a cluster of coral-associated riboclones (95.5% in average), whereas MZ-34.NAT and MZ-36.NAT were related to *Fusibacter paucivorans* and another coral-associated riboclone, BT60DS1BH4, 93.9% and 90.3% respectively.

Four clones (11.5%) were affiliated with ε-*Proteobacteria*. All but one, MZ-73.NAT, matched culturable *Arcobacter* sp. KT0913, isolated from the North Sea (97.0–98.5%). The most similar sequences found in GenBank for the MZ-73.NAT riboclone belong to environ-

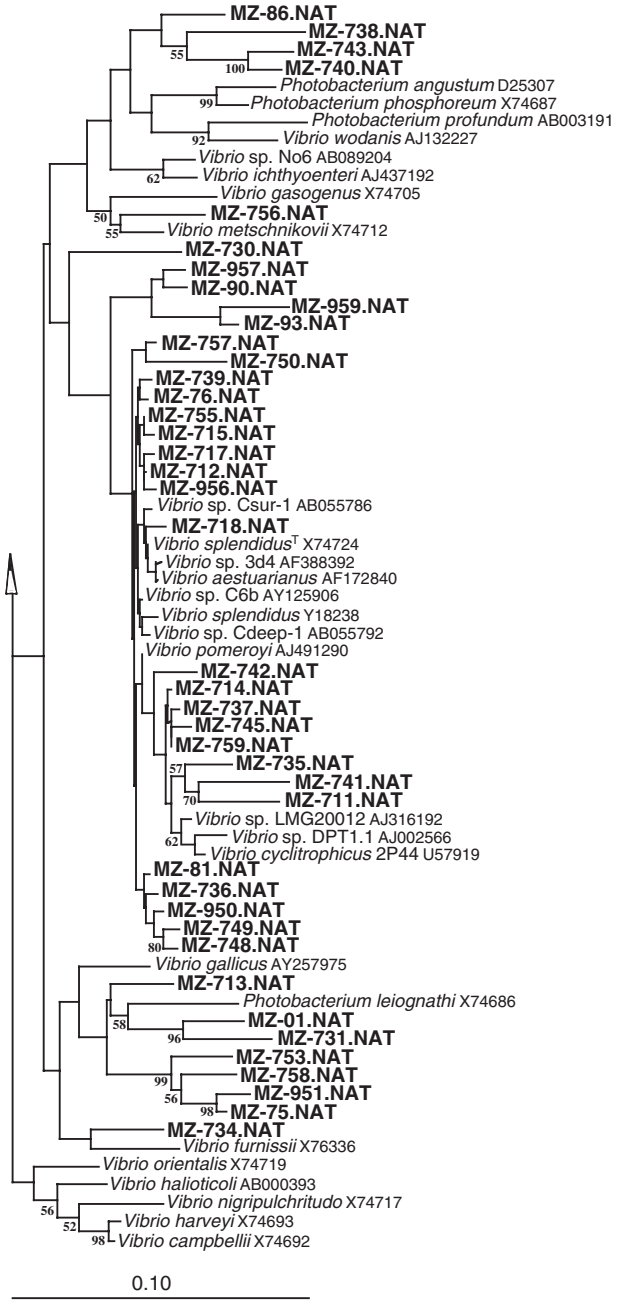

Fig. 3. Rooted phylogenetic tree of *Vibrio*-related NAT clones. The tree was constructed as in Fig. 2 and rooted and out-grouped with 16S rRNA gene sequences of *Psychroserpens burtonensis* (U62913) and *Cycloclasticus pugettii* (L34955) respectively.

mental clones found in deep marine sediments (97.6% identity to BD1-29).

Only one unique OTU and its sequence was affiliated with the *CFB* division. We have failed to associate clone MZ-961.NAT to any culturable member of the *CFB* division with a validly published name. The closest sequence (94.5% identity) was the environmental clone ZA2211c, recovered from Atlantic bacterioplankton.

The major group (64% of all NAT clones) consisted of γ-*Proteobacteria*. MZ-958.NAT was almost identical (99.6%) to *Pseudoalteromonas* sp. strain A28. MZ-78.NAT was associated with the sulfur-oxidizing bacterium sequence OBII5 (95%). MZ-761.NAT clustered with a deeply branching group of environmental clones BM89MF5BH10 (98.1% similarity) and Sva0091, which were isolated from Bermuda corals and permanently cold marine sediments respectively. The most frequently encountered group (62.5% of NAT clones from 26 RFLP-matching OTUs and 15 phylotypes; 41 sequenced clones) was apparently associated with the *Vibrio-Photobacterium* cluster with similarity values between 94.1% and 99.8% (Fig. 3). Eighteen riboclones showed a close relationship with the *Vibrio splendidus* cluster, with microdiversity at subspecies level (or rRNA operon variability). It should be noted that this cluster includes *Vibrio cyclitrophicus*, the only known PAH-degrading species within this genera (Hedlund and Staley, 2001). Species affiliation was estimated for four clones (MZ-01.NAT, MZ-731.NAT, MZ-734.NAT and MZ-756.NAT), whereas the other 19 clones formed separate groups with unclear taxanomic positions within the *Vibrio-Photobacterium* cluster.

Clone library analysis of microbial community in N, P-enriched microcosm

The initial sediment was characterized by low phosphate content, close to the lower detection limit (<5 nM). In the experimental microcosm is, hereinafter designated as 'ONR', named for ONR7a which is a mineral medium, described by Dyksterhouse and colleagues (1995) for isolation of *Cycloclasticus pugettii*. Minerals, phosphorus and nitrogen added at 0.25 mM and 5.0 mM, respectively, were consumed within the first 90 h (data not shown). The degradation of autochtonous hydrocarbons in the ONR microcosm was monitored by the consumption of alkanes and fatty acid methyl esters (FAME). As no mineralization of tracked hydrocarbons was observed during this period, the first peak of cell density detected (Fig. 4) was seemed to correspond to the consumption of other organic compounds present in the original sediment. The EUB388 and GVb oligonucleotide probes were used in dot-blot hybridization using community DNA to roughly quantify the contribution of the *Vibrio* group to the total pool of rRNA genes in our ONR microcosm at this time (96 h of the experiment). Hybridization data indicated that this group accounted for $8 \pm 2\%$ of the bacterial DNA, which is less than that observed in the authochtonous bacterial population. The second peak of microbial density was observed at 240 h (Fig. 4). A slight decrease was registered during next 48 h and the ONR microcosm experiment was concluded after 16 days when a stable plateau was reached for the last four days. The substrates for

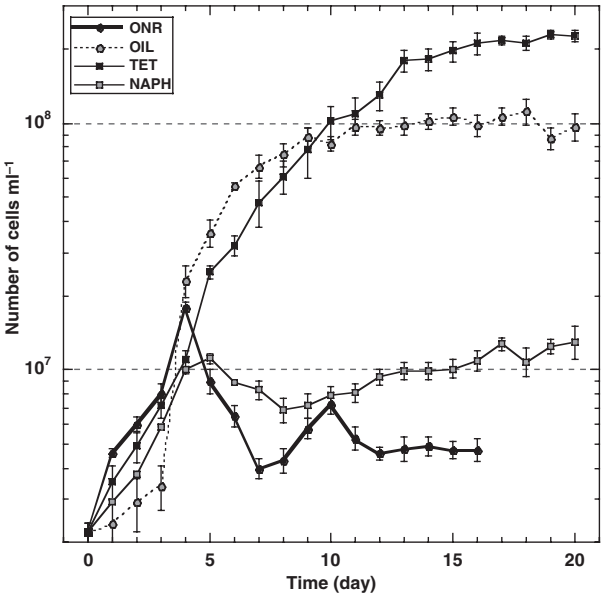

Fig. 4. Bacterial growth in microcosm experiments supplemented with nutrients, crude oil and hydrocarbons. Bacterial numbers in the microcosm supplemented only with nutrients (ONR, closed circles), with nutrients and crude oil (OIL, open circles), with nutrients and tetradecane (TET, closed squares) and with nutrients and naphthalene (NAPH, open squares) were determined after DAPI staining of bacterial cells. The values are averages of three independent measurements. Standard deviations obtained from three independent counting are shown as error bars.

bacterial growth during the second phase were assumed to be the intrinsic hydrocarbons, because a 92% (± 2%), 83% (± 6%) and 72% (± 9%) decreases alkanes, FAMEs and phytol concentrations, respectively, were recorded.

An ONR clone library based on universal primers was established. One hundred randomly-picked clones containing 16S rDNA inserts have been grouped using RFLP analysis and 20 different OTUs were obtained. RFLP profiling revealed that 58% of the clones belong to 5 OTUs, while RFLP patterns of four unique clones (each occurring only once in ONR library) gave a diversity coverage of 96%. Twenty-four clones representing 20 OTUs were sequenced from ONR library. No polymerase chain reaction (PCR)-amplification products with archaeal primers were detected. Figure 5 summarizes the phylogenetic positions of the sequenced clones.

Two clones (6% of the ONR clone library) were associated with the *CFB* division. MZ-33.ONR was most closely related to environmental clone JTB244 (94.5%; from the deep cold-seep area sediments) and to Antarctic isolate *Psychroserpens burtonensis* ACAM188 (94.3%), while the exact position of MZ-56.ONR remained uncertain (93% identity to the closest sequence of *Flexibacter* sp. SCB36).

Similarly to phylogenetic diversity of the NAT clone library, ONR was characterized by an abundance of clones belonging to γ-*Proteobacteria* (84%). MZ-16.ONR, MZ-26.ONR and MZ-50.ONR were most similar to marine methylotrophic organisms *Methylobacter* and *Methylophaga*. MZ-19.ONR was affiliated with the sequence of *Pseudomonas* sp. MBIC2027 (99.4%), isolated from coastal sediments. Almost identical clones MZ-32.ONR and MZ-36.ONR clustered together (96% identity) with members of *Oceanospirillum* sp. *MED92* and marine carbazol-degrading bacterium CAR-SF. Sequences of MZ-04.ONR and MZ-54.ONR were found to be identical to those of *Cycloclasticus pugetii* and *Alcanivorax borkumensis*, respectively, which are marine hydrocarbonoclastic bacteria highly specialized in the degradation of the polyaromatic and aliphatic fractions in oil (Dyksterhouse *et al*., 1995; Yakimov *et al*., 1998). Sixty-one per cent of the ONR clones (eight representative sequences) were apparently affiliated with different species related to *Marinobacter* spp. which are alkane- and PAH-degrading marine organisms (Gauthier *et al*., 1992; Hedlund *et al*., 2001). Interestingly, some strains of *Marinobacter* were shown to be able to degrade phytol found in the Milazzo sediments (Rontani *et al*., 1997, 1999).

Ten per cent of the ONR clones were classified as a subclass of α-*Proteobacteria* which was not detected in natural sediments. All clones except MZ-44.ONR appeared in groups related to the *Hyphomonas-Maricaula* cluster, while the last one has a 95% similarity to *Sphingomonas alaskensis*, an oligotrophic ultramicrobacterium, abundant in the northern Pacific Ocean (Eguchi *et al*., 2001).

Oil degradation and clone library analysis of microbial community in the OIL microcosm

Because of the continuous bacterial growth in the OIL microcosm, degradation of crude oil components was examined after 20 days of the experiment when a slight decline of cell density was observed (Fig. 4). The concentration of crude oil components was normalized using the 17α(H), 21β(H)-hopane, and the values obtained in triplicate subsamples were averaged. The concentration of each component in the control crude oil samples was defined as 100%. All GC-MS resolvable petroleum compounds were organized by structural similarity in separated groups (see *Experimental procedures*) and their degradation values were calculated. Significantly greater biodegradation of nC_{10}-to-nC_{35} and alkylcyclo-alkanes (41.3 ± 2.2% and 33.3 ± 4.6%, respectively) was observed than with branched alkanes (19.2 ± 4.3% degradation). Polyaromatic hydrocarbons were removed to a lesser extent and 84.4 ± 7.2% of these compounds remained in the OIL microcosm for more than 3 weeks from the beginning of the experiment.

DNA was extracted from the total microbial community

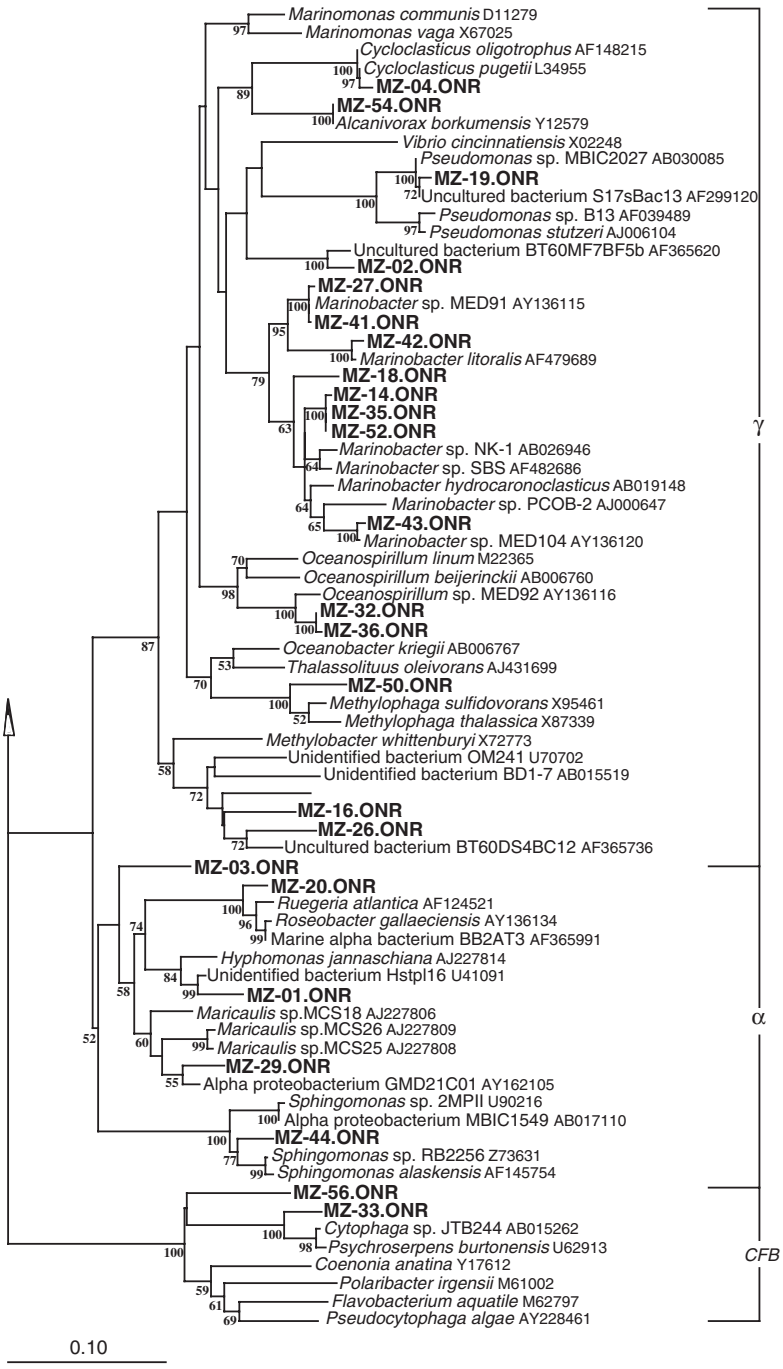

Fig. 5. Phylogenetic relationship of 16S rRNA gene sequences retrieved from the ONR microcosm. The tree was constructed as in Fig. 2 and rooted and out-grouped with 16S rRNA gene sequences of *Methanococcus jannaschii* (M59126) and *Thermus thermophilus* (L09659) respectively.

occurring in the OIL microcosm, then amplified with universal and archaeal-specific primers and the OIL clone library was further analysed. No archaeal amplification was observed.

Twenty-three different RFLP patterns were detected from 100 clones of the OIL library. The most abundant RFLP type constituted 44% of all clones. Ten singletons were recovered. The phylogenetic affiliation of 29 clones representing all RFLP patterns, including the five clones that were most abundant in the OTU library, were inferred (Fig. 6). Remarkably, 97% of the microbial community were represented by all five divisions of *Proteobacteria*, while the presence of the β and δ divisions was first detected in the OIL library. MZ-365.OIL, representing 7% of OIL clones, was identical to the phenanthrene- and 2,4-D-degrading *Burkholderia* strains TFD6 and S4.11 and closely affiliated to the PAH-degrading strain N3P2. MZ-61.OIL (9% of total OIL clones with identical RFLP profiles) was found to be identical to the toluene-degrading sulfate-reducing bacetrium *Desulfobacula toluolica*,

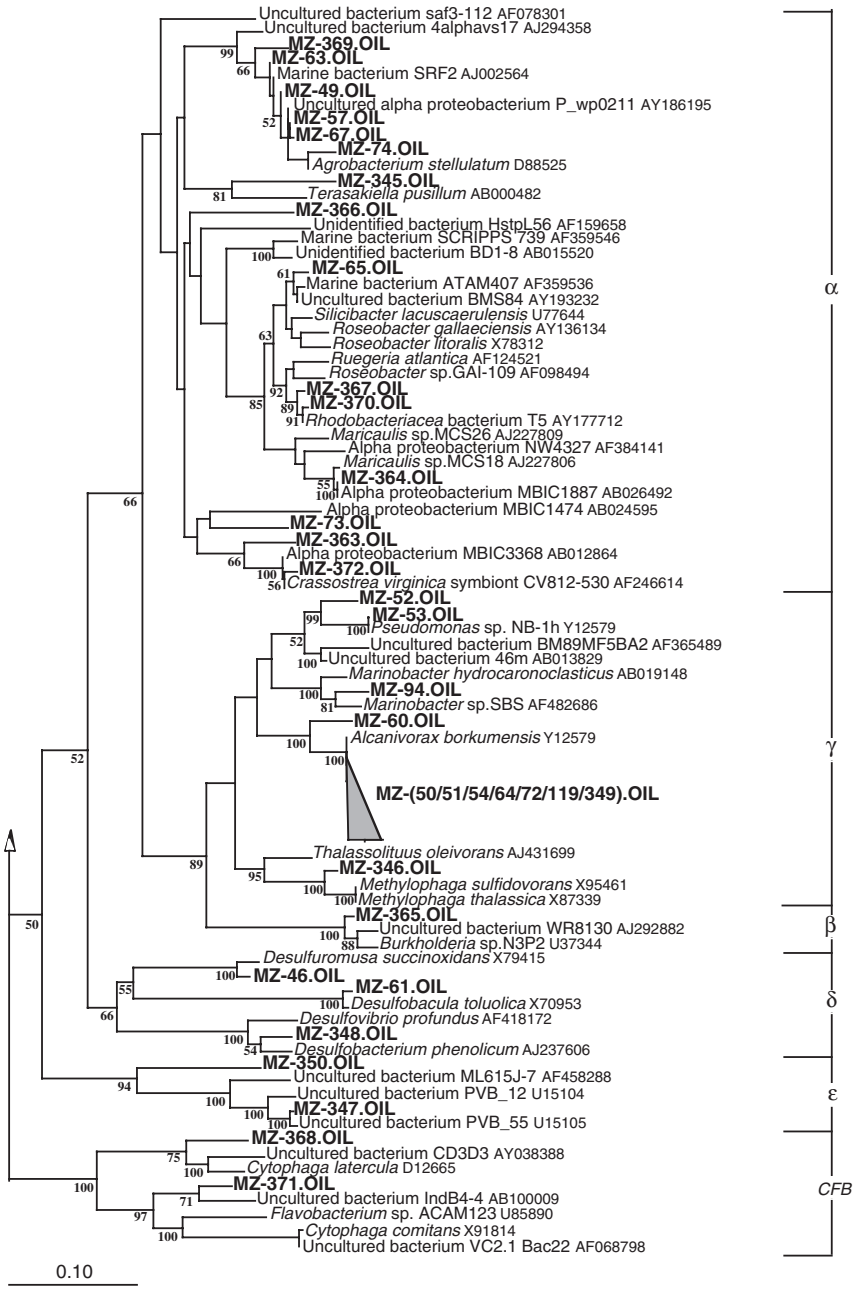

Fig. 6. Phylogenetic relationship of 16S rRNA gene sequences retrieved from the OIL microcosm. The tree was constructed as in Figs 2 and 5.

whereas the unique clone, MZ-348.OIL, was affiliated with *Desulfovibrio profundus* (99% similarity). The ∈-*Proteobacteria* division was represented in the OIL library only by singleton MZ-347.OIL, which was identical to the environmental riboclones PVB_OUT_3 and VC2.1 Bac32, both retrieved from a microbial mat at an active hydrothermal vent. Based on a combined analysis of RFLP clustering and DNA sequencing, we found that 30% of the OIL bacterial PCR amplicons were derived from α-*Proteobacteria*. Among them, a cluster of six sequences (11% of OIL clones) was closely affiliated with SRF-2, an environmental clone we had previously recovered from the Northern Mediterranean (Giuliano *et al.*, 1999). Another cluster of five sequenced clones, representing 9% of OIL clones, were classified within a group related to *Maricaulis-Roseobacter-Rhodobacter*. The phylogenetic placement of only one clone, MZ-73.OIL, remained unclear because similarity values of 91% with sequences of different α-proteobacterial riboclones, recovered from grassland soil, rhizosphere and sewage.

A small fraction of the sequences (3% of all clones) retrieved from the OIL microcosm could be assigned to the *CFB* phylum. Singleton MZ-371.OIL was found to be identical to environmental clone VC2.1 Bac22, recovered

from the deep Mid-Atlantic Ridge hydrothermal vent, while MZ-368.OIL was affiliated with *Cytophaga latercula*.

Almost 90% of all clones affiliated with γ-*Proteobacteria,* and 44% of all OIL clones sequenced grouped within an identical RFLP profile. Seven representative clones were sequenced and a 98–100% similarity to *Alc. borkumensis* SK2^T was found for all seven. Another *Alcanivorax*-related clone, MZ-60.OIL, revealed a similarity of 97%. MZ-346.OIL, whose RFLP pattern was found in 3% of all OIL clones, was closely associated with the marine methylotrophic bacterium *Methylophaga sulfidovorans*. Clones MZ-52.OIL and MZ-53.OIL showed a close relationship to deep sea isolate *Pseudomonas* sp. NB-1h.

Clone library analysis of microbial community in the tetradecane (TET) microcosm

Bacterial growth in the TET microcosm started immediately at the beginning of the experiment, suggesting that microorganisms in the sediments were preadapted to this pollutant and was characterized by a constant increase of cell density during the next 20 days. The experiment was terminated on the 20th day when the density plateau had been reached (Fig. 4). Estimated values of tetradecane degradation on that day were 94.7 ± 1.5%.

Very low diversity was detected for the microbial community in the tetradecane-enriched microcosm. Among the only 8 OTUs found, two similar OTUs accounted for 69 of the 100 clones analysed. One OTU was found to be present by a singleton, giving a very high index of diversity coverage of 99%. Fourteen clones representing all OTUs were sequenced from TET clone library and were shown to be members of four bacterial phylotypes, α- (20%), δ-*Proteobacteria* (3%) and the *CFB* division (8%) with an absolute abundance (69%) of γ-*Proteobacteria* (Fig. 7). The *CFB* phylum was represented by two phylotypes, MZ-28.TET and MZ-46.TET, which are distantly affiliated to a benzene-mineralizing uncultured eubacterium SB-1 (89%), while another three almost identical clones, MZ-12.TET, MZ-13.TET and MZ-70.TET, showed similarity values of 94% to *Flavobacterium columnare*. α-*Proteobacteria* were represented by clones closely affiliated with *Aquaspirillum peregrinum*, *Rhodospirillum rubrum* and *Terakasiella pusillum.* The clone MZ-16.TET was closely related (99%) to riboclone MN13BT4-48, found in the hydrocarbon-seeping Amsterdam mud volcano, in the eastern Mediterranean. The δ-proteobacterium MZ-31.TET had a sequence similarity of 98% to the sulfate-reducing acrylate-degrading bacterium *Desulfovibrio acrylicus*.

Five clones representing two abundant OTUs (69% of all TET clones) were sequenced. Two of them were identical to *Thalassolituus oleivorans*, recently isolated from the same site (Yakimov *et al*., 2004), whereas three others

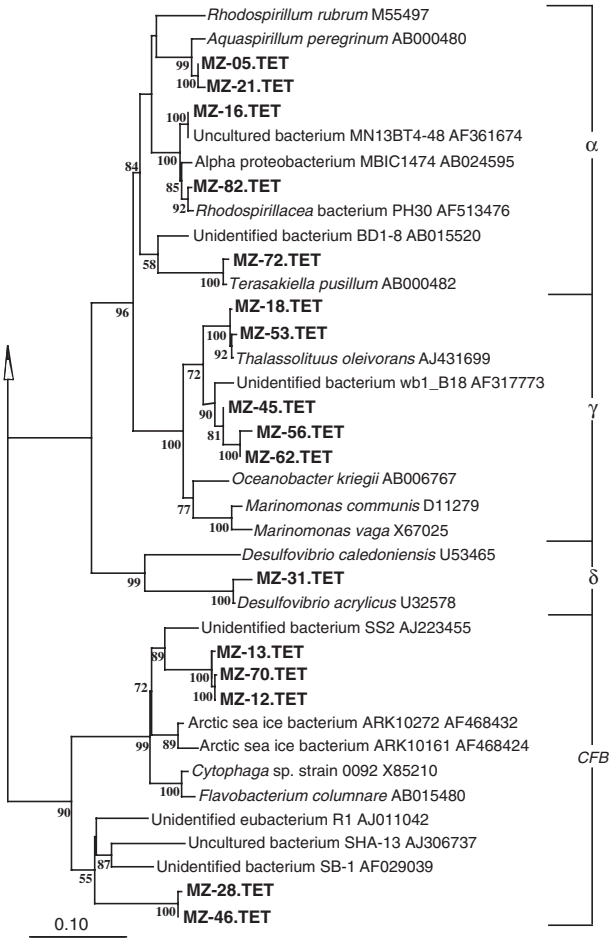

Fig. 7. Phylogenetic relationship of 16S rRNA gene sequences retrieved from the TET microcosm. The tree was constructed as in Figs 2 and 5.

revealed a 95% similarity to this hydrocarbonoclastic bacterium and could be assigned as a new species of *Thalassolituus*.

Clone library analysis of microbial community in the NAPH microcosm

The NAPH microcosm was characterized by a very small increase of microbial density comparable with the nutrient-supplied ONR microcosm and stabilized by the 6th day of the experiment ($P < 0.05$) (Fig. 4). The microcosm was terminated after 20 days for normalization of the data obtained from the other microcosms. GC-MS analysis revealed that 32.3 ± 5.6% of the naphthalene added was consumed.

The NAPH clone library was analysed by RFLP profiling of 100 clones and 8 different OTUs were found. More than 85% of the NAPH clones represented two abundant RFLP types, formed by 44 and 42 clones respectively. Four singletons were obtained giving the diversity coverage

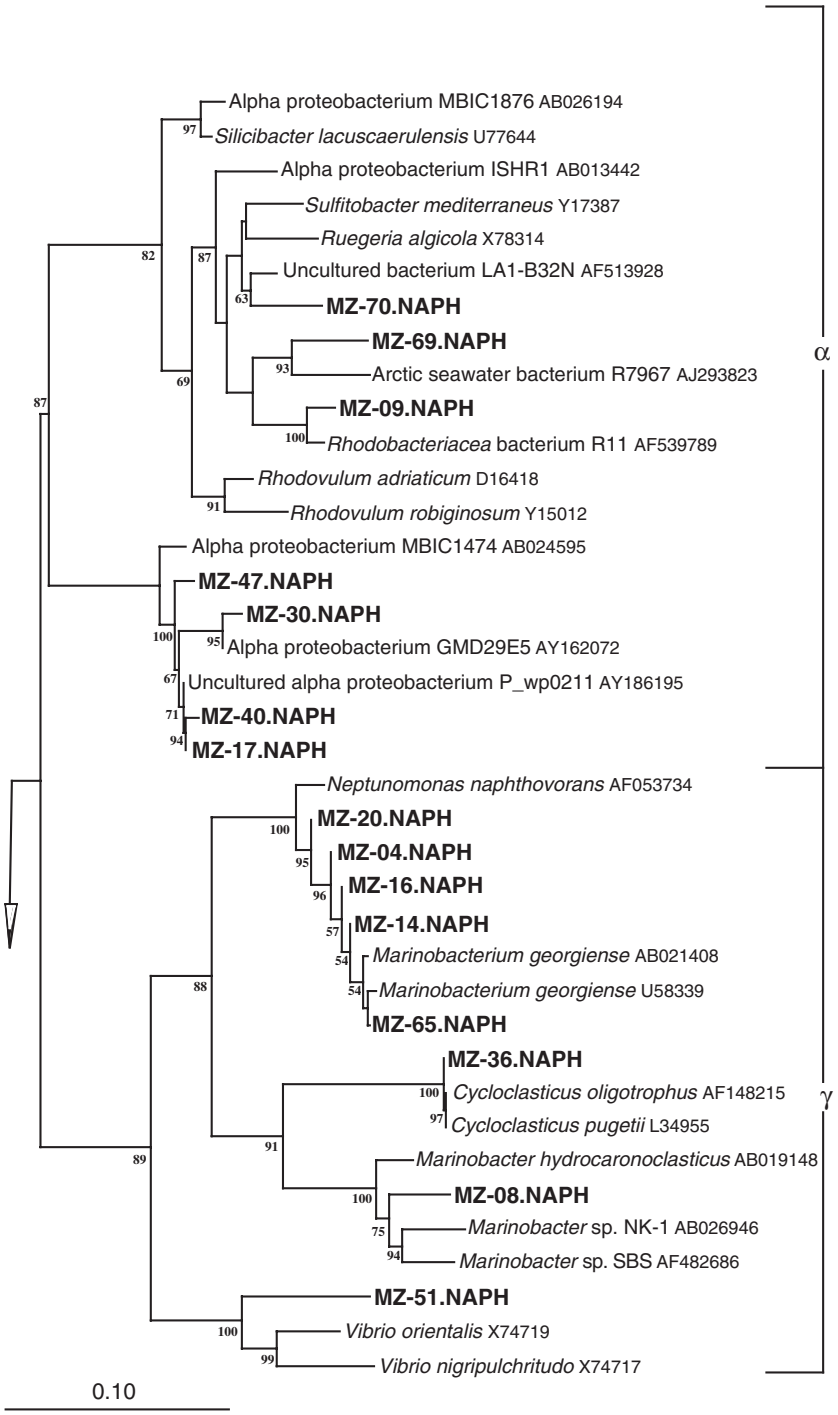

Fig. 8. Phylogenetic relationship of 16S rRNA gene sequences retrieved from the NAPH microcosm. The tree was constructed as in Figs 2 and 5.

index of 96%. Fifteen clones belonging to all OTUs, among which nine clones represented the major RFLP types, were sequenced and their phylogenetic positioning was inferred. The low diversity of the NAPH microbial assemblage was again confirmed as the sequenced clones were affiliated with only two bacterial phylotypes, α- (45%) and of γ-*Proteobacteria* (55%) (Fig. 8). All α-*Proteobacteria* clones, except singletons MZ-09.NAPH, MZ-69.NAPH and MZ-70.NAPH, formed a monotypic group closely related (99%) to riboclone P_wp0211, retrieved from a PAH-degrading enrichment of deep-sea sediments. Among NAPH clones related to γ-*Proteobacteria*, the distribution of type sequences was also highly disproportional. Singleton MZ-51.NAPH was related to genus *Vibrio* and had equal sequence similarity of 93% to *Vibrio niripulchritudo* and to *Vibrio orientalis*. MZ-08.NAPH covered 3% of NAPH clones (5.5% of all γ-*Proteobacteria*; Fig. 8) and had *Marinobacter* sp. NK-1 as

its closest relative (95% similarity). Seven percent of NAPH clones (12.7% of all NAPH γ-*Proteobacteria*) represented by MZ-36.NAPH were very closely affiliated with *C. pugetii* (100% of similarity). The most abundant NAPH OTU, represented by 44 clones (80% of all NAPH γ-*Proteobacteria*), had a monotypic sequence (five identical clones) closely related (99%) to a lineage of lignin-degrading *Marinobacterium georgiensis* which includes *Neptunomonas naphthovorans*, a naphthalene-degrading marine bacterium isolated from Puget Sound.

Clonal bacterial diversity in the libraries

As all archaeal clones were retrieved only from the autochthonous microbial community, they were withdrawn from this analysis and comparison of only bacterial diversity was performed. Distribution of 16S rDNA phylotypes from natural and microcosm microbial communities on the basis of phylogenetic grouping is shown in Table 1. Most bacterial phylotypes were affiliated with α- and γ-*Proteobacteria* and the *CFB* division. Members of γ-*Proteobac-*

Table 1. Distribution of bacterial phylotypes in the NAT, ONR, OIL, TET and NAPH 16S rRNA gene clone libraries.

	No. of phylotypes (no. of clones) in the following libraries				
Phylotyping or taxonomic group[a]	NAT	ONR	OIL	TET	NAPH
Bacteria					
α-Proteobacteria					
Agrobacterium/P_wp0211 cluster			2 (12)		1 (42)
Aquaspirillum group				1 (7)	
Beijerinckiaceae group			2 (5)		
Hyphomonas group		1 (1)			
Maricaulis group		1 (1)	1 (1)		
Rhodobacteriacea group			2 (8)		2 (2)
Rhodospirillacea group				1 (12)	
Ruegeria/Sulfitobacter cluster		2 (4)			1 (1)
Sphingomonas group		1 (4)			
Terasakiella group			1 (1)	1 (1)	
MBIC1474 group			1 (3)		
β-Proteobacteria					
Burkholderia cluster			1 (7)		
γ-Proteobacteria					
Alcanivorax group		1 (6)	2 (39)		
Cycloclasticus group		1 (6)			1 (7)
Marinobacter group		5 (56)	1 (3)		1 (3)
Methylobacter group		2 (5)			
Methylophaga group		1 (4)	1 (2)		
Neptunomonas/Marinobacterium group					1 (44)
Oceanospirillum/wb1_B18 cluster		1 (3)		1 (20)	
Pseudoalteromonas group	1 (1)				
Pseudomonas group		1 (2)	2 (4)		
Vibrio/Photobacterium group	15 (125)				1 (1)
Thalassolituus group				1 (49)	
BT60MF7BF5b cluster		1 (2)			
OBII5 group	1 (1)				
Sva0091/BPC022 cluster	1 (1)				
δ-Proteobacteria					
Desulfobacula toluolica			1 (6)		
Desulphuromusa succinooxidans			1 (1)		
Desulfovibrio group			1 (3)	1 (3)	
ε-Proteobacteria					
Arcobacter cluster	2 (23)				
PVB_OUT_3 cluster			2 (2)		
CFB					
Bacteroidetes CD3D3 group			1 (2)		
Cellulophaga/Psychroserpens group		2 (6)			
Cytophagales ML–1/IndB4-4 cluster			1 (1)		
Cytophagales SS2/ES13 cluster	1(1)			1 (6)	
SHA-13/SB-1 group				1 (2)	
Gram-positive					
Clostridium/Fusibacter group	9 (48)				
Total	30 (200)	20 (100)	23 (100)	8 (100)	8 (100)

a. Phylotype groups correspond to clusters of similar 16S rRNA genes shown in phylogenetic trees in Figs 2, 3 and 5–8.

Table 2. Biodiversity characteristics of the clone libraries analysed.

Clone library	Species richness[a]	Diversity coverage	Diversity estimates	Dominance	Dominance concentration	Dominant organisms[b]
NAT	30	94.0	2.593	0.131	0.869	*Vibrio*
ONR	20	96.0	2.494	0.122	0.878	*Marinobacter*
OIL	23	90.0	2.379	0.176	0.824	*Alcanivorax*
TET	8	99.0	2.097	0.164	0.836	*Thalassolituus*
NAPH	8	96.0	1.201	0.376	0.624	*Marinobacterium*

a. All sequences that clustered over 97% were taken as indicative of belonging to a single species/separate phylotype.
b. Identity over 97% to the sequence from the indicated organisms with validly published names.

teria were found to be dominant in all clone libraries: 49% (OIL); 55% (NAPH); 64% (NAT); 69% (TET); and 84% (ONR). The α-*Proteobacteria* division was not found in the natural microbial community in the harbour sediments, whereas its abundance in enriched microcosms was detected. All clone libraries except NAPH contained microorganisms belonging to the *CFB* division. The signatures of sulfate reducing heterotrophs were found in oil- and tetradecane-enriched mesocosms, although their relative proportions did not exceed 10% and 3% respectively. ε-*Proteobacteria* were only found in the natural community and in the OIL microcosms. Members of *Firmicutes* and β-*Proteobacteria* were found only once in the NAT and OIL libraries respectively.

To assess bacterial diversity between the five libraries, analysis of phylotype distribution was carried out using common criteria, as described elsewhere (Krebs, 1989; Mullins *et al*., 1995; Brown and Bowman, 2001). It should be noted that the inherent biases of DNA extraction and PCR can potentially distort clone library data and clone percentages probably do not accurately reflect the microbial diversity, but as all the samples were analysed in the same fashion and the predominant sequences in the clone libraries were so evident, it is likely that our results do reflect, at least partially, relative abundance in the studied microbial communities. Biodiversity coverage was relatively high ranging from 90.0% to 99.0%, suggesting that all bacterial assemblages were characterized by significantly decreased diversity. Based on the definition that each phylotype consists of either a unique clone or a group of clones that have sequence similarities of over 97% (Rossello-Mora and Amann, 2001), a total of 89 different phylotypes were discerned. Although the greatest species richness was observed in NAT library (Table 2), the natural community was characterized by a relatively lower diversity index mainly because: (i) only five major bacterial divisions were present; and (ii) 64% of all defined species derived from heterotrophic *Vibrio* and *Clostridium* species. The Milazzo Harbor sediments seems to be a system highly affected by chronic anthropogenic pollution which inhibits a more diverse microbial community. Interestingly, the diversity estimate observed in the OIL microcosm was equal to that of the natural microbial community (Table 2). This finding indirectly confirms an environmentally stressed system most probably caused by organic pollution. This could explain the contrast between the observations of other investigators, suggesting that the addition of crude oil to natural microbial populations results in a significant decrease in the diversity index mainly because of strong selection for petroleum-degrading bacteria (Harayama *et al*., 1999; Kasai *et al*., 2001; 2002b; Syutsubo *et al*., 2001). The nutrient-enriched microcosm had the same clone species richness as the OIL microcosm, and exhibited the same clonal diversity as well (Table 2), with strong dominance of *Marinobacter*-related sequences (58% of clones). Such a high prevalence of *Marinobactor* could be explained by recent observations suggesting that this group of marine hydrocarbonoclastic bacteria seem to play an important role in the biodegradation of phytol (Rontani *et al*., 1997, 1999) found at high concentrations in Milazzo Harbor sediments. Naphthalene and tetradecane addition in the microcosms drastically affected the diversity, either on a bacterial species or phylum level and obviously favoured the development of highly specialized microbial communities degrading these compounds.

Inter-sample similarity was measured numerically by pair wise comparisons of clone library data between each library (Table 3). Surprisingly, only a few phylotypes were shared between each and no one appeared in more than three separate libraries. No matches of NAT phylotypes with those of any other library were found. TET and ONR libraries did not share any of their clones. The highest level

Table 3. Inter-sample similarity between bacterial phylotypes found in the clone libraries constructed from natural sediment sample and from different microcosms.

Clone library	NAT	ONR	OIL	TET	NAPH
NAT	1.00				
ONR	0.00	1.00			
OIL	0.00	0.17	1.00		
TET	0.00	0.00	0.15	1.00	
NAPH	0.00	0.15	0.08	0.12	1.00

of inter-sample similarity of 17% was obtained by a comparison of bacterial phylotypes, recovered from OIL and ONR microcosms. Such a limited values may reflect the establishment of diverse microbial assemblages as a response to different kinds of pollutants, but may also be derived from a limitation of the clone library analysis technique.

Discussion

The harbour of Milazzo is a wide natural bay with low water turnover and runs along 15 km of the north-eastern coast of Sicily. This region is characterized by heavy industrialization, dense urbanization and heave large tanker traffic transporting crude and refined oil to and from the refinery located at this site. These antropogenic factors have strongly affected the marine environment of the harbour. The analysis of organic contamination in superficial sediments revealed high concentrations of five different classes of hydrocarbon pollutants clearly originating from human activities. The alkanes are principally attributed to the oil hydrocarbons probably from waste from the petrochemical refining plants located at the harbour. Two other contaminants found in the sediments, alkyl phthalates and methylated fatty acids, also seem to originate from oil refining activity and polymer production. Alkyl phthalates are widely used as plasticizers for many polymers, like polyvinyl chloride resins, polystyrene and cellulose ester plastic and may enter the environment via air emissions, aqueous effluent and solid waste products from processing plants. Fatty acid methyl esters are playing an increasingly important role in the petrochemical industry and are mostly used as chemical intermediates to produce a number of petrochemicals such as fatty alcohols, alkanolamides, sulfo-methyl esters, sucrose esters and other detergents. The last two groups of contaminants obtained from sediments, acyclic isoprenoids (phytol dominant) and sterols (coprostanol dominant) are relatively abundant and widespread components of harbour marine sediments. These compounds are often used as biological markers for primary production and urban wastes (Takada and Eganhouse, 1998) and mainly originate from the phytyl side-chain of chlorophyll a and from mammalian feces respectively.

Recently, many investigators have raised questions about possible problems associated with PCR of the 16S rRNA gene for phylogenetic analysis of microbial communities. Strong biases may be introduced by the copy number of 16S rRNA genes, varying from 1 to 14 copy number per cell depending on the species or even strains (Farrelly *et al.*, 1995), and by the differential PCR amplification efficacy of DNA from heterogeneous templates (Farrelly *et al.*, 1995; Chandler *et al.*, 1997). These possible effects may mean that the proportions found in the clone libraries do not always represent the 16S rDNA proportion within microbial communities. Keeping in mind this caveat, the molecular phylogenetic approach using PCR and 16S rDNA cloning is at the moment the most accurate technique available for describing the composition of complex microbial communities and gaining at the very least a descriptive overview of possible differences and comparability between controlled laboratory experiments and environmental samples.

While we found five major phylogenetic groups represented by NAT clones, it is unlikely that the exact proportions found in our study reflect the original *in situ* sediment diversity. It is possible that the complete dominance of *Vibrio*- and *Clostridium*-related organisms under controlled conditions could be a rough approximation of natural conditions in the harbour sediment. Moreover, with the calculated diversity coverage of 94% for NAT library, we failed to find α-, β- and δ-*Proteobacteria* in the natural sample, while bacteria belonging to these lineages were detected in experimental microcosms derived from same sediment. Surprisingly, sequences retrieved from microcosm experiments did not match those from the NAT library, suggesting that a major portion of the assemblage belongs to different phylogenetic lineages that do exist in the natural sample, but at undetectable levels, at least by the methods used.

The high levels of organic contamination, high abundance of the mud fraction in the sediments, and low water turnover in the harbour facilitate fast oxygen consumption at the surface and complete anoxic conditions in the sediment below 4 cm. This stratification favours the growth of anaerobic heterotrophic bacteria, such as *Clostridium,* which was found to be one of the major bacterial groups in the autochthonous microbial community. In general, the microbial community analysed from the sediment was different from that typically described from unpolluted sediment and seawater of the Mediterranean Sea (Boivin-Jahns *et al.*, 1996; Giuliano *et al.*, 1999; Schäfer *et al.*, 2001) and the observed decrease in diversity was obviously affected by anthropogenic impact.

The self-cleaning potential of naturally occurring bacterial assemblages and community successions resulting from sudden hydrocarbon pollution was analysed by means of the microcosm experiments. For the first time, we demonstrated that developed consortia were strongly dependent on the composition of hydrocarbon pollution and they had low inter-sample similarity. Microbial communities within contaminated ecosystems tend to be dominated by the organisms capable of consuming and/or tolerating toxic organic compounds. As a result, these communities are typically less diverse than those in non-stressed systems, although their diversity may be influenced by the complexity of contaminant mixtures present and the time of exposure. Each microcosm was charac-

terized by a strong dominance of different hydrocarbonoclastic bacteria, depending on the experiment. Simple inorganic nutrient addition to the sediments significantly enhanced the degradation of targeted hydrocarbons present in natural samples. Concordant absolute dominance of sequences in the ONR clone library, related to alkane- and phytol-degrading *Marinobacter* spp. was also observed.

Crude oil as a complex mixture of different classes of hydrocarbons could support the growth of various bacteria, each preferentially degrading one or several types of hydrocarbons. Alkanes are the major constituents of crude oil, which explains why *Alcanivorax*-related sequences are prominent in the OIL clone library. Population sizes of these alkane-degrading bacteria, usually low in unpolluted marine waters, increase significantly in response to oil pollution and fertilization (Kasai *et al.*, 2001; 2002b; Syutsubo *et al.*, 2001; Roling *et al.*, 2002). Absence of *Cycloclasticus*-related sequences detected in OIL clone library could be explained by the usage of weathered oil, where only 1% of GC-MS resolvable hydrocarbon fractions are represented by polyaromatic compounds, found to be almost single type of carbon source supported the growth of this highly specialized hydrocarbon-degrading bacterium (Dyksterhouse *et al.*, 1995; Kasai *et al.*, 2002a). These sequences were retrieved in clone library produced from ONR and NAPH microcosms, although their presence was not abundant. Another naphthalene-degrading bacterium, closely related to *Marinobacterium georginense* and *N. naphthovorans*, accounts for the majority of corresponding clones possibly because of its higher growth rate.

Surprisingly, *Alcanivorax*-related sequences were not found among the bacteria growing in tetradecane-enriched microcosms which is their preferred substrate. Almost 70% of the TET clone library consists of two related sequences belonging to a novel genus, *Thalassolituus*. The preliminary study performed in our laboratory demonstrated that *T. oleivorans* MII-1^T possessed higher rates of growth and tetradecane uptake than *Alc. borkumensis* SK-2T (data not shown). Another possible explanation is that *Thalassolituus* produces some secondary metabolites which could be toxic to other microorganisms. The study of interaction between these bacteria is currently in progress.

To our knowledge, the present study is the first attempt to characterize the succession of microbial assemblages derived from the same natural sample in response to differential contaminant load. The microcosm experiments indicated that various groups of marine hydrocarbonoclastic bacteria appeared to be involved in the primary degradation of petroleum hydrocarbons. This diversity presumably reflects the necessity of specific networks at the trophic and functional levels in hydrocarbon-degrading microbial communities to cope with the chemical complexity, the range of solubility, and toxicity of different hydrocarbon compounds. The present work is the initial phase of long-term study which is particularly relevant in light of the accidental oil spill which occurred in the Harbor of Milazzo at the beginning of January, 2003.

Experimental procedures

Field site, sampling and microcosm set-up

Sediment samples for the microcosms was collected from the upper part of the Milazzo Harbor, Italy, sediment at a depth of 8 m, from a site within a zone of heavy ship traffic (015°14.75E, 038°13.00N). Fifty grams from the upper 5 cm of sediment was transported to the laboratory in a cool box at ambient temperature and used for microcosm experiments within 1 day of collection. The sediment was composed of fine sand with a particle abundance in the range of 150–250 μm and contained more than 25% grey-black mud (sediment particles of <60 μm). The average total organic matter and total nitrogen content of sediment was 3.53 ± 0.15% and 0.32 ± 0.06%.

The sediment sample was homogenized with sterile seawater in the laboratory and four 5.0-g portions of wet sediment were placed into 200-ml flasks and held at 15°C, while another 5.0-g portion was used for direct extraction of total DNA. Additionally, 10 g of sediments were used for hydrocarbon analysis. Each microcosm received 45 ml of TAPS-buffered (5 mM) sterile seawater supplemented with NH_4Cl and $Na_2HPO_4x2H_2O$ to reach nitrogen and phosphorus concentrations of 5.0 and 0.25 mM respectively. Nutrients amounts and the type of buffer salts were based on those in Dyksterhouse and colleagues (1995), where the mineral medium ONR7a was applied for the cultivation of *C. pugetii*. One microcosm was not further modified (hereafter referred to as 'ONR' microcosm), while three others were supplemented with 0.4% (v/v) of crude oil and tetradecane and 0.2% (w/vol) of naphthalene respectively (hereafter referred to as the 'OIL', 'TET' and 'NAPH' microcosms). Weathering of crude oil (source was a small scale oil producing well of Lehrte, Germany) was simulated by distillation at 230°C in order to remove low-molecular-weight volatile compounds ($<C_{10}$ alkanes and non-substituted monoaromatic compounds). Total GC-MS resolvable hydrocarbons contributed 42.2 ± 2.8% of total hydrocarbon content in the weathered oil with relative concentrations of normal, branched, alkylcyclo-, C_{10}-to-C_{35}, alkanes and polyaromatic hydrocarbons of 77%, 16%, 6% and 1% respectively. The weathered oil and tetradecane were mixed vigorously with TAPS-buffered (5 mM) sterile seawater taken from the corresponding microcosm (1:4, v/v) and obtained water-in-oil emulsions were added. Microcosms were not shaken constantly during the experiment, simulating the low water turnover which occurs at the sampling site of the harbour.

Bacterial enumeration and biomass estimation

Total bacterial counts in seawater samples were taken from the microcosms at 24 h intervals and were determined by

fluorescence microscopy using 4,6-diamidino-2-phenylindole (DAPI) staining. Bacterial numbers in unamended sediments were counted by epifluorescence microscopy with SYBR Gold (× 10 000 in dimethyl sulfoxide; Molecular Probes) according to the procedure described by Weinbauer and colleagues (1998). This counting technique was not used throughout the microcosm experiments because of very low observed reproducibility. All microcosm experiments, excluding the ONR microcosm, were terminated on the 20th day when the total cell counts in the seawater overlying the sediments reached a plateau over a period of 48 h. The ONR microcosm was terminated earlier, on the 16th day of the experiment.

Dot-blot hybridization experiments were performed with non-radioactive, DIG-labelled universal EUB and *Vibrio*-specific G V oligonucleotide probes and total DNA bounded to QIABRANE nylon membrane (QIAGEN, Germany) as described elsewhere (Giuliano *et al.*, 1999). The analysis was performed on the microbial community of the ONR microcosm at the 96 h of the experiment (corresponding to the first peak of growth).

Total DNA extraction, PCR amplification, cloning and sequencing

Forty-seven millilitres from each microcosm was taken for molecular biological analyses. After centrifugation at 2500 *g* to settle the sediments, the water samples were filtered onto Durapore polyvinylidene fluoride filters with a pore size of 0.22 μm (Millipore, GVWP, 47-mm diameter). Subsequently, the filters were washed twice with 1 ml of 50 mM sodium phosphate buffer and the effluent was combined with the corresponding sediment for total DNA extraction.

Total DNA extraction was performed following the manufacturer's protocol of the FastDNA® SPIN® Kit (For Soil) (Q-BIOgene). Recent studies have shown that this bead beating protocol is suitable for obtaining DNA both from Gram-positive bacteria and from hypersaline mud samples (Yakimov *et al.*, 2002). Primers used for 16S rDNA amplification from the total DNA included primers 27F (5′-AGAGTTTGATC MTGGCTCAG-3′) and S-D-Bact-1492-a-A-21 (5′-ACG GYTACCTTGTTACGACTT-3′) (Lane, 1991). Specific primers, 8aF and S-D-Bact-1492-a-A-21 were used for PCR amplification of archaeal 16S rDNA (Lane, 1991). Polymerase chain reaction conditions utilized and methods used for clone library construction and sequencing have been described previously by Yakimov and colleagues (2002). All riboclones were checked by the CHECK_CHIMERA program (Maidak *et al.*, 1999) to determine the presence of any PCR-amplified hybrid sequences. Sequences were compared with the GenBank nucleotide database library by GAPPED BLAST on-line searches (Altschul *et al.*, 1997) (http://www.ncbi.nlm.nih.gov/BLAST/) and were then manually aligned using SeAl 2.0 alpha 1 program (Rambaut, 1996) together with the most important BLAST hits as well as with the homologous bacterial 16S rRNA primary structures obtained from Ribosomal RNA Project II database (http://rdp.cme.msu.edu/index.jsp) (Maidak *et al.*, 1999). Phylogenetic analysis was then performed using the program package PHYLIP (Felsenstein, 2001) as described by Yakimov and colleagues (2002). The sequences determined in this study have been deposited under GenBank Accession numbers AJ810552-AJ810706.

Diversity analysis

To assess changes in the genetic diversity of the bacterial communities in the microcosms, all five libraries were normalized to 100 clones each by using the RAREFACT.FOR program (Krebs, 1989) (http://www2.biology.ualberta.ca/jbrzusto/rarefact.php). Estimates of diversity (Shannon-Weaver Index), and dominance concentration (Simpson Index) were calculated as described elsewhere (Brown and Bowman, 2001). Pair wise comparison of clone libraries were carried out determining a similarity coefficient calculated by dividing the duplicate number of shared phylotypes by the total number of phylotypes detected in compared libraries (Odum, 1971).

Hydrocarbon analyses in natural sediments and microcosms

Hydrocarbons were extracted from natural sediments (10 g), analysed by high-resolution gas chromatography-mass spectrometry (GC-MS) and quantified according to previously described protocols (Dutta and Harayama, 2001; Wang *et al.*, 2002). Before proceeding, the samples were spiked with recovery surrogate squalane and decamethyl-anthracene (10 μg each added) to determine extraction efficiency. Subsequently, sediments were extracted using methanol followed by dichloromethane at ambient temperature on a shaker. The extract was concentrated by rotary evaporation (< 30°C) to a small volume (approximately 10 ml) and analysed for total extractable organic matter (TEO) content using a microbalance method. Thin-layer chromatography (TLC) was used for separation of TUC and alcohol (ALC) fractions, yields of which were determined gravimetrically, as described by Eganhouse and Sherblom (2001). The TUC fractions were evaporated under stream of nitrogen and taken up in a solution containing 1-phenyl $-C_{15}$ (2 μg ml^{-1}, internal standard). Sterols and phytols present in the ALC fraction were converted to corresponding trimethyl silyl derivates (Pierce) and taken up in a solution of 5α(H)-cholestane (2 μg ml^{-1}, internal standard). All these compounds were further determined by high resolution GC-MS.

To detect the biodegradation values in microcosms, 200 ng of the biomarker 17α(H),21β(H)-hopane (Chiron AS), a degradation resistant compound present in crude oil (Prince *et al.*, 1994), was added at the beginning of experiments. At the end of the experiment each microcosm was homogenized and a 1-ml sample was taken for GC-MS analysis three times. The percentage of biodegradation was calculated by normalizing the concentration of individual compounds relative to that of 17α(H),21β(H)-hopane at the end of the experiment and at the start of the experiment. Measurements made on replicate samples showed that precision was in all cases within 10%.

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