Biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium

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Abstract

Three bacterial isolates identified as \textit{Alcanivorax borkumensis} SK2, \textit{Rhodococcus erythropolis} HS4 and \textit{Pseudomonas stutzeri} SDM, based on 16S rRNA gene sequences, were isolated from crude oil enrichments of natural seawater. Single strains and four bacterial consortia designed by mixing the single bacterial cultures respectively in the following ratios: (\textit{Alcanivorax}: \textit{Pseudomonas}, 1:1), (\textit{Alcanivorax}: \textit{Rhodococcus}, 1:1), (\textit{Pseudomonas}: \textit{Rhodococcus}, 1:1), and (\textit{Alcanivorax}: \textit{Pseudomonas}: \textit{Rhodococcus}, 1:1:1), were analyzed in order to evaluate their oil degrading capability. All experiments were carried out in microcosms systems containing seawater (with and without addition of inorganic nutrients) and crude oil (unique carbon source). Measures of total and live bacterial abundance, Card-FISH and qualitative and quantitative analysis of hydrocarbons (GC-FID) were carried out in order to elucidate the co-operative action of mixed microbial populations in the process of biodegradation of crude oil. All data obtained confirmed the fundamental role of bacteria belonging to \textit{Alcanivorax} genus in the degradation of linear hydrocarbons in oil polluted environments.

Key words: \textit{Alcanivorax}, \textit{Pseudomonas}, \textit{Rhodococcus}, bioremediation, bioaugmentation.

Introduction

Petroleum hydrocarbons are the most widespread contaminants within the marine environment. Pollution by hydrocarbons in marine environments may be the consequence of various natural (natural seepages) and/or anthropogenic activities (discharge during tanks and/or ships transportation and/or pipeline failures) as well as the chronic pollution (ships, harbours, oil terminals, freshwater run-off, rivers and sewage systems).

The “fate” of petroleum in the sea water largely depends on mechanical (wave, wind), physical (temperature, UV) and chemical (pH, dissolved oxygen and nutrient concentration) factors which may differently influence its natural transformation (oil weathering) and bio-degradation (Nikolopoulou and Kalogeraki, 2010). At an early stage light fractions of oil are naturally removed; mostly by evaporation, thence by photo-oxidation and by geo-chemical reactions. Heavy fractions are instead dispersed or dissolved and only a small portion may be removed by the process of biodegradation. Although chemical-physical phenomena play an important role in the process of oil detoxification, the ultimate and complete degradation is mainly accomplished by marine microflora, dominant bacteria in this role (Della Torre \textit{et al.}, 2012).

As reported in different studies, a wide variety of marine bacteria are known to degrade petroleum hydrocarbons, and those, distributed over several (sub)phyla (\textit{α}, \textit{β}, and \textit{γ}-Proteobacteria; Bacteroidetes/Chlorobi group) have been described so far (Rooling \textit{et al.}, 2004; Cappello \textit{et al.}, 2007).
In the natural environment, biodegradation of crude oil involves a succession of species within the consortia of the present microbes (Alkatib et al., 2011). Indeed, since a single species can metabolize only a limited range of hydrocarbon substrates, a consortium of many different bacterial species, with broad enzymatic capacities, is usually involved in oil degradation (Rooling et al., 2002). Although some bacteria, belonging to *Pseudomonas* (Das and Chandar, 2011) and *Rhodococcus* genera (Hassanshahian et al., 2010 and 2012) have shown able to degrade hydrocarbons (Teramoto et al., 2010), in marine environments the key micro-organisms in the bio-degradation process has been identified as bacteria related to *Alcanivorax* genus (Yakimov et al., 2007; Cappello and Yakimov 2010).

On the above mentioned basis, bioremediation techniques have been developed and improved for cleaning up oil-polluted marine environments as an alternative to chemical and physical techniques (Alkatib et al., 2011). Bioremediation can be described as the conversion of pollutants (hydrocarbons) by micro-organisms (bacteria) into energy, cell mass and biological waste products (Nikolopoulou and Kalogeraki, 2010). Nevertheless, the rates of uptake and mineralization of many organic compounds (hydrocarbons) by bacteria in polluted seawater is limited due to the poor availability of nitrogen and phosphorus (Yakimov et al., 1998; Kasai et al., 2002a, b; Cappello and Guglielmino, 2006; Cefalì et al., 2002). For that reason, in the application of biostimulation techniques the growth of oil-degrading bacteria can be strongly enhanced by fertilization with inorganic nutrients (Nikolopoulou and Kalogeraki, 2010).

In order to elucidate the cooperative action of mixed microbial populations in the biodegradation of crude oil, we have built up artificial consortia made up of two/three bacteria. By using these consortia, we have been able to investigate the capability of efficient biodegradation of crude oil could be accomplished by the mixed populations. All experiments have been carried out into microcosms systems containing seawater (with and without inorganic nutrients); oil has been used as the only carbon source.

The knowledge of the efficiency and the activities of bacteria in oil-polluted sites may be helpful for the bioremediation of oil spills, since human action, by using specific microbial consortia, can be planned in order to clean up oil pollution (Denaro et al., 2005).

**Material and Methods**

**Bacterial strains**

Three bacterial strains named isoSS-01, corresponding to *Alcanivorax borkumensis* strain SK2\(^T\) (Genbank accession number Y12579; =DSM 11573\(^T\); 99%), isoSS-02 (*Rhodococcus erythropolis* HS4; Genebank accession number AY168582; 99%) and isoSS-03 (*Pseudomonas stutzeri* SDM; Genebank accession number DQ358054; 98%) were used in all the experiments (Fig. 1). Strain isoSS-01 belong to a collection of hydrocarbon-degrading bacteria hold at IAMC-Messina, strains isoSS-2 and iso-

![Figure 1 - Phylogenetic tree based on 16S rRNA gene sequences for bacterial strains (isolates isoSS-01, -02 and -03) used in this study. Percentages of 100 bootstrap resampling that supported the branching orders in each analysis are shown above or near the relevant nodes. The tree was rooted and outgrouped (black arrow) by using the 16S rRNA sequences of *Methanococcus jannaschii* (M59126). Evolutionary distance is indicated by vertical lines; each scale bar length corresponds to 0.05 fixed point mutations per sequence position.](image-url)
SS03 were isolated from natural seawater from crude oil enrichments in previous research. All strains used in this study were isolated from natural seawater from crude oil enrichments.

Analysis of 16S rRNA genes

Total DNA extraction of bacterial strains was performed by the MasterPure Complete DNA&RNA Purification Kit (Epicenter, Biotechnologies, Madison, WI) in accordance with manufacturer’s protocol. The 16S rDNA loci were amplified using 1 primer pair: the 27F (5’-AGAGTTTGATCCTGGCTCAG-3’, Lane, 1991) primer and the 1492R (5’-TACGGYTACCTTGTTACGACT-3’, Lane, 1991) universal primer. PCR (polymerase chain reaction) was carried out in 50 μL of reaction mixture containing 1x reaction buffer, 1x solution Q (both from QIAGEN), 1 μM of each primer, 200 μM dNTP (Gibco), 1 μL of template and 2.5 U of Qiagen Taq polymerase. The PCR reaction was carried out in Mastercycler Gradient (Eppendorf); the PCR conditions were as follows: 95 °C for 5 min (1 cycle); 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min (35 cycles); with a final extension step at 72 °C for 10 min. PCR products were sequenced using Macrogen Service (Macrogen, Korea). The analysis of the sequences (1400 bp of average length) was performed as previously described by Yakimov et al. (2005). The sequences similarity of individual inserts was analysed by the FASTA program Nucleotide Database Query available through the EMBL-European Bioinformatics Institute. The phylogenetic affiliation of the sequenced clones, was performed as described by Yakimov et al. (2006).

Growth conditions

Started cultures were prepared by inoculating one loop of microbial cells into 10 mL of ONR7a mineral medium based on the composition of seawater was used in this study (Dyksterhouse et al., 1995). Nitrogen was provided in the form of NH₄Cl, and was provided in the form of Na₂HPO₄. ONR7a contained (per liter of distilled or deionized water) 22.79 g of NaCl, 11.18 g of MgCl₂·6H₂O, 3.98 g of Na₂SO₄, 1.46 g of CaCl₂, -2H₂O, 1.3 g of TAPSO (3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid), 0.72 g of KCl, 0.27 g of NH₄Cl, 89 mg of NaH₂PO₄·7H₂O, 83 mg of NaBr, 31 mg of NaHCO₃, 27 mg of H₂BO₃, 24 mg of SrCl₂·6H₂O, 2.6 mg of NaF, and 2.0 mg of FeCl₃·4H₂O. To prevent precipitation of ONR7a during autoclaving, three separate solutions were prepared and then mixed together after autoclaving when the solutions had cooled to at least 50 °C; one solution contained NaCl, Na₂SO₄, KCl, NaBr, NaHCO₃, H₂BO₃, NaF, NH₄Cl, Na₂HPO₄, and TAPSO (pH adjusted to 7.6 with NaOH), the second solution contained MgCl₂, CaCl₂, and SrCl₂, (divalent cation salts), and the third solution contained FeCl₃; 0.1% (w/v) sterile tetradecane (C₁₄H₃₀, Sigma-Aldrich, Milan, Italy) was used as only energy and carbon source. After growing in a rotary shaker (New Brunswick C24KC, Edison NJ, USA; 150 rpm) at 25 °C for two days, 500 μL of the seed culture broth were transferred into a 250 mL Erlenmeyer flask containing 100 mL of ONR7a medium supplemented with 1% (w/v) sterile tetradecane. The culture was incubated in a rotary shaker (New Brunswick C24KC, Edison NJ, USA; 150 xg) at 25 °C for 5 days.

Consortia

At the beginning (T₀) of the experiments selected microorganisms (isoSS-01, A. borkumensis SK2; iso-SS-02, R. erythropolis HS4 and iso-SS-03 Ps. stutzeri SDM) were added at a final density of 10⁷ cell mL⁻¹, in experimental microcosms. Schematic representation of microbial consortia used in this study is indicated below (Fig. 2).

Experimental set-up of microcosms systems

The microcosms systems were performed in 250 mL sterilised Erlenmeyer flasks. Microcosms were incubated at 22 ± 1 °C for 15 days with shaking (100 g). All experiments were carried out in triplicate.

Two different series of experimentations were carried out. In the first experiment (identified as “SW”) bacterial cultures were carried out in natural seawater sterilized by...
filtration through a 0.2-μm syringe filter (Sartorius); in the second experiment (identified “SW+IN”) cultures were carried out in sterile natural seawater with addition of inorganic nutrients to reach higher concentrations than those obtained in natural water (final concentrations: KH2PO40.077 g L−1, NH4Cl 0.2 g L−1 and NaNO30.1 g L−1). Microcosms untreated (no bacteria inoculation) were used in each experiment series as negative (abiotic) control. Crude oil was added in all experimentation.

At the beginning (T0) of the experiments, 1000 ppm of sterile crude oil (Arabian Light Crude Oil; ENI Technology S.p.A.) were added into SW and SW+IN microcosms. Crude oil was introduced, in microcosm systems, after physical weathering (100 x g, 25 °C for 48 h); crude oil was supplemented with 0.1% (v/v) of squalene (C30H50, Sigma-Aldrich, Milan, Italy) as internal spike for measure of bio-degradation rate.

Sampling strategy and parameters assayed

At the beginning (T0) and at the end (T15) of the experimental period, sub-samples of each bacterial cultures were taken aseptically. Measures of direct bacterial count (DAPI), microbial viability (Live/Dead staining) and microbial activity (Card-FISH) were carried out. Measure of oil degradation was carried out as well. All experiments were carried out twice and all parameters detected were measured three times.

Total bacterial abundance (DAPI count)

After a short-time (30") ultrasonic treatment (Ultrasonic Bath Branson 1200, Branson, USA), the total bacterial cell counts were performed by DAPI (4’6-diamidino-2-phenylindole 2HCl, Sigma-Aldrich S.r.L., Milan, Italy) staining on samples fixed by formaldehyde (2% final concentration), according to Porter and Feig (1980) and Capsoni et al. (2012). Slides were examined by epifluorescence by using Axioplan 2 Imaging (Zeiss; Carl Zeiss Inc., Thornwood, N.Y., USA) microscope. Results were expressed as number of cells mL−1.

Determination of living and dead bacteria

Living and dead bacteria (L/D) were enumerated after staining with the Live/Dead (BacLight bacterial Viability Kit (Invitrogen Corp; Molecular Probes, Inc Eugene, OR, USA). The above mentioned method allowed discrimination, within the total bacterial community, of the living cells, labelled by SYTO 9 and green-fluorescing, from the dead ones, labelled by propidium iodide and red-fluorescing (Zampino et al., 2004). Cell counts, performed by an Axioskop epifluorescence microscope (Zeiss; Carl Zeiss Inc., Thornwood, N.Y., USA) equipped with a 100 W Hg lamp using fluoresein (BP 450-490; FT 510; LP 520) and rhodamine (BP 546/12; FT 580; LP 590) filter sets (for live and dead cells, respectively). Data obtained were reported as the mean value of (living and dead) cells mL−1.

Card-FISH

Card-FISH analysis was carried out according to protocol developed by Pernthaler et al. (2002). Aliquot of 1 mL of bacterial culture was filtered on 0.22 μm polycarbonate membranes (diameter 25 mm) by using a vacuum filtration device (Millipore, Milan, Italy). Filters for Card-FISH counts were embedded in low-gelling point agarose membranes (0.1% agarose, Sigma-Aldrich, Milan), dried at 37 °C for 20 min, and dehydrated with 95% ethanol. The bacteria on the polycarbonate membrane were then permeabilized by lysozyme (solution (EDTA 0.05 M; 1 M Tris-HCl, pH 8.0; MilliQ water and 10 mg mL−1 lysozyme) for 60 min at 37 °C and in some cases a treatment with a-chromopeptidase (60 U, 0.01 M NaCl, 0.01 M Tris-HCl [pH 8.0]) was performed. Filters were incubated at 37 °C for 30 min and hybridized with oligonucleotide probes modified at the 5’ end with horseradish peroxidase (HRP). Probes used in this work are listed in the Table 1.

After the hybridization and amplification steps, slides were examined by an Axioskop epifluorescence microscope (Zeiss; Carl Zeiss Inc., Thornwood, N.Y., USA) equipped with an appropriate filter sets for Card-FISH. Before counting, the slides were stored at -20 °C for several days without any loss of fluorescence intensity. Cell counts were reported as the mean value of cells mL−1.

Hydrocarbon analysis

The composition of the Total Extracted and Resolved Hydrocarbons and their derivates (TERHCs) were analysed by high-resolution GC-FID (DANI Master GC Fast Gas Chromatograph System, DANI Instruments Sp.A., Milan). After acidification, TERHCs from samples were extracted at room temperature on a shaking table by using dichloromethane (CH2Cl2, Sigma-Aldrich, Milan; 10% v/v). This procedure was repeated three times, and the CH2Cl2 phase was combined and treated with sodium sulfate anhydrous (Na2SO4, Sigma-Aldrich, Milan) in order to remove any residual water (Ehrhardt et al., 1991; Wang et al., 1998; Dutta

| Table 1 - Oligonucleotide probes used in Card-FISH for this study. |
|-----------------|-----------------|-----------------|-----------------|
| Probe           | Sequence (5’ to 3’) of probe | Specificity                | Source             |
| NON-Eub338      | ACA TCC TAC GGG AGG C          | Negative Control          | (Wallner et al., 1993) |
| Eub338          | GCT GCC TCC CGT AGG AGT        | Domain Bacteria           | (Amann et al., 1990) |
| Alk             | CGC ACG CGA GCT CAT CCA TCA    | Alcanivorax genus         | (Karner and Fuhrman, 1997) |
and Harayama 2001; Denaro et al., 2005). Extracts were concentrated by rotary evaporation (Rotavapor model R110; Büchi Labortechnik AG, Switzerland) at room temperature (< 30 °C), followed by evaporation under a stream of nitrogen and taken up into a solution containing heptamethyl-nonane as an internal standard (79 μg mL⁻¹). Indicators selected for this study were: n-C17/Pristane (nC17/Pr), n-C18/Phytane (nC18/Ph) in order to evaluate the relative biodegradation of n-alkanes.

Biodegradation efficiency (BE) of TERCHs

The degradation of TERCHs was expressed as the percentage of TERCHs degraded in relation to the amount of the remaining fractions in the appropriate abiotic control samples. The biodegradation efficiency (BE), based on the decrease in the total concentration of hydrocarbons, was calculated by using the expression described by Michaud et al., 2004:

\[ 100 - \left( \frac{As \times 100}{Aac} \right) \]

where As = total area of peaks in each sample, Aac = total area of peaks in the appropriate abiotic control, BE (%) = Biodegradation efficiency.

Statistical analysis and nMDS

The experimental data are presented in terms of arithmetic averages of at least three replicates and the standard deviations are indicated by error bars. The non-metric multi-dimensional (nMDS) scaling plot were done using PAST (PAleaeontological STatistics Software ver. 1.88; Hammer et al., 2001).

Results

Total bacterial abundance (DAPI count)

After 15 days of cultivation, the bacterial abundance was measured by direct DAPI count; and data obtained were compared with the quantity of cells present at the beginning of the experimental period (T0). The data obtained showed, how in seawater added with inorganic nutrients it was possible to observe a general increase of microbial abundance (systems “A”, “A + P”, “A + R,” “P + R”, and “A + P + R”) with mean values of 10⁸ cell mL⁻¹. In cultures performed using seawater (without inorganic nutrients), bacterial abundance present, at the end of experimental period, mean values of 10⁶ cell mL⁻¹ (systems “P”, “R”, “A + P” and “A + P + R”); in microcosms indicated as “A”, “A + R”, and “P + R”) values of ~10⁵ cell mL⁻¹ were observed (Fig. 3).

Determination of living and dead bacteria

Data of living and dead bacteria (L/D) enumerated using the Live/Dead staining are showed in Figure 4. The results obtained after 15 days of cultivation shown as the vital bacterial fraction, present in microcosm performed in seawater with addition of inorganic nutrients, was greater than that observed in the microcosms performed in sea water. In particular in microcosms indicated

![Figure 3](image-url)
as SW the percentage of dead cells was about four or six times greater than the initial time.

Card-FISH

The qualitative measure of microbial abundance, into the experimental systems named “A+P” and “A+R”, was carried out by using the card-FISH method. Values of abundance of cells hybridized using probes for Eubacteria (EUB338) resulted to be similar to the values obtained from the measure of total bacterial abundance (DAPI count) in the same conditions.

Data obtained put in evidence as almost total cells of experimentations carried out with seawater without inorganic nutrients were hybridized by probes for Eubacteria. The same result was not obtained during experimentations carried out with seawater added with inorganic nutrients (in such a case a number of cells of a lower logarithmic order has been obtained). The data obtained showed as the quantity of cells of *Alcanivorax borkumensis* (in “A + P”, SW + IN; “A + R”, SW and “A+ R”, SW + IN systems) present values lower (of a logarithmic order) those obtained in total cells (Fig. 5).

Rate of degradation of n-alkanes

The percentage degradation of n-alkanes (C_{12}-C_{30}) present in the crude oil was calculated by comparison of the gas chromatograms of the non degraded (abiotic) control and the degraded sample for each experimental conditions (Table 2 and Fig. 6).

During experiments performed with natural seawater the condition identified as “A+P+R” showed a better rate degradation (~ 90%); also in system “A+R” in other conditions is possible to observe a degradation of almost all n-alkanes (rate of degradation > of 60%).

The data obtained show that, during growth in natural seawater added with inorganic nutrients, conditions “A”, “R”, “A+P”, “A+ R” and “A+P+R” n-alkanes present in the crude oil were totally degraded; in contrast, conditions “P” and “P+R” present a low rate of degradation of n-alkanes.

For all strains, n-alkanes with a medium length (C_{12}-C_{18}) were degraded to a greater extent (rate of degradation > of ~ 70%) than and long chains (C_{19}-C_{30}) because long-chain n-alkanes are solid and their low solubility inhibits degradation by bacteria (Figs. 7 and 8).

Biodegradation efficiency (BE) of TERCHs

After 15 days of experimentation, measure of degradation of the TERHCs revealed as major rates of oil degradation are, in general, observed in systems carried out in natural seawater with inorganic nutrients (Table 3). In SW experiment the maximum rate of total oil degradation is observed in “A+P+R” (~ 97%) and “A+R” system (~ 83%). Other conditions present similar values. In system SW+IN the experimentations identified “A”, “A+P”, “A+R” and “A+P+R” the degradation of oil is total; values of ~ 90%,
~ 64% and ~ 30% of total oil degradation were observed for “P”, “R” and “P+R” experiments (Fig. 6).

Discussion

The recovery of petroleum contaminated sites could be achieved by either physicochemical or biological methods. Due to negative consequences of the physicochemical approach, more attention is now given to the exploitation of biological alternatives (Okoh, 2006).

Biological treatments are having more importance, mainly because of the low environmental impact, the costs (in general cheaper than other cleanup technologies), the capability to destroy organic contaminants, and the possibility of beneficial use of treated sediments (Rulkens and Bruning, 2005). Different studies have shown better results using bioremediation strategies (Beolchini et al., 2010; Rocchetti et al., 2011, 2012).

In general, bioremediation is often based on in-situ stimulation of the microbial community (biostimulation) or amending the microbial community with an inoculum of hydrocarbon-degrading bacteria (bioaugmentation). In both cases, the successful result of bioremediation depends on appropriate hydrocarbon-degrading consortia and environmental conditions.

In this study we have analyzed the cooperative action of mixed microbial populations in the biodegradation of crude oil during different culture conditions. All data obtained confirmed the fundamental role of bacteria belonging to Alcanivorax genus in degradation of linear hydrocarbons in oil polluted environments. Indeed, all experimentations carried out in seawater (with or without inorganic nutrients) whit presence of Alcanivorax showed maximum rates of oil degradation.

Capability of Alcanivorax genus to use hydrocarbons as the only sources of energy and organic carbon was widely (Yakimov et al., 1998; Scheiner et al., 2006). Kasai (2002) and Cappello (2012) explain these characteristics in ability of this strain to produce a lipidic bio-surfactant that increases the bioaviable of contaminant and the ability to use this (Yakimov et al., 1998; Scheiner et al., 2006). Alcanivorax borkumensis SK2 surfactant propose as one of the most efficient of bacterial surfactants; the possible presence of this surfactant can justify an increase in the rates of degradation by both the bacteria that possible microbial consortia. This defines an increment of rates of degradation by both the bacteria and possible microbial consortia (Yakimov et al., 1998; Scheiner et al., 2006).

The presence of Alcanivorax in natural environment or enrichment by laboratory is generally combined with the presence of other bacterial strains, such as Pseudomonas sp. and Rhodococcus sp., that participating in biodegradation phenomena. However, Pseudomonas sp. and Rhodococcus sp., can not be classified such as hydro-
carbonoclastic bacteria (Marine Obligate Hydrocarbonoclastic Bacteria, OMHCB; Yakimov et al., 2007), but these are heterotrophic bacteria that participate in the biodegradation processes via “syntrophy metabolic” in which the degradation of pollutant compounds takes place via a metabolic chain, in which the product of the catabolism of a bacterial species is identified as a source of carbon for metabolic another.

Analysis of microbial abundance in cultures in study showed, however, a divergence of the correlation between microbiological data and those of biodegradation. In experimentation carried out with *Alcanivorax* and *Pseudomonas* (*system “A”, “P” and “A+P”) was possible to observe after 15 days to incubation in seawater with and without inorganic nutrients an increase of microbial biomass. Data obtained during cultivation of *Rhodococcus erythropolis* (as single strain and/or as consortium) did not show, apparently, increment of microbial abundance. This condition may be due to an underestimation of the direct count (DAPI count) in cultures as result from an inefficiency of methodology used by us for the separation of microbial cells from oil remain (dislodging).

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Another important aspect was obtained to qualitative measures of microbial abundance. Card-FISH analysis carried out to estimate quantitative abundance of bacteria belonging to *Alcanivorax* genus in microbial consortia tested in this study. Therefore Card-FISH measures were realized for identified consortia A+P and A+R realized in seawater with and without inorganic nutrients. Hybridization with EUB-338 probe showed values similar to these obtained by direct DAPI count; for against assays carried out with ALK probe (specific probe to *Alcanivorax* genus) evidence as only the 15% of total cells were hybridized. This result can seem discordant with biodegradation results. However, it is important remember that the sample to Card-FISH was col-

| n-alkanes | Natural sea water | Natural sea water + inorganic nutrient |
|-----------|------------------|--------------------------------------|
|           | A    | P    | R    | A+P  | A+R  | P+R  | A+P+R |
| C12       | 100  | 87   | 100  | 100  | 100  | 100  | 100   |
| C13       | 100  | 88   | 97   | 100  | 100  | 100  | 100   |
| C14       | 100  | 89   | 95   | 100  | 100  | 100  | 100   |
| C15       | 100  | 82   | 91   | 100  | 100  | 100  | 100   |
| C16       | 100  | 84   | 95   | 100  | 100  | 90   | 100   |
| C17       | 100  | 85   | 95   | 100  | 100  | 81   | 100   |
| C18       | 100  | 83   | 94   | 100  | 100  | 94   | 100   |
| C19       | 100  | 83   | 90   | 100  | 100  | 80   | 100   |
| C20       | 100  | 82   | 92   | 100  | 100  | 80   | 100   |
| C21       | 100  | 81   | 90   | 100  | 100  | 77   | 100   |
| C22       | 100  | 81   | 92   | 100  | 100  | 72   | 100   |
| C23       | 100  | 83   | 90   | 100  | 100  | 71   | 100   |
| C24       | 100  | 76   | 87   | 100  | 100  | 63   | 100   |
| C25       | 100  | 77   | 90   | 100  | 100  | 68   | 100   |
| C26       | 100  | 77   | 86   | 100  | 100  | 70   | 100   |
| C27       | 100  | 75   | 87   | 100  | 100  | 71   | 100   |
| C28       | 100  | 75   | 86   | 100  | 100  | 69   | 100   |
| C29       | 100  | 75   | 81   | 100  | 100  | 71   | 100   |
| C30       | 100  | 77   | 87   | 100  | 100  | 62   | 100   |
Figure 6 - Relative values of major TERHC fractions of Arabian Light Crude Oil detected in SW and SW+IN cultures after 15 days of incubation; data expressed as the percentages compared to negative abiotic control (0). A, *Alcanivorax borkumensis* SK2; P, *Pseudomonas stutzeri* SMD; R, *Rhodococcus erythropolis* HS4. Experimentations carried out in natural seawater in absence (SW) and presence (SW + IN) of inorganic nutrients were indicated, respectively, with grey and dark grey bars.

Figure 7 - The non-metric multi-dimensional (nMDS) scaling plot related to the capability biodegradation of n-alkanes of different bacteria and consortia in study. A, *Alcanivorax borkumensis* SK2; P, *Pseudomonas stutzeri* SMD; R, *Rhodococcus erythropolis* HS4. Normal letter indicate the Natural Sea Water experimentation (SW), underlined letters indicate the Natural Sea Water + Inorganic Nutrients (SW + IN) experimentation.
lected after 15 days of incubation, therefore is possible that
the cells were collected in advance stationary phase and/or
not more active. Supposing that the oil degradation process
began early of the end of experiment, *Alcanivorax sp.* ,
dominant at the first experimental phase, tended to disap-
pear or decrease once hydrocarbons have been degraded,
while *Pseudomonas sp.* and *Rhodococcus sp.* cells could
become dominant using metabolic compounds or cellular
lysates like nutritional source.

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**Figure 8** - The non-metric multi-dimensional (nMDS) scaling plot related to the biodegradation efficiency (BE) of TERCHs of different bacteria and con-
sortia in study. A, *Alcanivorax borkumensis* SK2; P, *Pseudomonas stutzeri* SMD; R, *Rhodococcus erythropolis* HS4. Normal letter indicate the Natural Sea Water experimentation (SW), underlined letters indicate the Natural Sea Water + Inorganic Nutrients (SW + IN) experimentation.
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