Comparison the effects of bioaugmentation versus biostimulation on marine microbial community by PCR–DGGE: A mesocosm scale

Mehdi Hassanshahian1,⁎, Zeynab Bayat1, Simone Cappello2, Francesco Smedile2, Michail Yakimov2

1. Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran
2. Istituto per l’Ambiente Marino Costiero (IAMC)—CNR of Messina, Messina, Italy

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ABSTRACT
In order to better understand the effects of biostimulation and bioaugmentation processes on a marine microbial community, three different mesocosm experiments were planned. Natural seawater (10,000 L) was artificially polluted with crude oil (1 L) and (1) inorganic nutrients (Biostimulating Mesocosm, BM), (2) inorganic nutrients and an inoculum of Alcanivorax borkumensis SK2 (Single Bioaugmentation Mesocosm, SBM), (3) inorganic nutrients and inoculums of A. borkumensis SK2 and Thalassolituus oleivorans MIL-1 (Consortium Bioaugmentation Mesocosm, CBM). During the experimental period (20 days), samples were taken from each mesocosm and the community structure was analyzed by PCR–DGGE. The 16S rRNA gene DGGE banding patterns and sequence analysis demonstrated that biostimulation had the lowest effect on microbial biodiversity in the mesocosms; however, the biodiversity of the marine microbial community dramatically decreased in the CBM (Shannon index was 0.6 in T3). The community structures among the three mesocosms were also markedly different, and major bacteria derived from DGGE bands were related to uncultured Gamma Proteobacteria. The biodegradation results show that the Single Bioaugmentation Mesocosm (SBM) system had the highest percentage of degradation (95%) in comparison to the BM mesocosm (80%) and CBM (70%).

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Introduction
Organic pollutant contamination is a constant problem in many coastal waters adjacent to urban areas. In addition to occasional oil tanker accidents, there are many recurrent sources of marine oil pollution that introduce organic pollutants, particularly petroleum hydrocarbons: uncontrolled releases from crude oil plants, contaminated freshwater and terrestrial run-off, etc. (Head and Swannell, 1999; Bayat et al., 2015). Although the toxic effects of these contaminants on higher organisms such as fish, mollusks and other invertebrates are well known (Preston, 2002; Castle et al., 2006; Hassanshahian et al., 2014b), bacteria represent the predominant agents of hydrocarbon degradation in the marine environment and might be either stimulated or negatively affected by the hydrocarbons. A remarkable decrease in bacterial diversity has been frequently reported following exposure to hydrocarbons, as a consequence of a strong

⁎ Corresponding author. E-mail: mshahi@uk.ac.ir (Mehdi Hassanshahian).

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selection for hydrocarbon-degrading bacteria (Nyman, 1999; Röling et al., 2002; Castle et al., 2006; Hassanshahian et al., 2012b).

To date, a number of studies have investigated changes in bacterial composition associated with application of biostimulation and bioaugmentation techniques using molecular approaches such as DGGE (Denaturing Gradient Gel Electrophoresis) of polymerase chain reaction (PCR)-amplified 16S rRNA genes. However, no consistent pattern of variability has emerged from the application of these molecular tools (Macnaughton et al., 1999; Kasai et al., 2001; Ogino et al., 2001; Castle et al., 2006). Polymerase chain reaction-based techniques allow for a reasonably good characterization of the phylogenetic composition of a sample.

Molecular methods provide tools for analyzing the total bacterial community, covering bacteria that cannot be cultured in the laboratory. Therefore, such methods are becoming increasingly important in microbial ecology (Pickup, 1991; Stackebrandt et al., 1993; Amann et al., 1995; Holben and Harris, 1995). The amplification by PCR of rRNA genes from seawater DNA samples, combined with fingerprinting techniques such as DGGE, provides detailed information about the species composition of whole communities.

To understand how the composition of a microbial community affects the process of biodegradation, it is necessary to analyze the microbial response to oil pollution both at genetic and metabolic levels. It is also important to develop a multidisciplinary approach for the study of microorganisms having high specificity for recalcitrant compounds, dealing with their structure and function. Knowledge of microbial diversity and metabolism in oil-polluted sites can be helpful for bioremediation of oil spills, as human intervention by using specific microbial consortia can be planned for cleaning up oil pollution (Denaro et al., 2005; Hassanshahian and Emtiazi, 2008).

In this matter, the scale of the stimulating system can be particularly important; for example, the use of mesocosms. Mesocosms are middle-sized, constructed ecosystems that are used as tools for ecological research, applied research and development and education. Mesocosms represent extensions of the microcosm method. They are significant because their relatively large size allows the incorporation and use of much more ecological complexity than is possible in conventional microcosms (Cappello et al., 2006; Hassanshahian et al., 2013; Hassanshahian et al., 2014a).

The aim of the present study was to monitor in a mesocosm test the changes take place in the structure and composition of natural seawater microbial communities caused by addition of crude oil. Also, application of PCR-DGGE as a molecular method for understanding the changes in the microbial community after oil pollution was another aim of this study.

1. Material and methods

1.1. Site description and sampling

During the mesocosm experiments natural seawater (SW) was collected, in November 2011, from the station “Mare Sicilia” (38°12.23’N, 15°33.10’E) located at the harbor of Messina (Italy). In both the experiments the seawater was taken through use of a stiff pipeline connected to the sea that enabled the direct filling of the tanks of the experiment. Before introduction in the mesocosms, the natural seawater was filtered through a 100 μm nylon mesh in order to screen out large metazoans and detritus.

1.2. Set-up of experimental mesocosm systems

Experiments in mesocosms were carried out in a “Mesocosm Facility” planned and constructed at the Institute for Coastal Marine Environment (IAMC) — CNR of Messina (Italy). The experiments were carried out in a rectangular tank of 11,250-L capacity (5.000 cm long, 150 cm deep, 150 cm wide) filled with 10,000 L of SW collected as previously indicated. The mesocosm system was filled by SW collected directly from the Strait of Messina through a direct pipeline. The seawater in the study was aerated and stirred during the whole experimental period. The seawater was maintained at 18 ± 2 °C (Fig. 1).

1.3. Bacterial strains

Two bacterial strains named isoMH-01 (Alcanivorax borkumensis SK2; GenBank accession number NR_029340, 100%) and isoMH-02 (Thalassolituus oleivorans MIL-1; GenBank accession number AJ431699, 100%) were used in this study. All bacteria have been isolated from seawater crude oil enrichment. The enrichment was carried out in ONR7a medium (Dyksterhouse et al., 1995) with crude oil as the only energy and carbon source as previously described (Yakimov et al., 1998, 2004).

![Fig. 1 – Schematic representation of set-up and hydraulic system of the “Mesocosm Facility” of IAMC—CNR of Messina used in this study.](image-url)
1.4. Growth conditions and inoculate amendments

Initial cultures were prepared by inoculating one loop of microbial cells into 10 mL of ONR7a mineral medium (Dykerhouse et al., 1995) containing 0.1% (W/V) of sterile tetradecane (C14H30, Sigma-Aldrich, Milan, Italy). After growth in a rotary shaker (New Brunswick C24KC, USA; 150 r/min) at 25 °C for 2 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) of sterile crude oil. The culture was incubated in a rotary shaker (150 r/min) at 25 °C for 5 days. At the beginning (T0) of experiments, selected microorganisms (isoMH-01, SK2 and iso-MH-02, T. oleovorans MIL-1) were added at a final density of 108 cell/mL, corresponding to 0.05/g (dry weight), in the experimental mesocosms (Hassanshahian et al., 2014c).

1.5. Experimental planning for mesocosm experiments

Three different series of experiments were carried out. In all experiments, natural seawater was supplemented with Arabian Light Crude Oil and inorganic nutrients. 10 mg/L (1000 mg) of sterile Arabian Light Crude Oil (ENI Technology S.P.A) was added in all systems in the study after physical weathering (100 × g, 25 °C for 48 hr); 0.1% (V/V) of squalene (C30H50, Sigma-Aldrich, Milan) was added to the crude oil as an internal standard of bio-degradation. Inorganic nutrients (sterile) were added to reach concentrations higher than those in natural water (final concentrations: KH2PO4 0.077 g/L, NH4Cl 0.2 g/L and NaNO3 0.1 g/L). The microorganisms (isoMH-01, A. borkumensis SK2 and iso-MH-02, T. oleovorans MIL-1) were added at a final density of 108 cell/mL, corresponding to 0.05/g (dry weight), in the experimental mesocosms (Hassanshahian et al., 2014c).

1.6. Sampling strategy and parameters assayed

All experiments were conducted for 20 days. To monitor the succession of bacterial activity on fixed days (0, 3, 5, 10, 15 and 20) from time zero (T0; introduction of oil, inorganic nutrients and bacteria), sub-volumes (1000 mL) of sea-water were collected. Total microbial community DNA was extracted from each collected sample. The composition of total extracted and resolved hydrocarbons and their derivates (TERHC) was also analyzed.

1.7. DNA extraction

For DNA extraction, one liter of collected seawater samples at each sampling time was filtered with a 0.2 μm Millipore membrane filter. Seawater DNA extraction was performed using the Qiagen DNA extraction kit (ABN 75072) according to manufacturer’s instructions.

1.8. PCR amplification and DGGE analysis

The 16S rRNA gene based primers used in the PCR reactions were 1055F (5′-ATG GCT GTC GTC AGC T-3′) and 1392R (5′-ACG GGC GTG GTG TRC-3′). A GC clamp was added to the forward primer (F). The PCR thermo cycling (Eppendorf AG 22331 Hamburg) protocol consisted of: an initial denaturation of 1 min at 94 °C; 30 cycles of denaturation of 1 min at 94 °C, 1 min of annealing at temperature 53 °C, and elongation of 1 min at 72 °C; then a final extension step of 10 min at 72 °C. Reactions were performed in a total volume of 50 μL containing 2 mmol/L MgCl2, 10 × PCR reaction buffer (200 mmol/L Tris; 500 mmol/L KCl), 2 mmol/L of each dNTP, 0.15 mmol/L of each primer and 1 U of Taq DNA polymerase.

PCR products were run on a 6% polyacrylamide gel in a 40%-60% denaturing gradient of urea and formamide for 16S rDNA analysis. DGGE was carried out using a BioRad DCode Universal Mutation Detection System at 100 V at 60 °C for 15 hr, in 1.0 x TAE buffer (20 mmol/L Tris, 10 mmol/L acetate, 1 mmol/L EDTA pH 7.4). After electrophoresis, gels were stained for 30 min with SYBR gold nucleic acid gel stain (1:10,000 dilution; Molecular Probes, USA) (Silva et al., 2003). Stained gels were photographed under UV light with the Gel Doc 2000 system (Bio-Rad Laboratories, CA, USA). The digitized images of DGGE gels were analyzed by Image Quant (ver. 5.2) to generate a densitometric profile. Bands were considered significant when the peak height relative to total peak height exceeded 1% according to Iwamoto et al. (2000). The calculation of similarities was based on the Pearson (product-moment) correlation coefficient and resulted in a distance matrix. The Pearson correlation is an objective coefficient that does not suffer from typical peak/shoulder mismatches as often found when band-matching coefficients are applied and is recommended for use with data originating from DGGE profiles (Boon et al., 2002). The clustering algorithm of Ward was used to calculate the dendograms of each DGGE gel using a statistical software package (ver. 5.1, StatSoft).

1.9. Sequencing of DGGE fragments

Excised bands of DGGE gels were washed twice with 1 mL sterilized distilled water in a 1.5 mL tube. A portion of the gel piece (<1 mm3) was used as for the direct template for PCR to recover DNA fragments. The conditions for recovering 16S rRNA genes were the same as for the original PCR, except that the forward primer had no GC clamp attached.

The amplified 16S rRNA fragment was sequenced with a Big Dye Terminator v3.1 Cycle Sequencing Kit on an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). The similarity rank from the Ribosomal Database Project RDP) (Maidak et al., 1997) and FASTA Nucleotide Database Queries were used to estimate the degree of similarity to other 16S rRNA gene sequences. Phylogenetic analysis of the sequences was performed as previously described by Yakimov et al. (2006).

1.10. Hydrocarbon analysis

The composition of TERHC was extracted from 1 L aliquots, analyzed by high-resolution Gas Chromatography-Mass Spectroscopy (GC-MS) and quantified according to previously described protocols (Cappello et al., 2006; Dutta and Harayama, 2001). The TERHC composition was analyzed by high-resolution GC-MS using a Perkin-Elmer Turbo MS Auto System XL GC
Perkin-Elmer Biosystems, USA) equipped with a DB-TPH fused silica capillary column (30 m by 0.32 mm (inner diameter); J and W Scientific (USA)]. The samples were quantified according to previously described protocols (Dutta and Harayama, 2001) and as described by Denaro et al. (2005).

1.11. Statistical analysis

All data obtained from TERHC and DGGE analyses in different experiments were analyzed by Analysis of variance (ANOVA), PAST (PAleontological STatistics Software ver. 1.88) and Primer 6 software. For this purpose the data matrices were constructed as follows: the value (1) denoted the presence of a band that indicates a distinct phylogenetic group and value (0) denoted the absence of this band (phylogenetic group) in the other times of sampling. This matrix was analyzed in Primer 6 software for calculation of diversity indexes.

2. Results

2.1. Effects of biostimulation on marine microbial community in BM experiment

The marine microbial community dynamics during the mesocosm experiments were assayed by PCR–DGGE. The results are shown in Fig. 2. This DGGE gel image reveals that all phylogenetic groups decreased after oil contamination. Other results from the DGGE gel image were the emergence of some specific phylogenetic groups at some sampling times and disappearance at another times. For example (a) band was present from time zero (T0) until day 10 of the experiment (T10), but after 10 days of the experiment the density of this band decreased and disappeared completely by the end of the experiment (T20). The reverse pattern was also seen in the dynamics of the marine microbial community, such as (b) band being present at T0 (before oil contamination) but disappearing after 3 and 5 days of experiment (T3 and T5), although this band reappeared on day ten of the experiment (T10). The presence or absence of specific phylogenetic groups (bands) in the BM can be attributed to the conditions of this mesocosm and adaptation of these phylogenetic groups to oil contamination, because the supply of nitrogen and phosphorus can help the microbial community to better tolerate oil pollution in the marine environment.

A data matrix from the DGGE gel image was constructed, using the Primer-6 software, with the presence and absence of individual bands denoted by the numbers 1 and 0 respectively. The data matrix was analyzed by primer-6 software. The similarity of the marine microbial community in the BM is shown with cluster and two-dimensional graphs in Fig. 3a. Data analysis revealed the presence of three clusters identified as: Cluster 1 (constituted from the microbial population present at T0 and T3); Cluster 2 (microbial population at T5, T10) and Cluster 3 (microbial population at T15, T20). The similarity distance of one population is greater in comparison to other populations. These results mean that after oil pollution in a mesocosm, some changes take place in the marine microbial community.

Biodiversity indexes for the BM were calculated using the data matrix constructed from the DGGE image by Primer-6 software. Results are presented in Table 1. As shown in this table, the dominance (D) has a declining pattern from time zero (T0) until the end of the experiment (T20). The diversity index (H) has an increasing pattern from T0 until T20. These data demonstrated that biostimulation had a low effect on the biodiversity of the marine microbial community and that all phylogenetic groups had the same dominance in the mesocosm.

The DGGE bands were sequenced and some phylogenetic groups were determined after BLAST. The results of phylogenetic groups are presented in Table 2 and the phylogenetic tree constructed by MEGA-5 software is illustrated in Fig. 4. As shown in this figure, more phylogenetic groups are related to Uncultured Gamma Proteobacteria.

2.2. Effects of single bioaugmentation on marine microbial community in SBM experiment

The dynamics of the marine microbial community in the SBM are shown in Fig. 2. The *Alcanivorax* bacterium that was inoculated into the SBM had the highest density bands for all sampling times; this means that *Alcanivorax* predominated over other genera of the marine microbial community after oil pollution in the SBM. Also, after 3 days of the experiment (T3), the total bands in the DGGE gel in comparison to T0 (before inoculation) dramatically decreased, which confirmed the dominance of *Alcanivorax*. However on day 10 of sampling (T10), the number of bands increased until the end of the experiment (T20), which showed that other phylogenetic
The similarity of the marine microbial community in the SBM is shown with cluster and two-dimensional graphs in Fig. 3b. The structures of the marine microbial community before (T0) and after contamination (other times) were significantly different from each other. The microbial communities at T3 and T5 resemble each other, and the microbial population at T10 is different from the community structure at the end of the mesocosm experiment (T15, T20).

The Biodiversity indexes in the SBM are presented in Table 2. In this mesocosm, dominance (D) had the highest value at T5 (0.1429); this factor has an increasing trend from T0 until T5, but after this time decrement change is seen to a decreasing trend until the end of the experiment (T20), reaching the value of 0.066. In contrast, the diversity index (H) shows an opposite pattern, as this index had a decreasing trend from T0 to T5 and shifted to increasing after this time until the end of the experiment (T20), reaching the value of 2.7.

The results of phylogenetic groups are presented in Table 3 and a phylogenetic tree constructed by MEGA-5 software is illustrated in Fig. 5. The hydrocarbonoclasticus bacteria show higher frequency among the phylogenetic groups of the SBM.

### 2.3. Effects of consortium bioaugmentation on marine microbial community in CBM experiment

In this mesocosm, two important HCB: A. borkumensis SK2 and T. oleivorans MIL-1 were added into the mesocosm. Fig. 2 illustrates the PCR–DGGE results from different sampling times for this mesocosm. According to this figure and band patterns, it was concluded that the effect of consortium HCB inoculation on the marine microbial community is greater than inoculation with a single strain. As shown in Fig. 2, a and d bands correspond to Alcanivorax and Thalassolituus. The phylogenetic groups at T3 of the experiment dramatically decrease. This pattern is seen only in the CBM. Also, the total bands decrease from six bands at T0 to two bands at T3. The high density of the Alcanivorax band in comparison to the Thalassolituus band demonstrated that in competition in the marine microbial community, Alcanivorax dominates over Thalassolituus. A slow increment of phylogenetic groups was seen from T5 to T20, but the inoculated bacteria were present at all times.

The similarity of the marine microbial community in the CBM is shown with cluster and two-dimensional graphs in Fig. 3c. In this mesocosm, the structure of the community after contamination was different from its structure before contamination, similar to the SBM. However, the main difference

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**Table 1 – Diversity index in three designed mesocosms.**

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T3</th>
<th>T5</th>
<th>T10</th>
<th>T15</th>
<th>T20</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biostimulating Mesocosm (BM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taxa (S)</td>
<td>10</td>
<td>11</td>
<td>16</td>
<td>17</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>Dominance (D)</td>
<td>0.1</td>
<td>0.09091</td>
<td>0.0625</td>
<td>0.05882</td>
<td>0.05556</td>
<td>0.0625</td>
</tr>
<tr>
<td>Shannon (H)</td>
<td>2.303</td>
<td>2.398</td>
<td>2.773</td>
<td>2.833</td>
<td>2.89</td>
<td>2.773</td>
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<tr>
<td>Simpson (1-D)</td>
<td>0.9</td>
<td>0.9091</td>
<td>0.9375</td>
<td>0.9412</td>
<td>0.9444</td>
<td>0.9375</td>
</tr>
<tr>
<td><strong>Single Bioaugmentation Mesocosm (SBM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taxa (S)</td>
<td>9</td>
<td>8</td>
<td>7</td>
<td>12</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Dominance (D)</td>
<td>0.1111</td>
<td>0.125</td>
<td>0.1429</td>
<td>0.08333</td>
<td>0.0625</td>
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<tr>
<td>Shannon (H)</td>
<td>2.197</td>
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<td>2.485</td>
<td>2.773</td>
<td>2.708</td>
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<tr>
<td>Simpson (1-D)</td>
<td>0.8889</td>
<td>0.875</td>
<td>0.8571</td>
<td>0.9167</td>
<td>0.9375</td>
<td>0.9333</td>
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<td><strong>Consortium Bioaugmentation Mesocosm (CBM)</strong></td>
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<td></td>
</tr>
<tr>
<td>Taxa (S)</td>
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<td>2</td>
<td>5</td>
<td>7</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Dominance (D)</td>
<td>0.1429</td>
<td>0.5</td>
<td>0.2</td>
<td>0.1429</td>
<td>0.1111</td>
<td>0.09091</td>
</tr>
<tr>
<td>Shannon (H)</td>
<td>1.946</td>
<td>0.6931</td>
<td>1.609</td>
<td>1.946</td>
<td>2.197</td>
<td>2.398</td>
</tr>
<tr>
<td>Simpson (1-D)</td>
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<td>0.8</td>
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<td>0.9091</td>
</tr>
</tbody>
</table>
between the CBM and SBM was the higher similarity at T_{10} in the SBM to the natural microbial community (T_{0}); this model is never seen in the CBM. It can be concluded from these results that inoculation of a consortium of bacteria into a marine microbial community changes the composition of the natural marine microbial community such that the phylogenetic groups in this community cannot compete with the inoculated community; thus, a major shift was seen between the community before pollution and that after pollution, as illustrated in Fig. 3c.

The Biodiversity indexes in the CBM are presented in Table 1. According to this table, the dominance factor shows the maximum rate at T_{3} (0.5) and minimum at T_{20} (0.09). The diversity index is considerably decreased in this mesocosm; this decrement is only seen in this mesocosm, as this factor had minimum value at T_{3} (0.6) and maximum value at T_{20} (2.3).

The results of phylogenetic groups are presented in Table 4 and a phylogenetic tree constructed by MEGA-5 software is illustrated in Fig. 6. The low number of phylogenetic groups in this mesocosm confirmed the decrease of biodiversity in the marine microbial community caused by consortium bioaugmentation.

2.4. Comparisons of biostimulation versus bioaugmentation

A data matrix from the three designed mesocosms was input in Primer-6 software and the similarity between the microbial communities in the three mesocosms was calculated. The
results are presented in Fig. 7. As shown in this figure, the BM and SBM had more similarity in marine microbial community than the CBM, which was distinct from these mesocosms. These results confirmed that consortium bioaugmentation considerably altered the natural marine microbial community, as described previously.

The biodiversity graph of the three mesocosms is illustrated in Fig. 8. The lowest biodiversity and number of phylogenetic groups belong to the CBM, and the highest biodiversity is present in the BM. These data demonstrate that biostimulation increased the biodiversity of the natural marine microbial community more than bioaugmentation.

### 2.5. Crude oil biodegradation in mesocosms

The experiment on degradation of the crude oil components in the mesocosms was examined at 3, 5, 10, 15 and 20 days after the start of the experiments. Degradation of the crude oil in the mesocosm experiments was examined by GC-MS; the concentration of crude oil components was normalized using the pristane/phytane ratio, and the values obtained in triplicate sub-samples were averaged.

The results, expressed as percentage of oil degradation, are presented in Fig. 9. As shown in this figure, crude oil biodegradation shows an increasing trend in all mesocosms from the 5th day to the 20th day of the experiment. The SBM had the maximum degradation of crude oil (95% at the 20th day) among all mesocosms. It is notable that the CBM (70%) showed lower degradation of crude oil than the SBM or the BM (80%).

The effect of petroleum “weathering” during the microcosm experiment was monitored in a separate tank filled with sterile seawater and Arabian light oil. The obtained results indicated that evaporation of light petroleum hydrocarbons did occur and resulted in a loss of 5% of TERHC fraction (data not show).

### 3. Discussion

During the last two decades, many investigations have been performed to determine the persistence of hydrocarbons in different natural environments and the possible role of the indigenous microflora on the degradation rate of these contaminants (Kastner et al., 1994; Solano-Serena et al., 2000). The research into the degradation of petroleum in natural...
environments has intensified, and different alternative technologies have been proposed to treat oil polluted systems; however, bioremediation methods (which recruit bacteria to break down the hydrocarbons) are more attractive because of the low cost compared to other methods (Cappello et al., 2012; Hassanshahian, 2014d). Bioremediation is based on enhancing the activity of indigenous organisms (biostimulation) or the addition of microbial inoculates (bioaugmentation) to enhance the clean-up of spills (Syutsubo et al., 2001). The use of experimental mesocosms gives important information about the temporal effects of addition of various pollutants on the structure and functioning of the natural aquatic ecosystems (Hassanshahian et al., 2010; Ghanavati et al., 2008).

A major objective of our study was to determine how biostimulation and bioaugmentation affected the polluted marine microbial community at the mesocosm scale, and also to compare these two technologies. To achieve these aims, three different mesocosms were designed, one representing biostimulation and the others bioaugmentation with a single bacterium and a consortium of two bacteria. The results of this study show that biostimulation had the lowest effect on the biodiversity of the marine microbial community in comparison with the two bioaugmentation mesocosms (Tebyanian et al., 2013).

In this study, the Shannon–Simpson index of diversity was used in combination with correspondence analysis of the DGGE banding patterns based on the similarity coefficient to monitor a range of community responses after the addition of crude oil in the mesocosms. The changes in the community structure within the laboratory mesocosms during the experiment were confirmed, and the major bands in the DGGE profiles were identified. The results indicated that biostimulation with nitrogen and phosphorus only had a slight and short-time effect on community diversity. However, significant decreases in bacterial diversity were seen in the CBM. The bacterial community structures revealed by PCR–DGGE indicated that the Gamma-subgroup of Proteobacteria, especially HCB, plays an important role in crude-oil degradation in the mesocosms. This information is in accordance with data reported from other authors on degradation of crude oil in marine environments (Yakimov et al., 1998; Hassanshahian et al., 2012a).

In the analysis of PCR–DGGE, the number of DGGE bands was taken as an indicator of the species in each sample, and the relative surface intensity of each band was used to estimate species abundance. However, some researchers (Eichner et al., 1999) noted that the number and intensities of bands do not equal the number and abundance of genotypes within the microbial community due to features of 16S rDNA-based phylogeny and bias inherent to PCR amplification from complex template mixtures. Whether the bands represent the most

<table>
<thead>
<tr>
<th>Band name</th>
<th>Phylogenetic groups after sequencing</th>
<th>Percentage of similarity after BLAST (Basic Local Alignment Search Tool)</th>
<th>Accession number</th>
</tr>
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<tr>
<td>T0A</td>
<td>Thalassolitus oleivorans isolate SLHC162b</td>
<td>98</td>
<td>HF572908</td>
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<tr>
<td>T0B</td>
<td>Cycloclasticus spirillensus isolate F1</td>
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<td>HF572905</td>
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<tr>
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<td>Uncultured Cycloclasticus sp. clone B91</td>
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<td>HF572906</td>
</tr>
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</tr>
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<td>T0E</td>
<td>Marinobacter hydrocarbonoclasticus KT02ds19</td>
<td>95</td>
<td>HF572907</td>
</tr>
<tr>
<td>T0F</td>
<td>Alcanivorax sp. PA23 EU647559.1</td>
<td>98</td>
<td>HF572903</td>
</tr>
<tr>
<td>T20G</td>
<td>Uncultured Bacterium clone FS3</td>
<td>95</td>
<td>HF572909</td>
</tr>
</tbody>
</table>

Fig. 6 – Phylogenetic tree based on partial 16S rRNA: ribosomal ribo Nucleotide Acid gene sequenced data showing the location of DGGE gel bands in Consortium Bioaugmentation Mesocosm. The scale represents 0.1 substitutions per base position.

Fig. 7 – Cluster graph of comparisons of similarity between all mesocosms.
abundant species, the most easily extractable species, the most active species, or a combination of all these groups is uncertain. Nevertheless, DGGE may be a sensitive method for detecting differences in community diversity.

An unexpected discovery of our study was the maximum crude oil biodegradation in the SBM compared to the other mesocosms, and, surprisingly, the minimum biodegradation of crude oil took place in the consortium mesocosm. These results can be interpreted as indication that a competition or antagonism may be present between consortium bacteria, in this case Alcanivorax and Thalassolituus.

Scale-up and practical application of treatment technologies for biostimulation of native microbial communities and bioaugmentation with foreign bacteria are promising and have been demonstrated at some locations. Lendvay et al. (2003) compare biostimulation versus bioaugmentation in waters contaminated with chlorinated compounds. They concluded that bioaugmentation with Dehalococcoides had more efficiency than biostimulation with lactate for degradation of chlorinated compounds. In another study, Schafer et al. (2001) designed BMs for bioremediation of oil-contaminated Mediterranean Sea water. They conclude that biostimulation induced major changes in the marine microbial population and selected specific bacterial groups in the mesocosm.

One of the most remarkable facts observed in this study was the reversion of the phylogenetic groups to the native state after 10 days of oil pollution in the BM. Also, in the consortium mesocosm, a dramatic decrease in phylogenetic groups took place. Finally it was suggested that for better crude oil biodegradation in marine environments, a combination of bioremediation strategies must be selected, but this matter needs more investigation in the future.

4. Conclusions

The analysis of the response of the marine microbial community to oil pollution using the PCR–DGGE method during three different biostimulation and bioaugmentation assays (mesocosms) showed that the addition of crude oil decreased the biodiversity of the marine microbial community and that specific phylogenetic groups predominated after oil contamination. At the same time, the results obtained in this research suggest that using a single bacterium (Alcanivorax sp.) for biodegradation of an oil contaminated marine environment has advantages compared to using a consortium of bacteria; this is presumably due to the interaction between different genera of bacteria that possibly suppressed oil bioremediation in the marine environment.

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