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The effects of crude oil on marine microbial communities in sediments from the Persian Gulf and the Caspian Sea: A microcosm experiment

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Abstract

Changes in the microbial community in response to catastrophic oil spills in marine and fresh water environments have been well documented. Molecular methods provide tools for analyzing the entire bacterial community, covering also those bacteria that have not been cultured in the laboratory. In this study, four different microcosms were set up containing sediments collected from the Persian Gulf and the Caspian Sea. One from each location was experimentally contaminated with crude oil and the other left for control. PCR-DGGE analyses were used for understanding the effect of crude oil on marine microbial community in the sediments. The results of this research show that after oil pollution biodiversity decrease in contaminated ecosystems in compare to uncontaminated ecosystems as H index was 3.56 in uncontaminated sediments but it was decrease to 2.7 after contamination. Also different ecosystems have resembled in microbial community after contamination. These results confirmed that crude oil induce major shifts in the composition and biodiversity of marine microbial community in the sediments.

Key words: Caspian Sea, Community, Contamination, Crude oil, DGGE, Persian Gulf

Introduction

Petroleum hydrocarbons are the most common environmental pollutants and oil spills pose a great hazard to terrestrial and marine ecosystems. Oil pollution may arise either accidentally or operationally whenever oil is produced, transported, stored, processed or used at sea or on land. Oil spills are a major menace to the environment as they severely damage the surrounding ecosystems (Head et al. 2006). Persian Gulf is a marine environment that was polluted with crude oil during the 1991 Gulf war. The pollution impact of this episode has been evaluated in several studies, all indicated that crude oil accumulated and remained for long time in coastal area (Hassanshahian et al. 2010; Emtiazi el al., 2009). The oil pollution problem is particularly acute in an oil producing area such as the Persian Gulf, where about 60 percent of the marine transported oil in the world is carried out. The Caspian Sea is another important marine environment in Iran, although oil contamination in Caspian Sea is lower than Persian Gulf but in recent years oil pollution in this environment was increased (Radwan et al. 2005).

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Changes in microbial community in response to catastrophic oil spills in marine and fresh water environments have been well documented (Hara et al. 2003; Ghanavati et al., 2008). These changes include increases in bacterial abundance, alteration of enzyme kinetics and other physiological changes (Labud et al. 2007). Microbial communities can be adapted to contamination by enrichment of specific microorganisms which are able to use or resist the toxic effects of the contaminant. An increase in the total number of hydrocarbon-utilizing microorganisms and of their relative proportions within microbial community is commonly observed in microbial communities subjected to petroleum contaminations (Margesin et al. 2003; Cappello et al. 2007).

Various methods were used to characterize hydrocarbon-degrading populations in soils and sediments. Soil biological investigations such as soil respiration assays (Labud et al. 2007), enzyme activities (Margensin et al. 2003) and microbial counts (Wrenn and Venosa, 1996) can give information on the impact of environmental stresses on microbial community (Balba et al. 1998).

One gram of soil or sediment may contain more than 10^{10} bacteria as counted in fluorescence microscope after staining with a fluorescent dye (Fægri et al., 1977; Torsvik et al., 1996). A serious problem in microbial ecology is that the relative proportion of bacteria growing on agar plates (CFU) varies from 0.1 to 1% in pristine forest soils to 10% in environments like arable soil. This implies that investigations based on bacterial isolates may include only a minor part of the total bacterial diversity.

Molecular methods provide tools for analyzing the entire bacterial community, covering also those bacteria that have not been 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 gene from soil DNA samples, combined with fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE), provide detailed information about the species composition of whole communities.

DGGE separates PCR products of the same size but different sequences by chemical denaturizing. Following staining of the gel, banding patterns may be used to compare different communities or the same community following a perturbation (Prosser, 2002). In the present study, microcosm experiments were performed to evaluate the differences between oil degrading microbial communities inhabiting contaminated and uncontaminated sediments collected from the Persian Gulf and the Caspian Sea, respectively. The aim of this study was to compare the oil degrading microbial community from two different marine ecosystems of Iran in molecular level by PCR-DGGE method.

MATERIAL AND METHODS

Sediments sampling

Coastal sediments samples were collected from two marine environments in Iran. Three stations were located in the Persian Gulf (Khark, Siri and Hormoz islands; 26°15′ N; 54°15′ E) and the other Three stations were located in the Caspian Sea (Anzali, Tonkabon and Babolsar stations; 37°30′ N; 49°15′ E). Coastal sediments samples were collected in sterile mason jars, wrapped in aluminum foil, placed on ice and immediately transported to the laboratory for further analysis (Alef and Nanniper 1995).

Set-up of the microcosm systems and experimental planning

Four different microcosms were performed in glass tanks (50 cm long, 10 cm deep and 25 cm wide). Sediments were sieved (<2mm) to remove large particles of shells and then 1 kg of sieved sediments were used for each microcosms. Microcosms were incubated in the dark at 25°C for 120 days. The water content of the microcosms were adjusted and maintained at 60 % of its water holding capacity (WHC) during the whole incubation period. Aerobic condition was maintained by mixing of the microcosms content every day (Alef and Nanniper, 1995).

Four microcosms simulated natural and artificially polluted conditions were carried out. In particular, microcosms without oil contamination were used as control and are indicated as Uncontaminated Persian Gulf (UPG) and Uncontaminated Caspian Sea (UCS) respectively.

The other two microcosms were artificially contaminated with Iranian light crude oil (10 g kg^{-1}) and are indicated as Contaminated Persian Gulf (CPG) and Contaminated Caspian Sea (CCS), respectively.

MPN of heterotrophic and hydrocarbon degrading bacteria

Total heterotrophic and hydrocarbon-degrading, were enumerated by a miniaturized Most Probable Number (MPN) method according to Brown and Braddock 1990.

Marine Broth (MB) (Wrenn and Venosa, 1996) and ONR7a media (Dyksterhous et al. 1995) were used for enumeration of total heterotrophic bacteria and hydrocarbon-degrading bacteria, respectively.

Sterile Iranian light crude oil (1 %) was used as a selective growth substrate for the enumeration of hydrocarbon-degrading bacteria.

Sediments samples were diluted in a saline buffer solution that contained 0.1% sodium pyrophosphate (pH 7.5) and 4 % NaCl. Tenfold serials dilution were performed in microplates that were inoculated by adding 20 µL of each dilution to 1 of the 12 row wells. Iranian light crude oil (1%) was applied to the samples as described above. The first row of each plate was served as sterile control. Microplates were incubated at 20 ± 1 °C for 15 days. Two samples (dry weight) from each station were used for MPN count and MPN was carried out as triplicate. MPN counts were performed with the computer program MPN calculator (Klee, 1993; Wrenn and Venosa, 1996).

Extraction of total bacterial DNA from sediments samples

Total DNA of microbial population was extracted from 5 g of dry sediments by using the method described by Zhou et al, (1996) with the following modifications: (i) sediments samples were mixed vigorously with 14 ml of extraction buffer (100 mM Tris-HCl; 100 mM EDTA; 1.5 M NaCl; 1% CTAB, pH = 8) for 1 h at 30°C; (ii) precipitation was carried out with 0.6 volume of isopropanol at room temperature for 240 min.

Extracted DNA was purified with sephadex G-200 columns (Pharmacia) according to the manufacturer's instructions. The quality and quantity of the extracted DNA were checked by UV absorption spectrum measurements (Zhou et al. 1996).

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 GGT GTG TRC -3'). A GC clamp was added to the reverse primer (R). PCR thermo cycling (Eppendorf AG 22331 Hamburg) protocol consisted of: an initial denaturation of 1 min at 94°C; 30 cycle of denaturation of 1 min at 94°C, 1 min of annealing at temperature 53 °C , and elongation of 1 min at 72°C; final extension step of 10 min at 72°C. Reactions were performed in a total volume of 50 µl containing 2 mM $MgCl_2$, 10X PCR reaction buffer (200 mM Tris; 500 mM KCl), 2 mM of each dNTP, 0.15 mM of each primer and 1U 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 h, in 1.0 ×TAE buffer (20 mM Tris, 10 mM acetate, 1 mM EDTA pH 7.4). After electrophoresis, gels were stained for 30 min with SYBR gold nucleic acid gel stain (1:10000 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 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 originated from DGGE profiles (Boon et al., 2002). The clustering algorithm of Ward was used to calculate the dendrograms of each DGGE gel using the software package statistical (ver. 5.1, StatSoft).

Hydrocarbon analysis

The composition of total extracted and resolved hydrocarbons and their derivates (TERHC) was extracted from 10 g of sediments, analysed by high-resolution GC-MS and quantified according to previously described protocols (Dutta et al., 2001; Cappello et al., 2006). The TERHC composition was analysed by high-resolution GC-MS using a Perkin-Elmer Turbo MS Auto System XL GC (Perkin-Elmer Biosystems, Foster City, CA, USA) equipped with a DB-TPH fused silica capillary column [30 m by 0.32 mm (inner diameter); J and W Scientific (Folsom CA, USA)]. The samples were quantified according to previously described protocols (Dutta et al. 2001) and as described by Denaro et al. 2005.

Statistical analysis

All data obtained, in different experimentations, from MPN and DGGE analyses were detected by Analysis of variance (ANOVA) and PAST (PAlaeontological STatistics Software ver. 1.88).

Results

MPN count in microcosm experiments

The results of MPN counts (dry weight) used to estimate the density of total heterotrophic and hydrocarbon-degrader bacteria were shown in Table (1). ONR7a medium was used for enumeration of hydrocarbons degrading bacteria. The number of total heterotrophic bacteria in UPG and CPG microcosms were of the same order of magnitude, i.e, 2.7×10^5 (MPN g^{-1}) and 9.3×10^5 (MPN g^{-1}), respectively. The numbers of hydrocarbon-degraders bacteria in contaminated microcosms were

significantly higher than in uncontaminated microcosms (3.1×10^4 in CPG versus9.3×10^2 in UPG). While the contaminated Persian Gulf microcosm and contaminated Caspian Sea microcosm have the same densities of hydrocarbon degraders.

Total Petroleum Hydrocarbons (TPH) degradation in each Microcosm

Total Petroleum Hydrocarbons (TPH) was detected in each microcosm after incubation period (120 days) by GC-MS. The results were shown in table (2) and figure (1). Comparisons of remaining TPH in sediments of two ecosystems in contaminated and uncontaminated state indicated that Persian Gulf ecosystem have higher contamination than Caspian Sea. Table (2) show that UPG microcosm, natural sediments of Persian Gulf without pollution, have TPH content similar to CCS microcosm, Caspian Sea sediments with dummy pollution. This result may be attributed to chronic contamination of Persian Gulf sediments. As shown in GC-MS graphs (figure 1) total peaks of GC-MS were lower in Caspian Sea microcosms (contaminated or uncontaminated) than Persian Gulf microcosms.

Another result that obtained from GC-MS graphs is the presence of alcoholic and acidic compounds in contaminated sediments in compare to uncontaminated sediments. These compounds were intermediated of crude oil hydrocarbons biodegradation. Also there were some halogenated compounds (Chlor and brom) in sediments. These compounds have high electronegative property thus have high resistance to biodegradation and remain in microcosms after 120 days of incubation.

Effect of crude oil on marine microbial community of microcosms

The response of marine microbial community to oil pollution in sediments of the Persian Gulf and the Caspian Sea microcosms were analyzed by PCR-DGGE method with 16S rRNA genes. The results of these comparisons were shown in table (3) and figure (2).

As shown in table (3) both ecosystems have low biodiversity after oil pollution, in other hand, total bands present in UPG microcosm was 32 and in CPG microcosm was 20, this means that the decrement of 12 phylogenetic groups were take placed after oil contamination. In Caspian Sea microcosms biodiversity was lower than Persian Gulf but the decrement pattern of phylogenetic groups were also seen in these microcosms after oil pollution. As shown in table (3) in UCS microcosm 27 bands present but in CCS microcosm this valve was 19 bands indicated that decrease of 8 phylogenetic groups were disappear after oil contamination.

In according to figure (2) in UCS microcosm (a) and (b) bands were present only before contamination; these bands are equivalent to (e) and (f) bands in Persian Gulf microcosms in uncontaminated situation. In UPG microcosm (d) band were intensive but in CPG microcosm this band were disappeared also g and (h) bands in CPG microcosm have high intensity than UPG microcosm.

These results indicated that oil pollution cause decrease of biodiversity in marine sediments and enriched some specific phylogenetic groups. The reason of this phenomenon can be attributed to toxic effect of crude oil for marine microbial community in sediments thus some sensitive phylogenetic groups were damaged and disappeared but some microorganisms that can utilized crude oil as carbon and energy source remain and dominant in marine microbial community.

Biodiversity indexes

Some important biodiversity factors for each microcosm were calculated using constructed data matrix from DGGE image by PAST software. Results were presented in table (4).

Dominance (D) is the numerical index that variable between 0 and 1, zero number means that all microbial genus present same in microbial community and one number means that single phylum were dominant to all of the microbial community, thus increment of this number in contaminated microcosms indicated that dominance of some microbial genus take placed than other phylums of the microbial community. This index was highest in CPG microcosm.

Shannon (H) index illustrated biodiversity in microbial community and this is number between 0 and 10, thus if this number was larger means that diversity of bacterial species were higher in microbial community. As shown in table (4) uncontaminated microcosms have high diversity in compare to contaminated microcosms. These results indicated that oil pollution kill sensitive bacterial species in microbial community and decrease the biodiversity. The lowest biodiversity belong to CCS and CPG microcosms.

Simpson (1-D) index show the remaining of microbial community except dominance phyla. Thus if this number was larger that means the dominance between microbial community was lower. As shown in table (4) uncontaminated microcosms have lower dominance in compare to contaminated microcosms.

Similarity of marine microbial community between microcosms

Similarity of marine microbial community in sediments was shown with cluster and two dimensional graphs in figure (3). As shown in this figure contaminated microcosms from two ecosystems have more resemble with each other than uncontaminated microcosms from these two ecosystems. For example contaminated sediments from Persian Gulf (CPG) were located in the cluster of contaminated sediments of Caspian Sea (CCS). This pattern also coordinates for uncontaminated microcosms.

Statistical analysis of marine microbial community in microcosms sediments

Data matrix from DGGE gel image was constructed as presence and absence of individual bands that devoted number 1 and 0 to presence and absence respectively. Data matrix was analysis by PAST software and statistical factors was calculated. Table (5) shows the results of this analysis for each microcosm. The low value for standard error in this table confirmed the accuracy performance of DGGE method.

Discussion

The marine environment is subject to contamination by organic pollutants from a variety of sources. Organic contamination results from uncontrolled releases from manufacturing and refining installations, spillages during transportation, direct discharge from effluent treatment plants and run-off from terrestrial sources (Hassanshahian et al. 2012a; Hassanshahian et al. 2012b; Cappello et al. 2012a).

In quantitative terms, crude oil is one of the most important organic pollutants in marine environments (Hassanshahian and Emtiazi, 2008; Cappello et al. 2012b). Ecological effects of oil pollution on marine sediments illustrated that this pollution changed composition and diversity of microbial community and have some effects on activity of microorganisms and enzymes in the sediments (Caruso et al. 2004; Emtiazi et al. 2005). Microbial diversity in the sediments is a good indicator for evaluation the scale of

ecosystem perturbation with oil pollution. Many researchers evaluated the effects of petroleum hydrocarbons on marine microbial diversity in the sediments (Caruso et al. 2004).

Bacterial community inhabiting the sediments can adapt to oil contamination; such as oil pollution induced the enrichment of specific degrader as shown in other studies (Heissblanquet et al. 2005; Li hui et al. 2007). Delille and Delille, (2000) found that after 3 months of contamination heterotrophic bacteria numbers were of the same order of magnitude as the initial numbers. In contrast with total heterotrophic bacteria counts, oil contamination induced obvious increase of the number of hydrocarbon degrading bacteria. Similar results were obtained in this study.

The application of molecular biological techniques to detect and identify microorganisms by certain molecular markers, such as 16S rRNA or its encoding gene (Olsen et al. 1986; Amann et al. 1995), is now more and more frequently used to explore the microbial diversity and to analyses the structure of microbial communities (Muyzer and Ramsing, 1995). Genetic fingerprinting techniques provide a pattern or profile of the genetic diversity in a microbial community. One of the fingerprinting techniques that have been used in microbial ecology for more than a decade is the DGGE method.

DGGE of PCR amplified 16S rDNA fragments were first used to profile community complexity of a microbial mat and bacterial biofilms (Muyzer et al. 1993). Many researchers used PCR-DGGE for analysis the effect of pollutant on microbial community.

Andreoni et al (2004) used PCR-DGGE for study the biodiversity in three soils that contaminated with different concentrations of Poly Aromatic Hydrocarbons (PAH). Their results showed that soils with medium contaminations have high biodiversity and Shannon index (H) for these soils types were 2.87. Although soils with high contamination have low biodiversity and Shannon index were 0.85.

Li et al (2007) applied PCR-DGGE for study the response of marine sediments to diesel fuel contamination. Their results indicated that low contamination with diesel fuel have negligible effect on biodiversity of marine microbial community, however high level of contamination with diesel fuel were decrease dramatically biodiversity in marine sediments.

Watanabe et al (2000) study the microbial community in oil reservoirs by PCR-DGGE and they concluded that after oil pollution biodiversity in the reservoirs decrease and specific groups of microorganisms were prevalent.

In the present research marine microbial community from the Persian Gulf and the Caspian Sea in contaminated and uncontaminated states were investigated by PCR-DGGE method. Consistent with the results of other researchers, our data indicate that the bacterial biodiversity decreases in oil-contaminated compared to uncontaminated areas of an ecosystem. We found that different ecosystems have resemble in microbial community after contamination, as contaminated Persian Gulf microcosm has similarity to contaminated Caspian Sea microcosm. These results indicated that some specific phyla were predominant in contaminated environments after oil pollution.

Conclusion

The uses of molecular methods in microbial ecology were increased in the last decades. We use PCR-DGGE as a molecular fingerprinting method for study the effect of crude oil on marine microbial community. Our results showed that crude oil induce major shifts in the composition and diversity of

marine microbial community in the sediments. Crude oil contamination decrease dramatically biodiversity in the sediments that confirmed by disappear of bands in the DGGE gel image.

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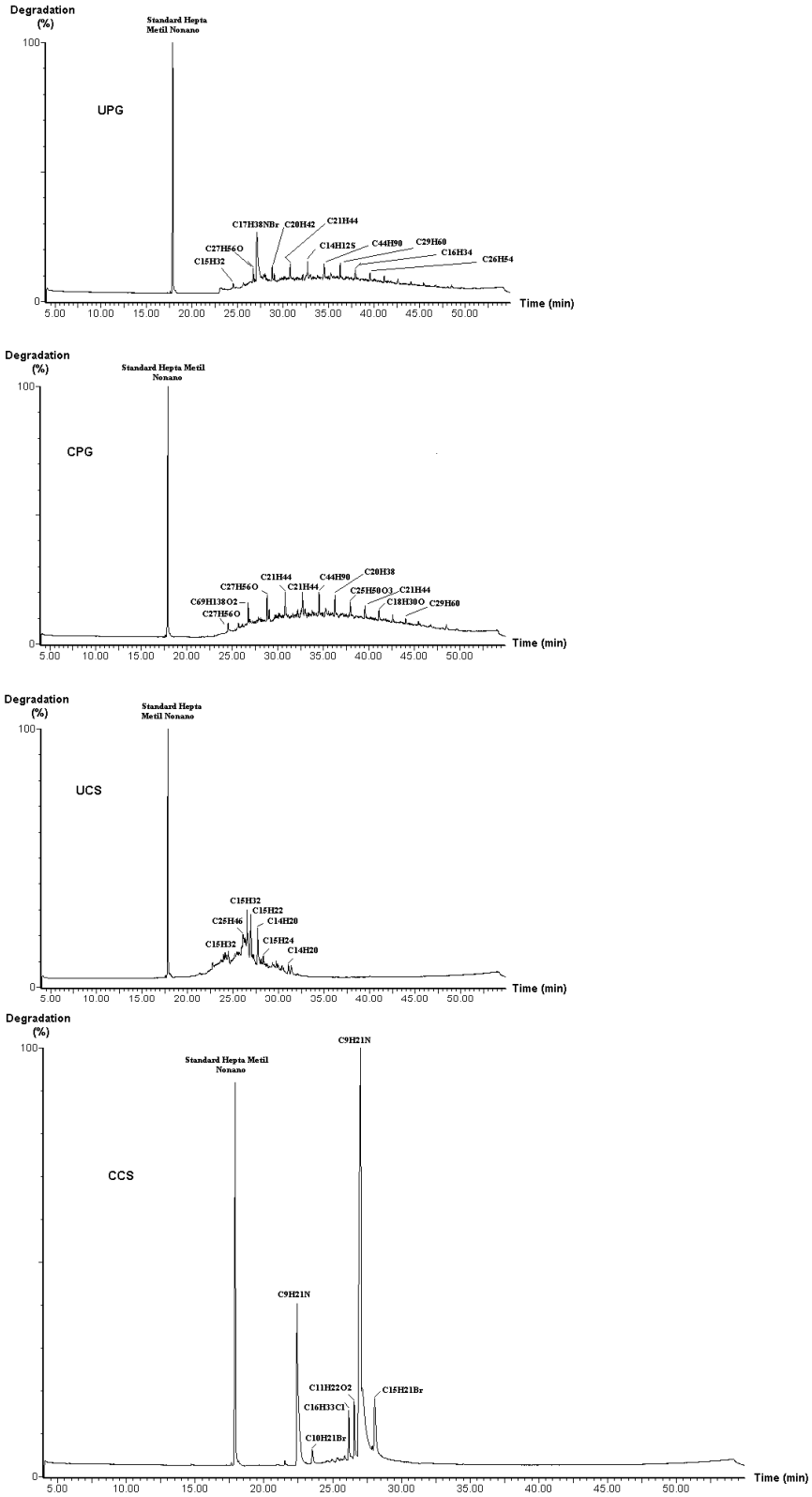

Figure1. The GC-MS chromatographs of crude oil degradation in microcosms after 120 days.

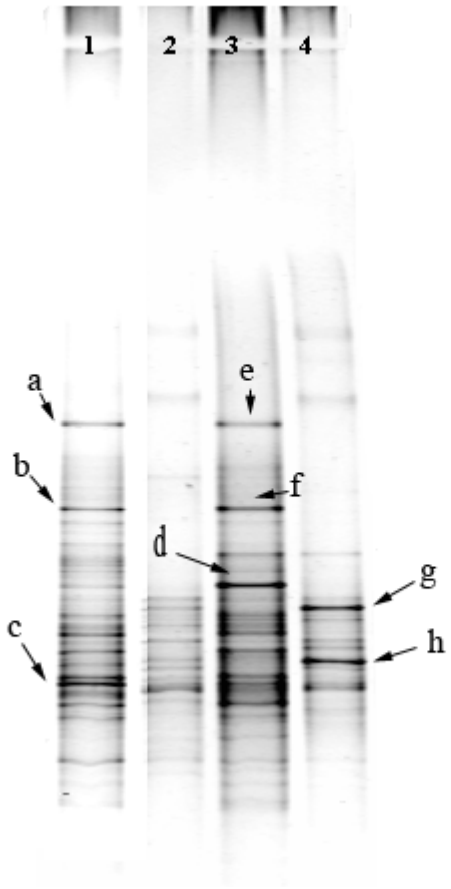

Figure2. DGGE analysis of PCR-amplified bacterial 16S rRNA gene fragments derived from DNA samples taken from microcosms. Lane (1): UCS microcosm (Uncontaminated Caspian Sea); Lane (2): CCS microcosm (Contaminated Caspian Sea); Lane (3): UPG (Uncontaminated Persian Gulf); Lane (4): CPG microcosm (Contaminated Persian Gulf).

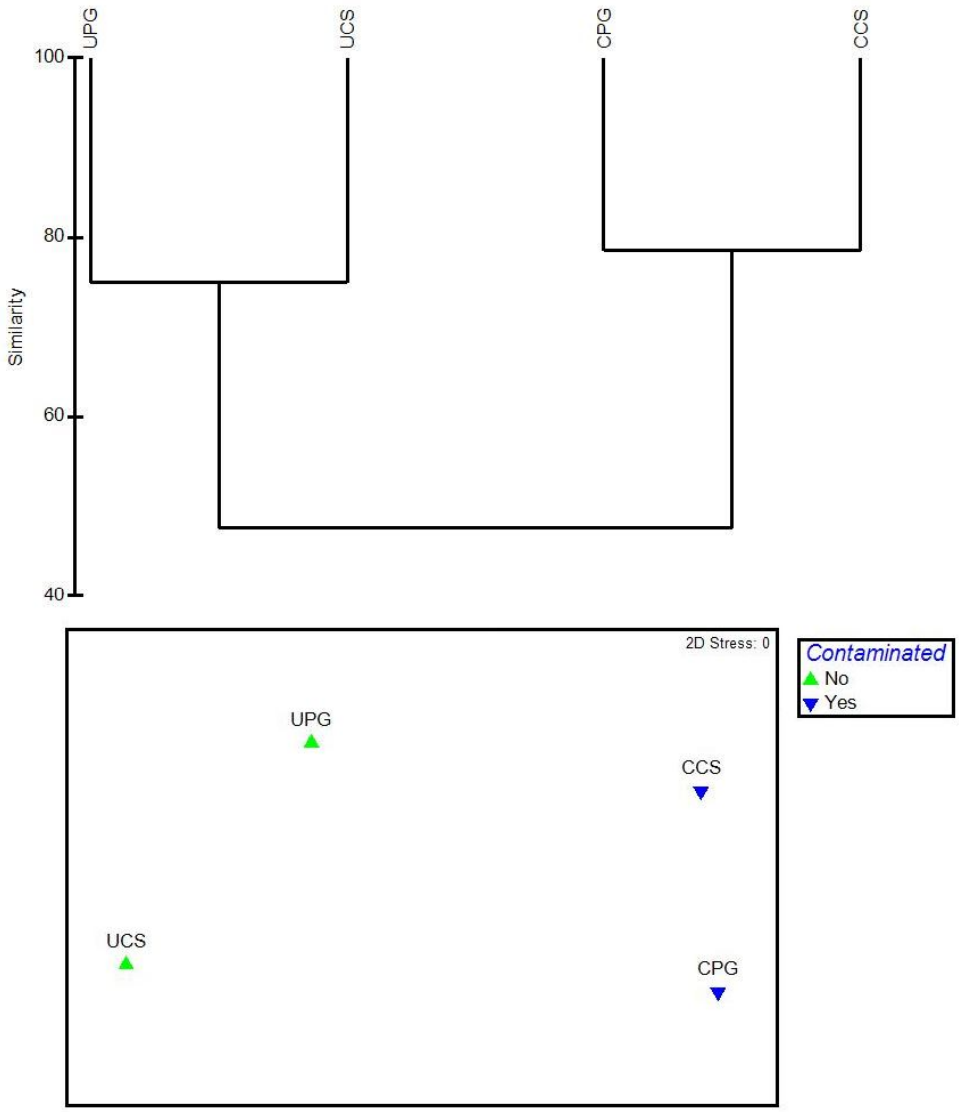

Figure3. The cluster and two dimensional graphs of community similarity in microcosms. These graphs were designed by PAST software.

Table 1. Measures of MPN (MPN g^{-1} dry weight) of hydrocarbon-degrading and total heterotrophic bacteria in the contaminated and uncontaminated microcosms. Two samples from each microcosm were used for MPN count and MPN carried out as triplicate. Abbreviations used: UPG (Uncontaminated Persian Gulf); CPG (Contaminated Persian Gulf); UCS (Uncontaminated Caspian Sea) and CCS (Contaminated Caspian Sea).

MPN / **Experiment** **EXPERIMENT**	Hydrocarbon-degrading bacteria (BH medium)	Hydrocarbonoclastic bacteria (ONR7a medium)	Total heterotrophic bacteria (TSB medium)
Microcosm UPG	2.7×10^2	9.3×10^2	2.7×10^5
Microcosm CPG	9.1×10^3	3.1×10^4	9.3×10^5
Microcosm UCS	1.3×10^2	5.3×10^2	1.2×10^5
Microcosm CCS	1.2×10^3	1.1×10^4	2×10^5

Table 2. Total Petroleum Hydrocarbon (TPH) extracted from sediments in each microcosms (ng g^{-1}). Remaining crude oil was extracted from each microcosm and analysis by GC-MS then calculation performed and TPH obtained. Abbreviations used: UPG (Uncontaminated Persian Gulf); CPG (Contaminated Persian Gulf); UCS (Uncontaminated Caspian Sea) and CCS (Contaminated Caspian Sea).

Microcosm Type	TPH (ng g^{-1})
UPG	12.6
CPG	15.9
UCS	9.4
CCS	13.2

Table 3. Total bands present in each microcosm. DGGE gel image were analyzed by Image Quant (ver. 5.2) software. Abbreviations used: UPG (Uncontaminated Persian Gulf); CPG (Contaminated Persian Gulf); UCS (Uncontaminated Caspian Sea) and CCS (Contaminated Caspian Sea).

Microcosm Type	Total band	Low density band	High density band
UPG	32	17	15
CPG	20	7	13
UCS	27	14	13
CCS	19	8	11

Table 4**.** Biodiversity indexes in each microcosm. These data calculated in PAST software after analysis of data matrix.

Microcosm Character	UPG	CPG	UCS	CCS
Dominance (D)	0.03704	0.08143	0.03448	0.07143
Shannon (H)	3.567	2.739	3.367	2.639
Simpson (1-D)	0.963	0.91857	0.9655	0.9286

Table 5. Statistical analysis of each microcosm obtained by PAST software.

Microcosm / Character	UPG	CPG	UCS	CCS
Total phylogenetic group	32	20	27	19
Mean	0.783	0.398	0.729	0.378
Standard Error	0.0686	0.0827	0.0740	0.0808
Variance	0.1741	0.2517	0.2027	0.2417
Standard deviation	0.417	0.502	0.450	0.491