The succession of Dominant Culturable Hydrocarbon-Utilizing Bacteria During Bioremediation of Oil-Based Drilling Waste

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ABSTRACT: *Drilling operations of petroleum generate oily wastes. The disposal of a significant amount of oil-based drill muds has caused soil contamination and critical environmental impacts in the last decades. The current study aimed to investigate the potential of microbial remediation for an aged oil-based drilling waste and to monitor the fluctuation in microbial population throughout a 60-day microcosm experiment. A representative aged oil-based drilling waste sample was obtained randomly from a contaminated mud pit in the Khangiran district, Iran. Respiration measurement was performed according to the method described by standard ISO 17155. Total Petroleum Hydrocarbon (TPH) was measured by the gravimetric method. Microbial counts were measured at 10-day intervals during 60 days of incubation. Total heterotrophic bacteria were enumerated by standard plate count using R_2A agar. Dominant heterotrophic and hydrocarbon-utilizing bacteria were selected for phylogenetic analysis. Statistical analyses of the experimental data, using one-way ANOVA were performed using Minitab 16. Following the biostimulation of the contaminated soil, both heterotrophic and hydrocarbon-utilizing bacterial counts increased to above three orders of magnitude in less than 20 days. The highest respiration level and hydrocarbon degradation efficiency were correlated and measured between the 10th and 20th days of the experiment to be 70.7 µg/g.soil.h and 23.13% respectively. Phylogenetic analyses indicated that the members of Actinobacteria (Georgenia, Brevibacterium, Micromonospora, and Streptomyces) were the major hydrocarbon-utilizing bacteria in the microcosm, among which the species of genus Georgenia were dominant throughout the experiments. Furthermore, the population of Alcanivorax species increased promptly and thrived in the microcosm during the active bioremediation phase which indicated their vital role for remediation of diesel range hydrocarbons in saline environments. In an overall view, elegant diversity of hydrocarbon utilizing bacteria along with the accomplished TPH removal efficiency of 45.4% (w/w) in the microcosms, confirmed the potential of indigenous microorganisms for bioremediation of the aged oil-based drilling waste.*

KEYWORDS: *Alcanivorax; Bioremediation; Georgenia; Hydrocarbon-utilizing bacteria; Oil-based drilling waste.*

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INTRODUCTION

During oil and gas production activities, the seepage of hydrocarbons occurs occasionally due to accidents or process failures [1]. For example, large amounts of oil-based wastes are generated during drilling in the early stages of oil and gas production [2]. The inappropriate disposal of this high amount of hazardous wastes has led to serious contamination of natural ecosystems [3, 4]. Therefore, the management of drilling wastes has been focused in the industry by applying physical, chemical, and biological methods and various technologies have been exploited including solidification and stabilization, thermal and microwave technologies, extraction, phytoremediation, and biodegradation [4, 5].

Among various methods, bioremediation is a cost-effective and environmentally approved cleanup technology for the treatment of organic pollutants especially petroleum hydrocarbons [6]. In previous studies on drilling waste bioremediation, the effects of nutrient, surfactant, and H_2O_2 [7], agroindustrial wastes [8], bulking agent [9, 10], water content [8, 11], nitrogen fertilizer [12], biostimulation and bioaugmentation [10, 13-15], Kitchen Effluent [16], C:N:P ratio [11, 17-19] and combined plant-microbe System [20] have been investigated. Also, various strategies including composting, biopiling, and slurry bioreactors were appraised for drilling waste bioremediation [17].

The use of respirometry provides valuable information about microbial activity in the bioremediation process [5]. This method has been used in the monitoring of crude oil contaminated soil [21, 22] and also diesel, bitumen, and petroleum-contaminated slurries [23]. To the knowledge of the authors, there is no report on the use of the soil respirometry technique in monitoring bioremediation of oil-based drilling waste.

Nevertheless, scarce information is available concerning the microbiology of drilling wastes bioremediation which plays the main role in the process. *Nnubia* and *Okpokwasili* [24] isolated *bacillus* and *Staphylococcus* from mud cuttings as drilling-fluid-utilizing bacteria. Similarly *Benka-Coker* and *Olumagin* [25] isolated *Serratia*, *Staphylococcus*, *Acinetobacter*, and *Alcaligenes* from drilling wastes. *Chaineau et al.* [26] isolated and identified several hydrocarbons utilizing bacteria including *Pseudomonas*, *Micrococcus*, *Xanthomonas*, *Acinetobacter*, *Flavobacterium*, *Agrobacterium*, *Rhodococcus*, and *Arthrobacter* in oil-based drilling waste bioremediation experiment. The study of *Steliga et al.* [13] also indicated that aged drilling waste (about 70 years old) contains diverse genera of hydrocarbon utilizing bacteria including *Acinetobacter*, *Agrobacterium*, *Bacillus*, *Gordonia*, *Micrococcus*, *Klebsiella*, *Mycobacterium*, *Nocardia*, *Rhizobium*, *Rhodococcus*, *Pseudomonas*, *Flavobacterium* and *Streptomyces*.

In a similar work, a hydrocarbon-utilizing microbial consortium including *Bacillus*, *Klebsiella*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Rhizobium*, *Rhodococcus*, and *Pseudomonas* was enriched from a drilling waste pit [27]. It has been shown that *Bacillus* species isolated from drilling waste have a promising capacity in bioremediation of drilling oily waste [28]. In a recent study, *Napp et al.* [29] showed that diverse hydrocarbon-utilizing bacteria were prominent in an enriched oil-drilling waste. The most abundant genera were *Pseudomonas*, *Bacillus*, *Bordetella*, *Achromobacter*, *Stenotrophomonas*, *Pantoea*, *Brevundimonas*, and *Xanthomonas*.

Although there are many researches on the bacterial community succession associated with bioremediation of hydrocarbon-contaminated environments [30-32], little is known about microbiology and bacterial succession in oil-based drilling waste bioremediation. In this study, the potential of biological remediation of an aged oil-based drilling waste was investigated and the succession of dominant culturable hydrocarbon-utilizing bacteria was monitored in microcosms. The applicability of respirometry in the monitoring of drilling wastes bioremediation was investigated and new strains with high capacity in the process were introduced.

EXPERIMENTAL SECTION

Sample characterization

A representative aged oil-based drilling waste sample was obtained by random sampling from a contaminated mud pit in the Khangiran district, Northeast of Iran. The sample was sieved to remove large particles and well mixed before physical and chemical analysis. The contaminated sample had a sandy texture with pH 7.6. Based on primary investigations, 250 ppm industrial nitrogen source (in the form of urea) was added to the sample to improve the sample texture and microbial activity.

Soil respirometry

Respiration measurement was performed according to the method described by standard ISO 17155. The method was based on the measurement of the evolved carbon dioxide (CO_2) by the microorganisms. The respiration analysis system is shown in Fig. 1. To carry out this test, approximately 500 g of homogenized soil samples were placed in the reactor vessels. Aeration by a CO_2-free stream was performed from the bottom of the reactor at a flow rate of 0.1 L/min. Carbon dioxide content in the outlet of the reactor was measured by a carbon dioxide analyzer (TES 1370 NDIR CO_2 Meter). The analysis was performed in ambient temperature (25±5° C) for 60 days and sample moisture content was kept around 15% during the test. The average results of carbon dioxide evolution in vessels were represented as the mass (µg) of CO_2 per gram of dry soil per hour.

Hydrocarbon analysis

Representative soil samples were obtained from microcosm at 10-day intervals. Soil was extracted by n-hexane in the soxhlet apparatus as described by standard method 5520F. Following polar compounds clean-up through the Florisil filter, Total Petroleum Hydrocarbon (TPH) was measured by the gravimetric Method [33].

To determine the carbon distribution of extracted hydrocarbons, simulated-distillation GC was used. Based on ASTM D2887 [34], a portion of the extracted hydrocarbons was injected into a CPC sil8/CB capillary column (length, 25 m) in an Agilent gas chromatograph equipped with FID detector. GC temperature program started at -70°C and reached 425 °C by a ramp rate of 15 °C/min and kept there for 1 minute.

Monitoring microbial population

Microbial counts were measured at 10-day intervals during 60 days of incubation. Total heterotrophic bacteria were enumerated by standard plate count using R2A agar. The inoculated plates were incubated at 30° C for 7 days. The bacterial population was enumerated and presented as CFU/g of sample. Bushnell-Haas broth amended with 0.5% NaCl (w/v) and 2 mL/L Gasoil was used for enumeration and isolation of hydrocarbon-utilizing bacteria. Hydrocarbon-utilizing bacteria were enumerated by the three-tube Most-Probable Number (MPN) method [35]. The inoculated tubes were incubated at 30 °C for

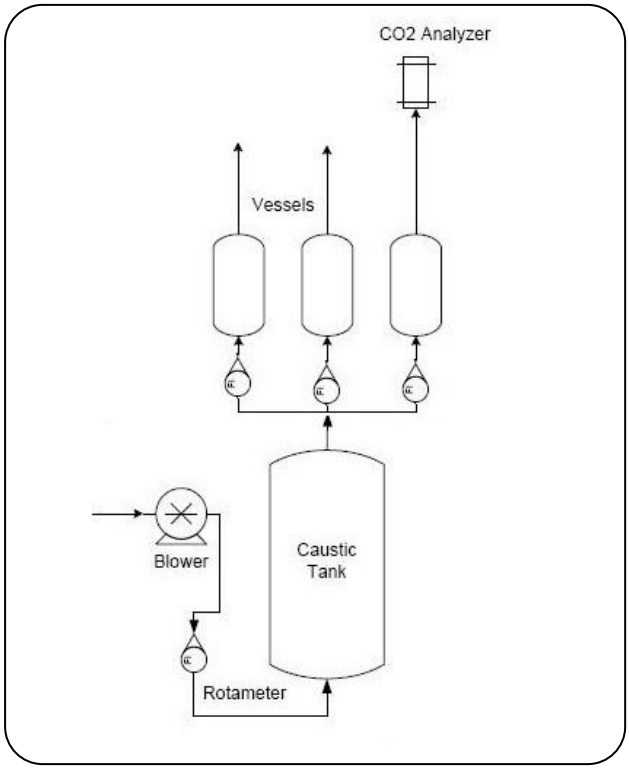

Fig. 1: Schematic illustration of the respiration analysis system.

1 month and bacterial growth was monitored by turbidity survey in comparison with un-inoculated controls. The MPN index was determined from statistical tables published by the U.S. Food and Drug Administration [36]. For isolating dominant hydrocarbon-utilizing bacteria, the highest positive dilutions were streaked on R2A agar plates and distinct colonies were purified by successive cultivations. To verify the hydrocarbon utilization the ability of the isolates, pure cultures were grown in minimal media amended with 2 mL/L Gasoil as the sole carbon source.

Phylogenetic analysis

Dominant heterotrophic and hydrocarbon-utilizing bacteria at days 0, 20, and 60, were selected for phylogenetic analysis. Genomic DNA's of the selected bacteria were extracted according to *Wilson* [37]. 16s rRNA genes were amplified using 9F (5' -AAGAGTTTGATCATGGCTCAG-3 ') and 1541R (5 ' -AGGAGGTGATCCAACCGCA-3 ') universal primers. The 16S rRNA gene sequences of hydrocarbon-utilizing bacteria were obtained and used for phylogenetic analysis. The identification of phylogenetic neighbors

and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; [38]). MEGA5 software was used for phylogenetic analyses [39] and the ClustalW algorithm of this software was used for sequence alignments. The neighbor-joining method in the MEGA5 software was used to construct a phylogenetic tree.

Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences of isolates have been deposited in the GenBank database under accession no. KP337604 through KP337611.

Statistical analysis

Statistical analyses of the experimental data, using one-way ANOVA (analysis of variance) were performed using Minitab 16. This parametric test compares the means of two or more independent groups to determine whether there is statistical evidence that the associated population means are significantly different. Statistical significance between samples was determined at $P<0.05$.

RESULTS AND DISCUSSION

Bioremediation efficacy and microbial activity of the consortium

As shown in Fig. 2, after starting the bioremediation process, both heterotrophic and hydrocarbon-utilizing bacterial counts increased significantly ($p<0.05$). Both groups reached maximum counts on day 20 and their count increased 3 orders of magnitude between day 0 and day 20. In this period, culturable heterotrophic bacterial count raised from 3.1×10^6 to 4×10^9 CFU/g. The population of culturable hydrocarbon-utilizing bacteria increased rapidly from 1.5×10^3 to 7.8×10^6 CFU/g during the first 20 days of treatment, and simultaneously, the highest rate of hydrocarbon degradation($p<0.05$) occurred during this period (Fig. 2).

The contaminated soil contained 26.5 g/kg of total petroleum hydrocarbon. Based on simulated distillation, the carbon distribution analysis showed that at the beginning of the process the hydrocarbons in the soil ranged from C_{10} to C_{30} with C_{22} constituting the highest peak (Fig. 3). After 60 days remained hydrocarbons in the soil ranged from C_{15} to C_{30} with C_{22} constituting the highest peak (Fig. 3). As shown in Fig. 2 the partial TPH removal efficiency was 45.4% after 60 days.

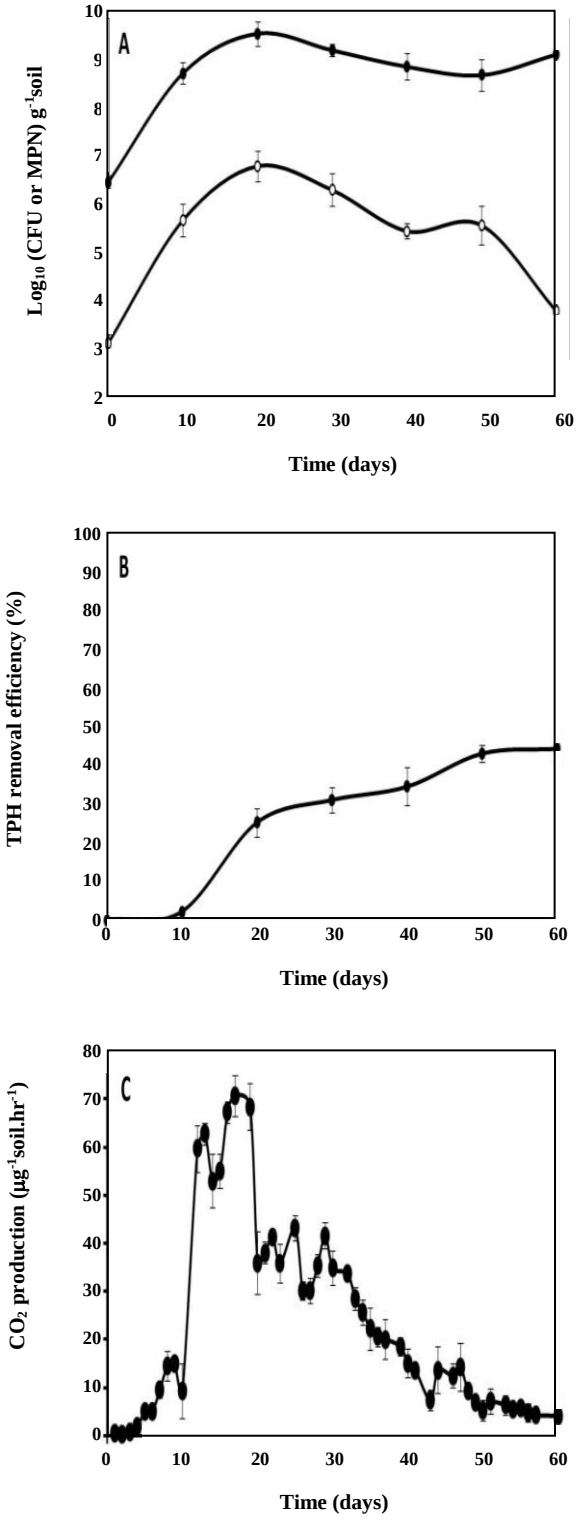

Fig. 2: Heterotrophic (closed circle) and hydrocarbon-utilizing bacterial count (open circle) (A); TPH removal efficiency (B) and respiration curve of the microcosm (C) during 60 days of the experiment.

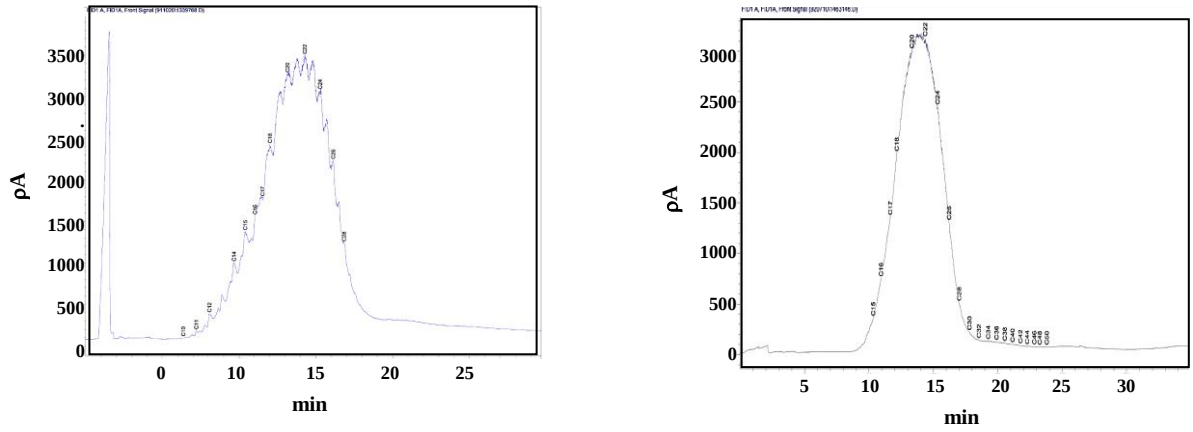

Fig. 3: Simulated distillation of hydrocarbons in the beginning (A) and after 60 days (B) of process.

CO_2 production of microcosms was expressed as µg/g.soil.h (Fig. 2). In the initial 4 days of the process, respiration rate was low and hardly detectable however the respiration rate increased henceforth and maximal respiration rate occurred between days 10 to 20 which correlated well with bacterial count and TPH degradation ($p<0.05$). Following this period, the respiration rate decreased, by total bacterial count.

Phylogenetic analysis of hydrocarbon-utilizing bacteria

Hydrocarbon-utilizing bacteria were isolated from microcosms and phylogenetically analyzed at three stages; beginning (day 0), active bioremediation phase (day 20), and the end of the process (day 60). As shown in Table 1, 11 dominant hydrocarbon-utilizing bacteria were isolated (5 in day 0, 4 in day 20, and 2 in day 60). At the beginning of the process, *Actinobacteria* (genera *Georgenia*, *Brevibacterium* and *Streptomyces*) and *Alphaproteobacteria* (genus *Mesorhizobium*) were the dominant hydrocarbon-utilizing bacteria. On day 20, *Gammaproteobacteria* (genus *Alcanivorax*) became dominant. Similar to day 0, at this time *Actinobacteria* (genera *Georgenia* and *Brevibacterium*) were also prevalent. At the final stages of the remediation process, only *Actinobacteria* (genera *Georgenia* and *Micromonospora*) were dominant. In Fig. 4, the phylogenetic tree based on 16S rRNA gene sequences shows the position of dominant hydrocarbon utilizing bacteria and closely related species of the related genera.

Discussion

The prepared microcosms were composed of drilling waste and sandy soil and encompassed a trivial quantity of hydrocarbon-utilizing bacteria. However following the startup of the bioremediation process, the population of both heterotrophic and hydrocarbon-utilizing bacteria increased remarkably by 3 orders of magnitude and reached the highest amount in 20 days.

Noticeably the maximal respiration rate (70.7 µg/g.h) and TPH degradation occurred between days 10 to 20 ($p<0.05$) accounting for 23.13% of TPH removal. By consumption of readily available hydrocarbon during In the active phase, both respiration and hydrocarbon utilizing microbial counts decreased to finally reach a state comparable to the primary conditions of the microcosms. The correlation between respiration rate, bacterial count and TPH analyses, showed the usefulness of respirometry in the monitoring of bioremediation of oil-based drilling wastes which has been shown in other situations such as crude oil contaminated soil [21, 22]; diesel, bitumen, and petroleum-contaminated soil slurries [23].

The results indicated that the existing microorganisms had the adequate potential for bioremediation of aged oil-based drilling waste resulting in a TPH removal efficiency of 45.4% during 60 days. In the study of *Steliga et al.* [13] about 25% of TPH in drilling waste was removed after 8 weeks of bioremediation but this efficacy could be enhanced up to 90% after 20 weeks by bioaugmentation with a bacterial consortium. The investigation of *Alavi et al.* [16] showed that TPH

Table 1: Dominant culturable hydrocarbon utilizing bacteria.

Strain	Isolation Time (Day)	GenBank accession number	Most Related Strain	GenBank accession number	Similarity (%)	Taxonomical position of isolate
A	0	KP337604	*Georgenia sediminis* SCSIO 15020	JX555983	97.98	*Actinobacteria, Actinobacteria, Micrococcales, Bogoriellaceae*
Q	0	KP337605	*Georgenia muralis* 1A-C	X94155	100	*Actinobacteria, Actinobacteria, Micrococcales, Bogoriellaceae*
D	0	KP337606	*Mesorhizobium albiziae* CCBAU 61158	NR043549	99.42	*Proteobacteria, Alphaproteobacteria, Rhizobiales, Phyllobacteriaceae*
P	0	KP337607	*Brevibacterium epidermidis* NCDO 2286	X76565	99.86	*Actinobacteria, Actinobacteria, Micrococcales, Brevibacteriaceae*
H	0	KP337608	*Streptomyces griseoplanus* NBRC 12779	AB184138	98.96	*Actinobacteria, Actinobacteria, Streptomycetales, Streptomycetaceae*
B	20	KP337609	*Alcanivorax dieselolei* B5	NR074734	99.43	*Proteobacteria,Gammaproteobacteria, Oceanospirillales, Alcanivoracaceae*
K	20	KP337610	*Alcanivorax marinus* R8-12	KC415169	99.60	*Proteobacteria,Gammaproteobacteria, Oceanospirillales, Alcanivoracaceae*
I	20	KP337605	*Georgenia muralis* 1A-C	X94155	100	*Actinobacteria, Actinobacteria, Micrococcales, Bogoriellaceae*
F	20	KP337607	*Brevibacterium epidermidis* NCDO 2286	X76565	99.86	*Actinobacteria, Actinobacteria, Micrococcales, Brevibacteriaceae*
E	60	KP337604	*Georgenia sediminis* SCSIO 15020	JX555983	97.98	*Actinobacteria, Actinobacteria, Micrococcales, Bogoriellaceae*
M	60	KP337611	*Micromonospora aurantiaca* ATCC 27029	CP002162	100	*Actinobacteria, Actinobacteria, Micromonosporales, Micromonosporaceae*

removal efficiency in drilling waste bioremediation could be as high as 90% in 21 days by using a slurry bioreactor. However, the slurry bioreactor could not be economically feasible, in cases encountering a high volume of contaminated soil. The study of *Ma et al.* [10] showed that TPH removal in bioremediation of oil-field drilling waste was between 30 to 80% in biopiles with different treatments after 60 days.

A vivid succession of bacteria occurred throughout the process in the microcosm. As expected, *Actinobacteria* which are well described for the decomposition of organic matter in the soil media [40] played a central role in the studied microcosm. Similarly, studies of *Steliga* [27] and *Stelgia et al.* [11] showed the importance of *Actinobacteria* in drilling waste bioremediation. Phylogenetic analyses indicated that members of this group especially genus *Georgenia* were thriving as dominant hydrocarbon-utilizing bacteria in the microcosms throughout the experiment. The *Actinobacteria* comprise a morphologically and physiologically diverse class of microorganisms and approximately 220 genera of *Actinobacteria* have been reported so far to degrade hydrocarbons [40], however, few studies have demonstrated the capability of *Georgenia* in hydrocarbon degradation. *Georgenia* spp. had been found among halophilic hydrocarbon-utilizing

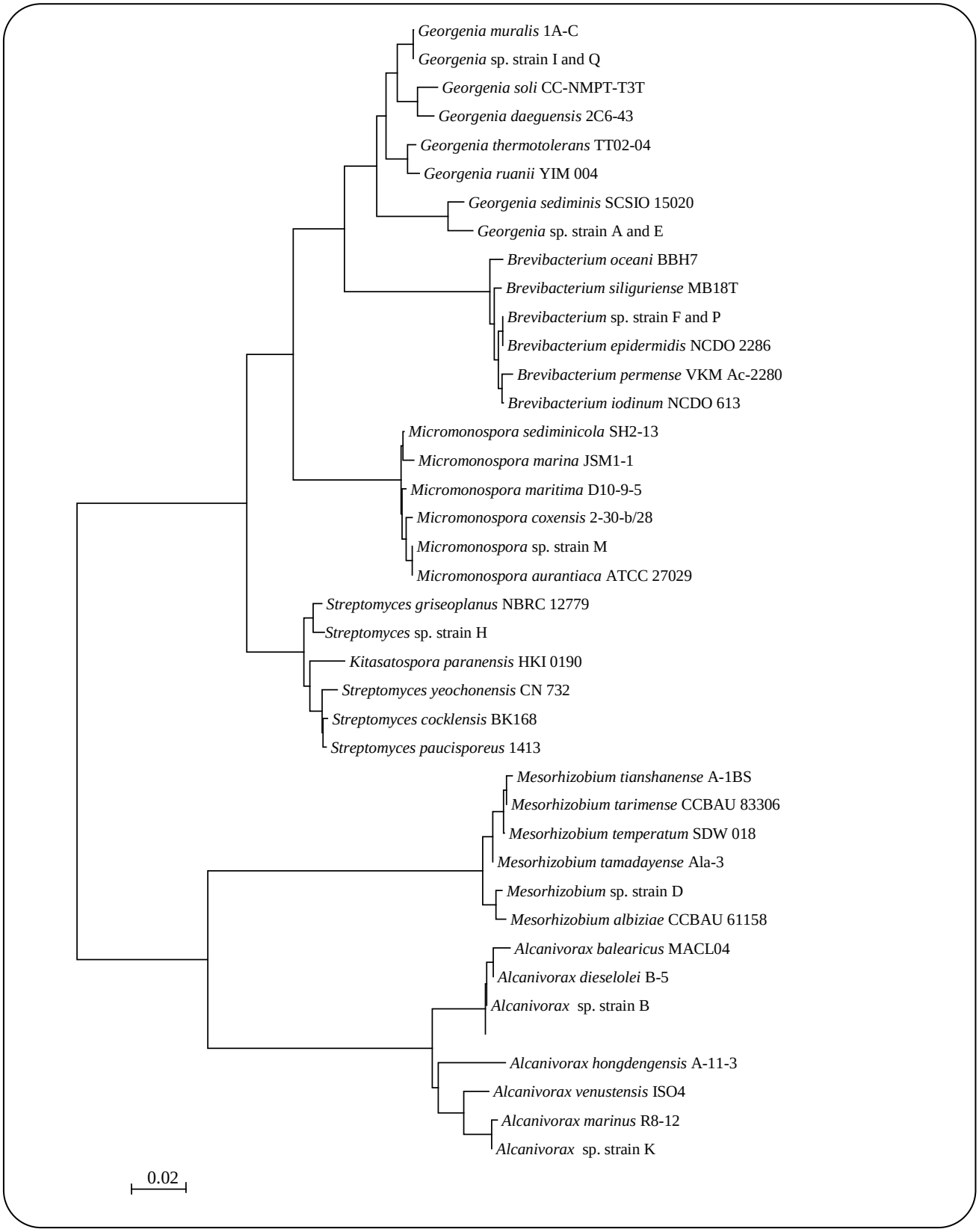

Fig. 4: Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of dominant hydrocarbon utilizing bacteria and closely related species of the related genera. Bar, 0.02 substitutions per nucleotide position.

bacteria from Kuwaiti coasts of the Persian Gulf [41] and had been demonstrated by *Pizarro-Tobías et al.* [42] to be involved in petroleum-hydrocarbon removal in a field study. On the other hand, two other genera of *actinobacteria* namely *Streptomyces* and *Brevibacterium* were also prevailing in the prepared microcosm, whose capability to degrade different types of hydrocarbon have been copiously reported [40].

Considering the bloom of the hydrocarbon degraders' population, the prevalence of *Brevibacterium* and *Georgenia* strains in the active bioremediation phase confirmed that they were capable of adapting and playing a vital role during bioremediation.

Genus *Mesorhizobium*, a member of *Alphaproteobacteria*, was another prevailing hydrocarbon-utilizing bacterium in the beginning of the process. Although Hydrocarbon degradation capability of Genus *Mesorhizobium* was indicated in previous studies [43, 44], it was not detected in the subsequent stages of bioremediation in the microcosms.

Prominently, through activation by aeration and nutrient amendment, the microbial population shifted to *Gammaproteobacteria*, especially genus *Alcanivorax*, in the active bioremediation phase. *Alcanivorax* is a well-known marine bacterium cable of growing efficiently on a restricted spectrum of substrates, predominantly linear and branched alkanes [40]. In the studied microcosms, both diesel range of hydrocarbons and moderate salinity were appropriate for the growth and activity of *Alcanivorax* species, and consequently, they became dominant in the active phase of the bioremediation process. Although the pivotal role of *Alcanivorax* genus has been fully established in hydrocarbon biodegradation in marine environments and coastal areas [40, 45-49], a few reports have focused on its presence and activity in terrestrial environments.

Previously, *Dastgheib et al.* [50] showed the potential of a halotolerant *Alcanivorax dieselolei* for augmentation of a saline soil contaminated by drilling fluid. The current study revealed that even though *Alcanivorax* species were not prevalent at the beginning of the remediation process, they could rise to an important constituent of the microbial community during the active degradation phase and their population would diminish at the final stage of bioremediation. Another notable phenomenon observed in the succession of hydrocarbon degraders after the active phase was that *Georgenia* sp. strain A restored its dominant state in the microbial community accompanying with another *actinobacterium* named *Micromonospora* sp. strain M. This could highlight the role of *actinobacteria* specially *Georgenia* species in attenuating hydrocarbon contamination and soil recycling in natural conditions. It should be noted that different drilling wastes may have different chemical composition leading into various groups of dominant hydrocarbon-utilizing bacteria. As an indication for this, in our study similar to the reports of *Steliga* [27] and *Stelgia et al.* [11], the genera belonging to *Actinobacteria* were dominant in microcosms, which was in contrast with the results of *Napp et al.* [29] in which *Proteobacteria* were prevailing.

All dominant isolates including *Alcanivorax*, *Georgenia*, *Brevibacterium*, *Streptomyces* [40] *Micromonospora* [51, 52], and *Mesorhizobium* [43, 44] were known hydrocarbon degraders. Since fresh oil-based drilling waste does not seem to have adequate active microbial flora, these hydrocarbon utilizing bacteria could be originated from the soil. Since bioaugmentation could be a promising approach for drilling fluid bioremediation [14], isolated strains in the present study are good candidates for augmentation in fresh oil-based drilling wastes bioremediation.

CONCLUSIONS

Although there are much research works on the bacterial community succession associated with bioremediation of hydrocarbon contaminated environments [30-32], little is known about bacterial succession in oil-based drilling waste bioremediation. In this study, the succession of dominant culturable hydrocarbon-utilizing bacteria in oil-based drilling waste bioremediation process investigated in microcosm experiments. The result showed that the soil sample encompassed potent microbial flora and biological treatment was promising for the cleanup of the oil-based mud contamination.

Following biostimulation, a rapid uprising in the population of hydrocarbon degraders occurred during the first twenty days of the bioremediation process which resulted in a sharp peak in the respiration level, along with a pronounced biodegradation rate of hydrocarbon. In the course of this critical period, *Gammaproteobacteria* especially genus *Alcanivorax* were dominant in the microcosm. We assume that *Alcanivorax* is one of

the key hydrocarbon-utilizing bacteria in active phase of hydrocarbon bioremediation in salt-containing terrestrial environments which could be greatly promising for cleanup of oil-based drilling waste.

The obtained results also emphasized the role of *Actinobacteria* in hydrocarbon degradation throughout the remediation process following previous studies, however, on the other hand, suggested that the importance of the genus *Georgenia* in the bioremediation was greater than previously established. Further studies by molecular techniques are on the way to elucidate the source and fate of the determined key bacterial species during bioremediation of a drilling waste contaminated soil.

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REFERENCES

[1] Das N., Chandran P., Microbial Degradation of Petroleum Hydrocarbon Contaminants: an Overview, *Biotechn. Res. Int.*, **Article ID: 941810** (2011).

[2] Xie S., Jiang G., Chen M., Li Z., Huang X., Liang C., Jia X.P., Harmless Treatment Technology of Waste Oil–Based Drilling Fluids, *Pet. Sci. Technol*, **32**(9): 1043-1049 (2014).

[3] Holdway D.A., The Acute and Chronic Effects of Wastes Associated with Offshore Oil and Gas Production on Temperate and Tropical Marine Ecological Processes, *Mar. Pollut. Bull.*, **44**(3): 185-203 (2002).

[4] Ji G., Yang Y., Zhou Q., Sun T., Ni J., Phytodegradation of Extra Heavy Oil-Based Drill Cuttings Using Mature Reed Wetland: an in Situ Pilot Study, *Environ. Int.*,**30**(4): 509-517 (2004).

[5] Yan P., Lu M., Guan Y., Zhang W., Zhang Z., Remediation of Oil-Based Drill Cuttings Through a Biosurfactant-Based Washing Followed by a Biodegradation Treatment, *Bioresource. Technol.*, **102**(22): 10252-10259 (2011).

[6] Balba M., Al-Awadhi N., Al-Daher R., Bioremediation of Oil-Contaminated Soil: Microbiological Methods for Feasibility Assessment and Field Evaluation, *J. Microbiol. Methods.*, **32**(2): 155-164 (1998).

[7] Rojas-Avelizapa N., Olvera-Barrera E., Fernández-Linares L., Feasibility Study of Bioremediation of a Drilling-Waste-Polluted Soil: Stimulation of Microbial Activities and Hydrocarbon Removal, *J. Environ. Sci. Heal.*, Part A, **40**(12): 2189-2201 (2005).

[8] Rojas-Avelizapa N., Roldan-Carrillo T., Arce-Ortega J., Ramirez-Islas M., Zegarra-Martinez H., Fernandez-Linares L., Enhancement of Hydrocarbon Removal in a Clay and Drilling-Waste Polluted Soil, *Soil. Sediment. Contam.*, **15**(4): 417-428 (2006).

[9] Rojas-Avelizapa N., Roldan-Carrillo T., Zegarra-Martinez H., Munoz-Colunga A., Fernandez-Linares L., A Field Trial for an Ex-Situ Bioremediation of a Drilling Mud-Polluted Site, *Chemosphere*, **66**(9): 1595-1600 (2007).

[10] Ma J., Yang Y., Dai X., Chen Y., Deng H., Zhou H., Guo S., Yan G., Effects of Adding Bulking Agent, Inorganic Nutrient and Microbial Inocula on Biopile Treatment for Oil-Field Drilling Waste, *Chemosphere*, **150**: 17-23 (2016).

[11] Ramírez M., Zapién B., Zegarra H., Rojas N., Fernandez L., Assessment of Hydrocarbon Biodegradability in Clayed and Weathered Polluted Soils, *Int. Biodet. Biodegr.*, **63**(3): 347-353 (2009).

[12] Choi W.-J., Chang S.X., Technical Note: Nitrogen Fertilization Effects on the Degradation of Aged Diesel Oil in Composted Drilling Wastes, *Int. J. Phytorem.*, **11**(5): 441-450 (2009).

[13] Steliga T., Jakubowicz P., Kapusta P., Changes in Toxicity During in Situ Bioremediation of Weathered Drill Wastes Contaminated with Petroleum Hydrocarbons, *Bioresource. technol.*, **125**: 1-10 (2012).

[14] Fan Y.Y., Wang G.C., Fu J.H., Zheng X.H., Bioremediation of Waste Drilling Fluid: Comparison of Biostimulation and Bioaugmentation, *Desalin. Water. Treat.*, 2012. **48**(1-3): p. 329-334.

[15] Okparanma R.N., A.J.M., Araka P.P., Bioremediation of Hydrocarbon Contaminated-Oil Field Drill-Cuttings with Bacterial Isolates, *Afr. J. Environ. Sci. Technol.*, **3**(5): 131-140 (2009).

[16] Goddey U., The Use of Kitchen Effluent as Alternative Nutrient Source for Bioremediation of Oil Based Drilling Muds, *J. Appl. Sci. Environ. Manag.*, **14**(4): 5-11 (2010).

[17] Alavi N., Mesdaghinia A.R., Naddafi K., Mohebali G., Daraei H., Maleki A., Alaei L., Biodegradation of Petroleum Hydrocarbons in a Soil Polluted Sample by Oil-Based Drilling Cuttings. *Soil. Sediment. Contam.*, **23**(5): 586-597 (2014).

[18] Al-Razaq A.A.A., Al-Joubori M.H., AI-Hiti A.H., Biotreatment Technique to Treat Oil Wells Drilling Wastes. *Iraqi. J. Chem. Petrol. Eng.*, **8**(3): 37-41 (2007).

[19] Shaeyan M., Tirandaz H., Ghanbarpour S., Seyedipour N., Shavandi M., Dastgheib S.M.M., Bioremediation of a Drilling Waste-Contaminated Soil; Biotreatability Assessment and Microcosm Optimization for Developing a Field-Scale Remediation Process. *Iran. J. Biotechnol.*, **16**(3):193-199 (2018).

[20] Fan Y-Y., Wang G-C., Fu J-H., Zheng X-H., The Remediation of Waste Drilling Muds by a Combined Plant-microbe System. *Petrol. Sci. Technol.*, **32**(17): 2086-2092 (2014).

[21] Fiúza A.M., Vila M.C.C., An Insight into Soil Bioremediation Through Respirometry, *Environ. Int.*, **31**(2): 179-183 (2005).

[22] Plaza G., Ulfig K., Worsztynowicz A., Malina G., Krzeminska B., Brigmon R., Respirometry for Assessing the Biodegradation of Petroleum Hydrocarbons, *Environ. Technol.*, **26**(2): 161-170 (2005).

[23] Aspray T., Carvalho D., Philp J., Application of Soil Slurry Respirometry to Optimise and Subsequently Monitor ex Situ Bioremediation of Hydrocarbon-Contaminated Soils, *Int. Biodeter. Biodegr.*, **60**(4): 279-284 (2007).

[24] Nnubia C., Okpokwasili G., The Microbiology of Drill Mud Cuttings from a New Off-Shore Oilfield in Nigeria, *Environ. Pollut.*, **82**(2): 153-156 (1993).

[25] Benka-Coker M., Olumagin A., Waste Drilling-Fluid-Utilising Microorganisms in a Tropical Mangrove Swamp Oilfield Location, *Bioresource. Technol.*, **53**(3): 211-215 (1995).

[26] ChaIneau C.-H., Morel J.-L., Oudot J., Microbial Degradation in Soil Microcosms of Fuel Oil Hydrocarbons from Drilling Cuttings, *Environ. Sci. Technol.*, **29**(6): 1615-1621 (1995).

[27] Steliga T., Role of Fungi in Biodegradation of Petroleum Hydrocarbons in Drill Waste, *Pol. J. Environ. Stud*., **21**(2):471-479 (2012).

[28] Turner K.P., "Bioremediation of Drill Cuttings From Oil Based Muds", University of Nottingham (2002).

[29] Napp A.P., Pereira J.E.S., Oliveira J.S., Silva-Portela R.C.B., Agnez-Lima L.F., Peralba M.C.R., Bento F.M., Passaglia L.M.P., Thompson C.E., Vainstein M.H., "Comparative Metagenomics Reveals Different Hydrocarbon Degradative Abilities from Enriched Oil-Drilling Waste", Chemosphere, 209: 7-16 (2018).

[30] Kaplan C.W., Kitts C.L., Bacterial Succession in a Petroleum Land Treatment Unit, *Appl. Environ. Microb.*, **70**(3): 1777-1786 (2004).

[31] Militon C., Boucher D., Vachelard C., Perchet G., Barra V., Troquet J., Peyretaillade E., Peyret P., Bacterial Community Changes During Bioremediation of Aliphatic Hydrocarbon-Contaminated Soil, *FEMS. Microbiol. Ecol.*, **74**(3): 669-681 (2010).

[32] Vinas M., Sabaté J., Espuny M.J., Solanas A.M., Bacterial Community Dynamics and Polycyclic Aromatic Hydrocarbon Degradation During Bioremediation of Heavily Creosote-Contaminated Soil, *Appl. Environ. Microb*, **71**(11): 7008-7018 (2005).

[33] Federation W.E., "Association A.P.H., Standard Methods for the Examination of Water and Wastewater", American Public Health Association (APHA): Washington, DC, USA, (2005).

[34] ASTM, "ASTM D2887, Standard Test Method for Boiling Range Distribution of Petroleum Fractions by Gas Chromatography", ASTM International West Conshohocken, PA (2008).

[35] Margesin R., Schinner F., "Manual for Soil Analysis-Monitoring and Assessing Soil Bioremediation", Vol. 5. Springer Science & Business Media (2005).

[36] Blodgett R., "Bacteriological Analytical Manual Appendix 2, Most Probable Number from Serial Dilutions, US Food and Drug Administration, Washington, DC, (2010).

[37] Wilson K., Preparation of Genomic DNA from Bacteria, *Curr. Protoc. Mol. Biol.*, **56**(1): 2.4.1-2.4.5 (2001).

[38] Kim O-S., Cho Y-J., Lee K., Yoon S-H., Kim M., Na H., Park S-C., Jeon Y-S., Lee J-H., Yi H., Won S., Chun J., Introducing EzTaxon-e: a Prokaryotic 16S rRNA Gene Sequence Database with Phylotypes that Represent Uncultured Species, *Int. J. Syst. Evol. Micr*., **62**(3): 716-721 (2012).

[39] Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S., MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods, *Mol. Biol. Evol.*, **28**(10): 2731-2739 (2011).

[40] Timmis K.N., McGenity T., Van Der Meer J., De Lorenzo V., "Handbook of Hydrocarbon and Lipid Microbiology", Springer Berlin (2010).

[41] Al-Awadhi H., Sulaiman R.H., Mahmoud H.M., Radwan S., Alkaliphilic and Halophilic Hydrocarbon-Utilizing Bacteria from Kuwaiti Coasts of the Arabian Gulf, *Appl. Environ. Microb.*, **77**(1): 183-186 (2007).

[42] Pizarro-Tobías P., Niqui J.L., Roca A., Solano J., Fernández M., Bastida F., Garcia C., Ramos J.L., Field Trial on Removal of Petroleum-Hydrocarbon Pollutants Using a Microbial Consortium for Bioremediation and Rhizoremediation, *Environ. Microbiol. Rep.*, **7**(1): 85-94 (2015).

[43] Jiménez N., Viñas M., Guiu-Aragonés C., Bayona J.M., Albaigés J., Solanas A.M., Polyphasic Approach for Assessing Changes in an Autochthonous Marine Bacterial Community in the Presence of Prestige Fuel oil and Its Biodegradation Potential, *Appl. Environ. Microb.*, **91**(3): 823-834 (2011).

[44] Wang L., Wang W., Lai Q., Shao Z., Gene Diversity of CYP153A and AlkB Alkane Hydroxylases in Oil-Degrading Bacteria Isolated from the Atlantic Ocean, *Environ. Microbiol.*, **12**(5): 1230-1242 (2010).

[45] Cappello S., Denaro R., Genovese M., Giuliano L., Yakimov MM., Predominant Growth of Alcanivorax During Experiments on "Oil Spill Bioremediation" in Mesocosms, *Microbiol. Res.*, **162**(2): 185-190 (2007).

[46] Kasai Y., Kishira H., Sasaki T., Syutsubo K., Watanabe K., Harayama S., Predominant Growth of Alcanivorax Strains in Oil-Contaminated and Nutrient-Supplemented Sea Water, *Environ. Microbiol.*, **4**(3): 141-147 (2002).

[47] Kleinsteuber S., Riis V., Fetzer I., Harms H., Müller S., Population Dynamics Within a Microbial Consortium During Growth on Diesel Fuel in Saline Environments, *Appl. Environ. Microb.*, **72**(5): 3531-3542 (2006).

[48] Kostka J.E., Prakash O., Overholt W.A., Green S.J., Freyer G., Canion A., Delgardio J., Norton N., Hazen T.C., Huettel M., Hydrocarbon-Degrading Bacteria and the Bacterial Community Response in Gulf of Mexico Beach Sands Impacted by the Deepwater Horizon Oil Spill, *Appl. Environ. Microb.*, **77**(22): 7962-7974 (2011).

[49] Shibata A.K., Robert F.M., Shifts in Alkane-Degrading Bacteria Genotypes During Bioremediation of a Vegetated Coastal Soil, *World. J. Microbiol. Biotechnol.*, **25**(9): 1667-1675 (2009).

[50] Dastgheib S.M.M., Amoozegar M.A., Khajeh K., Shavandi M., Ventosa A., Biodegradation of Polycyclic Aromatic Hydrocarbons by a Halophilic Microbial Consortium, *Appl. Microbil. Biotechnol.*, **95**(3): 789-798 (2012).

[51] Ali N., Dashti N., Al-Mailem D., Eliyas M., Radwan S., Indigenous Soil Bacteria with the Combined Potential for Hydrocarbon Consumption and Heavy Metal Resistance, *Environ. Sci. Pollut. R.*, **19**(3): 812-820 (2012).

[52] Gojgic-Cvijovic G.D., Milic J.S., Solevic T.M., Beskoski V.P., Ilic M.V., Djokic L.S., Narancic T.M., Vrvic M.M., Biodegradation of Petroleum Sludge and Petroleum Polluted Soil by a Bacterial Consortium: A Laboratory Study, *Biodegradation*, **23**(1): 1-14 (2012).