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Phospholipid fatty acids analysis-fatty acid methyl ester (PLFA-FAME) changes during bioremediation of crude oil contamination soil

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This study aims to develop certain perspectives based on the principle of on-site remediation of the soil through biological means known as "bioremediation" against soil pollution issues resulting from fuel contamination in our country and to reveal the fatty acid profile in the final soils. The fatty acid profile of the soils was pointed out by testing the activity of three basic bioremediation applications (biological multiplication, biological excitation and the combined application of these two approaches) established in the laboratory condition. Under biological multiplication applications, six of the selected bacterial strains *(Pseudomons aeruginosa, Pseudomonas putida biotype A, Citrobacter amalonaticus-GC subgroup A*, *Acinetobacter genomospecies)* exhibit the highest growth in crude oil environment isolated from oil-contaminated soils of Adana, Batman and Adıyaman, and they also have the highest levels of crude oil degradation. Under biological excitation applications, the organic materials being humic-fulvic acid and, in combined applications, different combinations of bacteria mixture and organic materials were examined as to the amount of crude oil they degrade in an incubation period of 120 days by qualitative hydrocarbon-type analyses. The highest level of oil degradation, being 56%, occurred under biological multiplication applications where the bacteria mixture was applied. Under biological excitation conditions where various organic materials were applied to the contaminated soil, degradation to 18% was observed. In combined applications, oil degradation was achieved to 30%. The most common fatty acids were found to be 15:0 iso, 15:0 anteiso, 16:0, 16:1 w7c, 17:0ai, 18:2w6,9 and 18:1w9c fatty acids detected in both unpolluted and oil-contaminated soils. Determination of high level 18:1w9c fatty acid in oil contaminated and clean soils may indicate the presence of *Pseudomonas* spp. However, fatty acid 15:0 anteiso was determined to be higher in oil-contaminated soils than in unpolluted soils. It may be explained that Gram positive bacteria were predominant in oil-contaminated environment.

Key words: Soil, crude oil, bacteria, bioremediation, phospholipid fatty acids analysis-fatty acid methyl ester (PLFA-FAME).

INTRODUCTION

It is well known that in soil, number and types of existing microorganisms are affected by biological and physico-chemical events including soil properties such as suitable conditions for microbial decomposition (oxygen, food substance, temperature and pH), microbial decomposition of hydrocarbons, quantity and quality of contaminants and its biological usefulness and particle distribution (Atlas, 1981; Atlas and Bartha, 1992; Steffan et al., 1997; Morgan and Watkinson, 1989; Margesin and Schinner, 1997a). Kapley et al. (1999) demonstrated that fungi can

decompose hydrocarbons, particularly *Emericella nidulans*, *Graphiwn putredinis*, *Eupenicillum javanicum* and *Aspergillus flavipes* are active in the assimilation of aromatic hydrocarbons. They showed that some soil-originated bacteria such as *Pseudomonas* spp. have the capability to decompose some fractions of crude oil. In addition, Jùrgensen et al. (2000) isolated and identified *Enterobacter sakazakii, Bacillus mycoides, Klebsiella oxytaca* and *Acinetobacter calcoaceticus*, from the compost application no. 3 on petroleum-contaminated soils; *Bacillus megaterium, Pseudomonas diminuta, Gluconobacter cerenius* and *Pasteurella caballi* from the compost application No. 1; and *Sphingomonas paucimobilis* and *Sphingobacterium multivorum* and some unknown bacteria from compost application No. 2. Obire and Okudo (1997), Bailey et al. (2002) showed existence of different microbial populations in petroleum-contaminated environment compared with the ones in cleaner environment. These changes in microbial community are the result of food cycle and movement in soil and can be determined by the method of total extractable phospholipid fatty acid.

In spite of the fact that there are many studies conducted on microorganisms which remove particular hydrocarbons or hydrocarbon groups forming structure of petroleum and petroleum products (PPP) in a short time, our knowledge about the role of soil microorganisms in hydrocarbon decomposition is very limited. In the present study, the selected microorganisms and mixture of microorganisms isolated from various soil ecosystems under different environmental conditions were evaluated for elimination of PPP-related contamination in laboratory and field studies in Turkey. For this purpose, an economical and environment-friendly "biological improvement (bioremediation)" approach was taken as a model in the elimination of petroleum and similar organic contaminants. Then fatty acid profiles of the soils were determined by testing the efficiency of three basic bioremediation applications established in the laboratory conditions (bio-augmentation, bio-stimulation and the combined application of these two approaches) in elimination of crude oil based contamination.

MATERIALS AND METHODS

Procurement of soil material in which experimental contamination conditions are to be established

Soil material from trial lands of AUZF Research and Application Farm (approximately 40 kg, taken from 0-20 cm depth) were brought to laboratory after sieved through a sieve of 2 mm.

Contaminating material

Crude oil was procured from Kırıkkale Refinery Premises of Turkish Petroleum Corporation (TPAO).

Composition by weight of hydrocarbon are: alkanes (paraffins) 30%, naphthenes 49% aromatics15%, asphaltics 6%.

Procurement of the bio-augmentation material (bacteria-bac)

Batman Refinery waste accumulating field, samples were taken from 0-20 cm depth: (waste 1) and samples were taken from 20-40 cm depth: (waste 2). Samples were also taken from Adiyaman TPAO petroleum wells inner station petroleum water accumulating area and "BTC (Bakü - Tiflis - Ceyhan) crude oil loading terminal area in Adana. All samples were kept at +4°C until analyses were made.

Bio-stimulation material (humic-fulvic- acid)

As bio-stimulation material, soil regulator sold in the market, coded as HFA (humic-fulvic- acid) (K- Humate) was used.

Preparation of the soil used for bioremediation purposes

The soil used in this study (about 40 kg), was collected one week before the setting of last trial and was kept at room temperature.

Isolation of bacteria from petroleum-contaminated soils

The following procedures were followed with isolation purposes on the samples taken from i) Batman refinery, refinery waste accumulating area, ii) from Adıyaman TPAO petroleum wells inner station petroleum water accumulating area and iii) from "BTC" Crude oil loading terminal area in Adana. 1% crude oil and Triton-X-100 emulsifier (1:1) were added into 1 L broth medium which include 10 g soil ,1 g KNO_3, 0,2 g MgSO4, 0,1 g NaCl, 0,1 g $CaCl_2$ g, 1 g K_2HPO_4 . Then they were left for incubation at 180 cyc/min at 28°C for 3 days. At the end of the 3rd day, 10 ml was taken out from incubated broth medium and put in a fresh environment again which has the same components (Erdoğan et al., 2011, Rojas-Avelizapa et al., 1999).

Microbial Identification System (MIS) identification of isolated bacteria

Miller and Berger (1985) carried phospholipid fatty acids analysis (PLFA) analysis made on pure bioremediation bacteria cultures out using MIS. This system is based on the fact that number, variety and quantity as percentage (fatty acid profile) of fatty acids in the cells of microorganisms with same genetics and that they do not change as long as environment conditions remain the same (Şahin, 1997; Şahin et al., 1999; Erdoğan et al., 2011).

Bioremediation applications

Preparation of experimental contamination conditions

A 3000 g of soil sample having a moisture content of 50% of its water holding capacity was placed into a plastic pot incubated 25°C for 10 days. After three days of pre-incubation, soil sample was contaminated by applying crude oil of 1% on weight basis (w/w)

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Abbreviations: PPP, Petroleum and petroleum products; **HFA,** humic-fulvic- acid; **PLFA,** phospholipid fatty acids analysis; **MIS,** microbial identification system; **TPH,** total petroleum hydrocarbons; **FAME,** fatty acid methyl ester; **GC,** gas chromatographic.

Table 1. Experimental design.

Treatment number	Treatment	Treatment of content
1	*BAC + *N	Biological multiplication
2	*HFA + *N	Biological excitation
3	*BAC + *HFA + *N	Combined application (1+2)
4	C + *N	Control (basic fertilization)

*BAC, Bacteria mixture; *N, nutrient; *HFA, humic fulvic acid.

homogeneously. Clean soil material (as a control) was left in room conditions for the same duration.

Preparation of bioremediation bacteria mixture cultures

Six bacterial strains, their petroleum decomposition abilities in liquid culture determined were applied homogeneously to clean and contaminated soils by spraying. The applied bacteria density of each strain was 10^{10} CFU/ml. (Erdogan, 2010; Erdoğan et al., 2011).

Experimental design

Trial was set in 5 kg plastic mouth capped cups (Table 1). Samplings have been done on 1st, 30th, 60th, 90th and 120th day. Total petroleum hydrocarbons (TPH) analyses and cultural count on the1st, 30th, 60th, 90th and 120th day was done for monitoring of crude oil decomposition. PLFA-fatty acid methyl ester (FAME) analysis on the 1st, 30th, 60th, 90th and 120th day using direct extraction method has been done for investigating soil community structure in each sampling. In this way, it allowed determination of "indicator-fatty acid methyl ester" with respect to bioremediation bacteria which is to be used (Erdogan, 2010).

Determination of efficiency of bioremediation applications

Monitoring crude oil decomposition in soil

In soil samplings taken on the 1st, 30th, 60th, 90th and 120th day of the trial, petroleum analyses were conducted with ASE device (Dionex ASE 300) in TPAO-Research Central Geochemistry Laboratory (EPA method 3545).

Ascertainment of determinative features of bioremediation bacteria used with PLFA-FAME analysis

"Indicator-fatty acid methyl ester" of bioremediation bacteria which is to be used was ascertained by making FAME analysis with direct extraction method in order to monitor life status of petroleum decomposing bacteria, which were made resistant to crude oil, in petroleum-free and petroleum contaminated soil.

Essentials of this procedure can be defined as i) Extracted microbial living from soil and having been treated to them with a medium level alkali hydrolysis and break up of their cells; ii)ester binds breaking and fatty acids being separated from lipids and fatty acids; iii) having been transformed into methyl ester form, being analyzed in gas chromatographic (GC) system in quantitative (%) and qualitative respects. Bligh and Dyer (1959), Sasser (1990), Zelles et al. (1992), Frostegard et al. (1993), Bossio et al. (1998), Ibekwe and Kennedy (1998) also used this analysis method in their study.

Statistical analyses

Results of the study were evaluated with repeated measures variance analysis method with respect to features focused on. Repeated measures were made at the levels of time factor and conducted in three replicates "SPSS 12.0", MSTAT software packages were used for calculations. In addition, correlations between investigated parameters were evaluated using Pearson Correlation Test (Winer et al., 1991; Gürbüz et al., 2003).

RESULTS

Species-genus-order-families of isolated bacteria

Results of the isolated bacteria are given collectively in Table 2.

Changes in total petroleum hydrocarbons (TPH)

TPH values of Pet+BAC+N,Pet+HFA+N and Pet+BAC+HFA+N applications applied on petroleum-contaminated (Pet.) soils for 120-day incubation period are given in Figure 1. When variance analysis table relating to TPH in petroleum-contaminated soils was examined, time-bacteria-HFA triple interaction was found statistically significant ($P<0.05$). When we compare petroleum-contaminated soils in respect of their TPH values, time-dependent reduction is observed in all applications except control. While the biggest reduction was observed in Pet+BAC+N soils, the smallest reduction was observed in control soils. If we are to compare application types with each other, the highest TPH value (7998 mg.kg-1) was determined in soil Pet+BAC+N on the 1st day and the lowest one (4500 mg.kg-1) was determined in soil Pet+BAC+N on the 120th day; the difference between applications was found to be significant statistically at level of $P<0.05$.

Filauro et al. (1998) stated in their study that TPH concentration was decomposed by 48% with bacteria application, Peressuttia et al. (2003) indicated that TPH quantity was reduced by 45.48%. Pokethitiyook et al. (2002) found out that *A. Calcoaceticus* isone among those isolated from a petroleum-contaminated area in Bangok, and three Pseudomonas strains provided high level of decomposition at 0.5% crude oil level.

Table 2. Genus-species-order-families of isolated bacteria.

Bacteria (Genus-species)	Order - Family
Pseudomonas putida	Pseudomonadales - Pseudomonadaceae
Pseudomonas aeruginosa	Pseudomonadales - Pseudomonadaceae
Pseudomonas mucidolens	Pseudomonadales- Pseudomonadaceae
Acinetobacter genomospecies	Pseudomonadales - Moraxellaceae
Stenotrophomonas maltophilia	Xanthomonadales - Xanthomonadaceae
Enterobacter hormaechei	Enterobacteriales - Enterobacteriaceae
Enterobacter sakazakii	Enterobacteriales - Enterobacteriaceae
Citrobacter amalonaticus	Enterobacteriales - Enterobacteriaceae
Eschericha coli	Enterobacteriales - Enterobacteriaceae
Sphingobacterium multivorum	Sphingobacteriales - Sphingobacteriaceae
Aeromonas caviae	Aeromonadales - Aeromonadaceae
Paucimonas-lemoignei	Burkholderiales - Burkholderiaceae

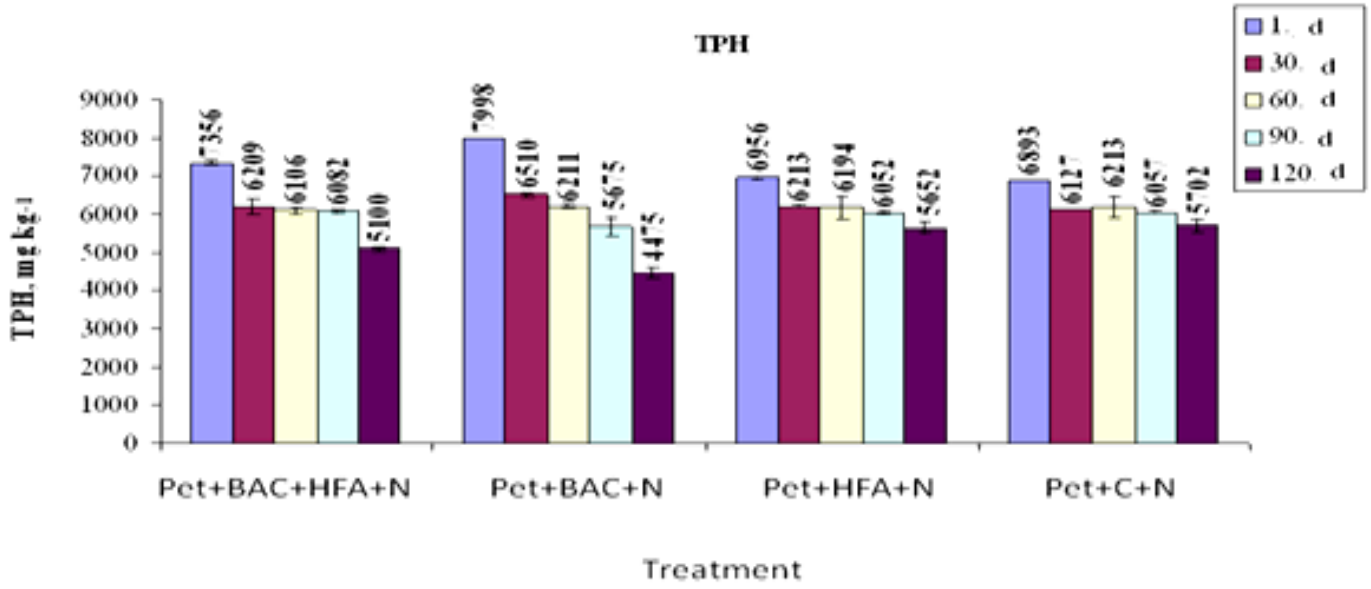

Figure 1. TPH values relating to applications Pet+BAC+N, Pet+HFA+N and Pet+BAC+HFA+N applied on petroleum-contaminated soils.

A. calcoaceticus, the best among four bacteria, provided decomposition values of 80.76, 68.86 and 65.18% in contaminated soil at different temperatures of 20, 40 and 30°C, respectively.

The data of another study conducted by Ghazali et al. (2004) is very similar and supporting our results. Researchers found out that two different bacterial mixtures isolated from contaminated soil showed similar and high levels of petroleum decomposition under soil-free *in vitro* conditions. They indicated that a bacterial strain isolated from contaminated soil and bacterial mixtures composed of *Pseudomonas* spp and *Bacillus* spp provided a higher level of decomposition tested under conditions of diesel, petroleum and machine oil-contaminated soil for a 60-day incubation process. Rambeloarisoa et al. (1984) found out that a bacterial mixture, which is composed of 8 species belonging to six different bacterial genera, effectively decomposed crude oil.

In another study, Kishore and Ashis (2007) tested *Bacillus subtilis*(DM-04), and *Pseudomonas aeruginosa* (M) isolated from a petroleum-contaminated area at southeastern India under soil-free *in vitro* and contaminated soil conditions, and showed that *P. aerugionasa* (M) was more effective than *B. subtilis* (DM-04) in soil-free environment. After a 120-day incubation period in the petroleum-containing soil experiment, it was observed that while *B. subtilis* DM-04 eliminated 50% of the crude

Table 3. Change over time of measurable fatty acids of humic acid and bacteria (Pet+HFA+BAC+N) application under petroleum containing conditions.

Pet+HFA+BAC+N	Time				
PLFA-FAME	**1st day**	**30th day**	**60th day**	**90th day**	**120th day**
10:0	3.36	13.3	-	-	-
11:0	4.07	-	-	-	-
12:0	1.88	-	-	-	-
12:0 iso 3OH	3.39	-	-	-	-
12:0 anteiso	1.22	30.64	-	-	-
13:0 anteiso	2.43	21.68	-	-	-
15:0 iso	4.77	-	-	-	-
15:0 anteiso	13.35	34.38	100.00	-	-
16:0	6.23	-	-	-	-
16:0 anteiso	11.35	-	-	-	-
16:0 iso	7.56	-	-	-	-
16:0 10-methyl	5.47	-	-	-	-
17:1 w5c	7.5	-	-	-	-
17:0 anteiso	6.53	-	-	-	-
18:0 anteiso	10.42	-	-	-	-
18:1 w9c	3.91	-	-	-	-
18:3 w6c(6.9.12)	4.18	-	-	-	-

Table 4. Change over time of measurable fatty acids of bacteria (Pet+BAC+N) application under petroleum containing conditions.

Pet+BAC+N	Time				
PLFA-FAME	**1st day**	**30th day**	**60th day**	**90th day**	**120th day**
9:0 3OH	12.04	-	-	-	-
10:0	11.96	28.99	-	-	-
12:0 anteiso	12.03	71.01	-	-	-
13:0 anteiso	10.54	-	-	-	-
14:0 anteiso	12.44	-	-	-	-
15:0 anteiso	16.93	-	100.00	-	-
18:1 w9c	17.63	-	-	-	-
17:0 anteiso	9.01	-	-	-	-
18:1 w6.9c	7.53	-	-	-	-
18:3 w6c(6.9.12)	7.29	-	-	-	-

oil, *P. aerugionasa* (M) and a bacterial strain isolated from contaminated soil were able to decompose up to 100% of those. Porta et al. (1998) also reported similar results. On the other hand, other researchers indicated that complex hydrocarbons, phenols, phenanthrene and benzopyrene show a high metabolic capability in gram-positive bacteria with respect to decomposing oxidation products (Sextone et al., 1978; Song and Barta, 1990).

Phospholipid fatty acid methyl ester (FAME)

Evaluation of soil PLFA-FAME analysis is shown in Table 3 to 6 and the number of indicator fatty acid, which could be measured at different times, was different in soil at PLFA-FAME analysis results.

Based on this information, we can see that the most intensive fatty acid in all applications was on the 1st day and it reduced over time; and finally, on the 90th and 120th day fatty acid could not be found (Tables 3 to 6). While in Pet+HFA+BAC+N application, at the beginning of the incubation (1st day), the number of PLFA was 17, it reduced to 4 on the 30th day and to 1 on the 60th day, and later no fatty acid could be found (Table 3). The number of PLFA-FAME in this application was more than that of other applications. This could be due to the

Table 5. Change over time of measurable fatty acids of humic acid (Pet+HFA+N) application under petroleum containing conditions.

Pet+HFA+N	Time				
PLFA-FAME	**1st day**	**30th day**	**60th day**	**90th day**	**120th day**
12:0 anteiso	20.21	24.88	-	-	-
13:0 anteiso	18.42	16.55	-	-	-
14:0 anteiso	21.65	8.99	-	-	-
15:0 anteiso	22.67	22.83	100.00	-	-
16:0 anteiso	23.53	22.54	-	-	-

Table 6. Change over time of measurable fatty acids of control (Pet+C+N) application under petroleum containing conditions.

Pet+C+N	Time				
PLFA-FAME	**1st day**	**30th day**	**60th day**	**90th day**	**120th day**
11:0 anteiso	-	21.59	-	-	-
12:0 anteiso	-	29.57	-	-	-
13:0 anteiso	-	21.03	-	-	-
15:0 anteiso	21.58	27.82	100.00	-	-
16:0 anteiso	20.9	-	-	-	-
17:0 anteiso	11.8	-	-	-	-
18:2 w6.9c	16.3	-	-	-	-
18:3 w6c(6.9.12)	10.61	-	-	-	-
19:1 w6.9c	18.80	-	-	-	-

addition of bacteria. This quantity fell over time and after the 90th day no PLFA-FAME could be found. While in Pet+BAC+N application, at the beginning of the incubation (1st day), the number of FAME was 10, it reduced to 2 on the 30th day and to 1 on the 60th day, and later no fatty acid could be found (Table 4).

In this application as well, quantity of PLFA-FAME was higher on the 1st day than that of other applications due to the addition of bacteria. While in Pet+HFA+N application, at the beginning of the incubation (1st day), the number of FAME was 5, on the 30th day number of fatty acid was same, the 60th day it reduced to 1, and later no fatty acid could be found (Table 5). While in Pet+C+N application, at the beginning of the incubation (1st day), the number of FAME was 6, it reduced to 4 on the 30th day and to 1 on the 60th day, and later no fatty acid could be found (Table 6).

DISCUSSION

One of the basic intentions of this study is to determine the microorganisms which can eliminate contamination in soil stemming from petroleum and that kind of materials, and to evaluate their efficiency in laboratory conditions. Applications known as bioremediation are based on use of microorganisms, which have different levels of hydrocarbon decomposing capability, in petroleum-contaminated environments. The most important measure that is taken as basis in revealing bioremediation potential of applications used (bio-augmentation, bio-stimulation, bio-augmentation + bio-stimulation) is soil TPH analysis results based on total hydrocarbons in soil. The highest decrease in total petroleum hydrocarbon rate over time among applications was found in bacteria (Pet+BAC+N) application, which is followed by bacteria + humic fulvic acid (Pet+BAC+HFA+N) application and the lowest decrease was found in the control application. When we look at the 1st and 120th days of the study with respect to TPH, bioremediation process proceeded fastest in bacteria (Pet+BAC+N) application by 56%, which is followed by bacteria + humic acid (Pet+BAC+HFA+N) application by 30%, only humic fulvic acid (Pet+HFA+N) application by 18% and control application by 17%. The data showed that mixture of bacterial strains (*P. aeruginosa, Pseudomonas putida* biotype A, *Citrobacter-amalonaticus-GC subgroup* A, *Acinetobacter-genomospecies*) gave the best result. In the case of decrease level in TPH values in bacteria (Pet+BAC+N) application, the fastest degradation was measured between 1-30 days and 90-120 days. It is seen that between 30-90 days of incubation, there is a very little change. This result indicated that petroleum decomposition is not continuous but has some inactive periods. It was observed that other applications also have the same inactive phase. Different PLFA bio-indicators were used to gather information about many

members of soil micro flora such as bacteria, fungi, algae, gram-negative bacteria, gram-positive bacteria, sphingomonas, actinomycetes and sulphate-reducing bacteria. It was indicated that these agent materials provide clues about lipid synthesis that soil microorganisms make in connection with many environmental events (White et al., 1996a, b; Venosa et al., 2000). Olsson and Persson (1999) reported that 18:1ω9c is also an indicator representing *Pseudomonas* spp. bacteria. Since fatty acid 18:1w9c showed high values in bacteria (Pet+BAC+N ve Pet+BAC+HFA+N) applications we conducted in clean and contaminated soils, we can put emphasis on that it is a specific fatty acid of *Pseudomonas* spp. bacteria. 15:0 iso is an indicator for gram-positive and sulphur bacteria (Olsson and Persson, 1999). It showed reduction over time in all applications. Other researchers indicated that for (i15:0 and a15:0) gram-positive bacteria, their biomarkers increase (Ringelberg et al., 2008). It was determined that the level of the 15:0 anteiso fatty acid is higher in petroleum-contaminated soils than in unpolluted soils. We may emphasize that the existence of gram-positive bacteria increases in petroleum-contaminated environment. It was indicated that saturated fatty acid 16:0 is an indicator which can be found in all bacteria generally (Pelz et al., 2001; Keinanen et al., 2003), however, Kneif et al. (2006) stated that PLFA 16:0 is only specific to methane-oxidizing bacteria (metanotroph) as a source of carbon and energy. Some researchers indicated that biomarkers of fatty acids n16:1w7c, n18:1w9c and n18:1w7c increase for gram-negative bacteria (Ringelberg et al., 2008). When investigated in respect of 18:1ω9c concentrations ascertained in our study, the common point is that it is a fatty acid occurring in bacteria (Pet+BAC+N) applications, and this PLFA agent had a high value at the beginning of the incubation while at the end of it, it decreased and on the 120th day it could not be found. If 18:1ω9c is an indicator of species *Pseudomonas* spp. as it is claimed by Olsson and Persson (1999), then we can relate the reduction of this indicator fatty acid concentration over time to the decrease of hydrocarbon sources in the environment. According to the study of Parker and Taylor (1983) and Guckert et al. (1985) 18:1ω7c and 18:1ω9c are agents specific to aerobic bacteria and also Fierer et al. (2003) grouped these fatty acids as gram-negative bacteria indicator. Bundy et al. (2002) related the fatty acids 18:1w9 and 17:1w8, which are extracted from petroleum-contaminated soil, to gram-positive bacteria. Fatty acid 17:0ai was observed more in number in clean soil than petroleum-contaminated soil.

PLFA agents (fatty acids) determined are biomarker for gram-negative bacteria and the fact that this PLFA agent has a high value at the beginning of the incubation and it decreases at the end of the incubation is related to the reduction in hydrocarbon sources in the environment. In clean and petroleum-contaminated soils, mostly fatty acids 15:0 iso, 15:0 anteiso, 16:0, 16:1 w7c, 17:0ai, 18:2w6, 9, and 18:1w9c were detected. Because the fatty acid 18:1w9c showed high levels, we may state that it is a fatty acid specific to the bacteria *Pseudomonas* spp. We could not yet reach a clear answer about how to use bioremediation applications, a new subject in Turkey, for the elimination of contamination, despite the scientific efforts made in our research institutions. The present study will cast light on the solution of a potential problem in Turkey. In this regard, in medium-level lime, light alkali, 1% (w/w) petroleum-contaminated soils, the highest petroleum decomposition, that is, 56%, occurs under bio-augmentation applications in which bacteria mixture is applied and what should be kept in mind is that the applied augmentation material consists of local bacteria.

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