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# Bacterial Community Composition in Produced Water of Diyarbakır Oil Fields in Turkey

**Bacterial communities in produced waters of south-eastern Turkey reported in detail for the first time**

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Oil fields harbour a wide variety of microorganisms with different metabolic capabilities. To examine the microbial ecology of petroleum reservoirs, a molecular-based approach was used to assess the composition of bacterial communities in produced water of Diyarbakır oil fields in Turkey. Denaturing gradient gel electrophoresis (DGGE) of polymerase chain reaction (PCR)-amplified 16S rRNA gene fragments was performed to characterise the bacterial community structure of produced water samples and to identify predominant community members after sequencing of separated DGGE bands. The majority of bacterial sequences retrieved from DGGE analysis of produced water samples belonged to unclassified bacteria (50%).

Among the classified bacteria, *Proteobacteria* (29.2%), *Firmicutes* (8.3%), *Bacteroidetes* (8.3%) and *Actinobacteria* (4.2%) groups were identified. *Pseudomonas* was the dominant genus detected in the produced water samples. The results of this research provide, for the first time, insight into the complexity of microbial communities in the Diyarbakır oil reservoirs and their dominant constituents.

## 1. Introduction

Although much progress has been made in the use of renewable energy in recent years, fossil fuels (especially oil and gas) still meet most of the global energy demand, and they will continue to be the dominant source of energy worldwide over the next few decades (1).

Petroleum is a naturally occurring material found in various geological formations (reservoirs) worldwide. Crude oil, the liquid part of petroleum, is primarily composed of hydrocarbons (2). However, it may also include compounds of nitrogen, sulfur, oxygen and metals (3). Because crude oil in reservoirs is found as a mixture containing varying constituents and proportions, each crude oil has its own unique properties. The most important specified properties are density and sulfur content (4). The density of crude oil is reported in terms of American Petroleum Institute (API) gravity (specific gravity). Based on the API gravity, crude oils can be classified into light, medium, heavy and extra heavy oils (3). Depending on the amount of sulfur content (elemental sulfur or sulfur compounds such as hydrogen sulfide), the crude oil is categorised as 'sweet' or 'sour'. In addition to chemical composition and physical properties, crude oil typically is also identified by underground

reservoir (4). Reservoir characteristics (depth, temperature, pressure and other factors) vary significantly from one location to another, even in the same geologic formation (5, 6). The fact that microbial community composition and reservoir conditions vary dramatically not only between the different geographical areas, but also among different oil fields in the same region, makes each oil reservoir ecosystem unique.

Despite the extreme environmental conditions in the oil-bearing formations (i.e. anoxic, high temperature, high salinity), many microorganisms are capable of surviving in the oil and water phases of the oil wells (7, 8). Oil fields harbour mainly facultative aerobic and strictly anaerobic microorganisms due to the low redox potential in the reservoirs (8). These ecosystems contain different types of microbial communities (such as mesophiles, thermophiles and halophiles) which adapt to the reservoir conditions (9). Bacterial and archaeal groups identified in oil fields include sulfate-reducing bacteria (10), sulfur-oxidising bacteria (11), methanogens (12), fermentative microorganisms (13), acetogens (14), nitrate reducers (15), manganese and iron reducers (16) and hydrocarbon degraders (17). Among these microbes, sulfate-reducing bacteria have attracted much attention due to their detrimental effects such as reservoir souring and biocorrosion (7). In addition, different members of the oil microbial community are involved in syntrophic interactions. Fermenting bacteria and methanogenic archaea are involved in methanogenic hydrocarbon biodegradation through their close syntrophic associations (18). This microbial process is undesirable in oil reservoirs because it causes a decrease in oil quality and value (19). Syntrophic microorganisms in oil reservoirs also play important roles in the global biogeochemical cycling of sulfur, carbon and nitrogen. For instance, sulfate-reducing bacteria and sulfur-oxidising bacteria, the key drivers in sulfur transformations, are involved in the sulfur cycle (11). Thus, knowledge of the microbial groups and microbial dynamics in oil fields enable us to obtain detailed insights into the microbial ecology of oil associated environments.

Understanding the microbial ecology of oil reservoirs is crucial to the petroleum industry because the success of oilfield operations is strongly influenced by the activity of microorganisms. Oil microbes with different metabolic capabilities have significant negative and positive impacts on the petroleum resources and the extraction processes (7). Microbial activity may lead to severe problems such as

reservoir souring and microbial corrosion. Reservoir souring, which is characterised by an increase in production of H<sub>2</sub>S in the reservoir fluids, most commonly occurs when sulfidogenic microorganisms reduce sulfate to sulfide, a toxic and corrosive product (20). Undesirable accumulation of sulfide minerals in reservoirs is one of the major challenging problems in oil production because it causes plugging of reservoirs, decreasing the oil quality and value and increasing the refining costs. Moreover, exposure to H<sub>2</sub>S can be dangerous in terms of worker health and safety due to its high toxicity. Additionally, the produced H<sub>2</sub>S promotes corrosion of the metallic equipment and structures used for oil production and processing (21). Another destructive phenomenon is biocorrosion, which is defined as microbial attack on the surface of the metal infrastructure leading to disruption of the material (22). In addition to sulfate-reducing bacteria, which play a major role in biocorrosion, other corrosive microbes, such as acetogenic bacteria and methanogenic archaea, are also associated with corrosion failures (23). Biocorrosion is a great concern because it leads to loss of material, large economic losses and safety issues in the oil industry (24). In contrast, hydrocarbon-degrading bacteria may be used for environmental clean-up processes (6). Bacterial degradation of hydrocarbons was carried out by both aerobic (for example, *Rhodococcus* sp., *Sphingomonas* sp., *Pseudomonas putida*, *Pseudomonas stutzeri*, *Acinetobacter* sp.) and anaerobic bacteria (such as Fe(III)-reducing bacteria, sulfate-reducing bacteria) (6, 17). Furthermore, microbial products such as biopolymers and biosurfactants can be used for facilitating oil movement in a widely used technology, known as microbial enhanced oil recovery (MEOR) (1). Compared with other conventional oil recovery techniques, MEOR has advantages such as low cost, wide application, high efficiency and low environmental pollution (25). Therefore, diversity, metabolic processes and habitat conditions of microbial communities in oil reservoirs should be investigated, so that their negative effects can be decreased and their positive effects can be exploited.

This study aimed to determine the bacterial community composition and to identify the predominant community members in produced water from oil fields located in the Diyarbakır region in Turkey. To this end, we used PCR-DGGE to analyse 20 produced water samples from the Diyarbakır region. There are limited studies on produced water from the Diyarbakır region and this paper represents the only *in situ* study available. The results of this study provide not only new

data about the microbial ecology of the Diyarbakır oil fields, but also information on the bacterial populations which may have potential roles in terms of increasing or decreasing the efficiency of industrial applications.

## 2. Materials and Methods

### 2.1 Sampling Procedure

The sampling site, the Diyarbakır region, is located at the boundary of the Anatolian plate and the Middle Eastern oil region in south-eastern Turkey. A total of 20 crude oil samples (B1, B6, B8, B14, B23, B32, B56, GK8, GS6, GS15, M3, K2, K3, K32, K35, K44, S4, S15, Y18 and Y30) consisting of an oil/water mixture were collected from the production wells of Diyarbakır oil fields (**Figure 1**). These wells produced oils withdrawn from the oil sandstone deposits (depths from 1600 m to 2620 m, API gravity from 24.3° to 42.3°, water content around 94%, an average pH of 7.0 and salinity from 2966 mg l<sup>-1</sup> to 26,961 mg l<sup>-1</sup>). The samples were aseptically taken at the wellhead and put into sterile 500 ml serum bottles sealed with rubber stoppers and aluminium caps. The samples were shipped at ambient temperature. Upon arrival at the laboratory, the samples were immediately analysed. All samples were treated within 48 h after collection. Decantation was used to separate produced water from the oil/water mixture.

### 2.2 DNA Extraction

Bacteria in the produced water samples were collected by filtration over 0.20 µm pore size polyamide filters (Sartolon®, Sartorius AG, Germany). Genomic DNA was extracted with the UltraClean® Microbial DNA isolation kit (MO BIO Laboratories Inc, USA) according to the manufacturer's protocol.

### 2.3 Polymerase Chain Reaction Amplification

Extracted DNA was used as the template for PCR amplification of partial 16S rRNA fragments. Primer pair consisting of 341F with a GC clamp and 907R was used for DGGE analysis (26). A 40-base GC clamp was used to prevent complete denaturation of the fragment during DGGE (27).

Due to the low DNA yield, a two-step PCR strategy was used. At the first step, a real-time PCR (quantitative PCR, qPCR) approach was applied to the produced water samples. The reaction mixture in a final volume of 22.5 µl contained 0.2 µl of each primer, 12.5 µl iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc, USA), 9.6 µl RNase-Free Water (Qiagen, Germany) and 0.5 µl DNA template. qPCR was performed in iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc, USA) using the following conditions: 5 min at 95°C; 40 cycles of 95°C for 30 s, 57°C for 40 s,

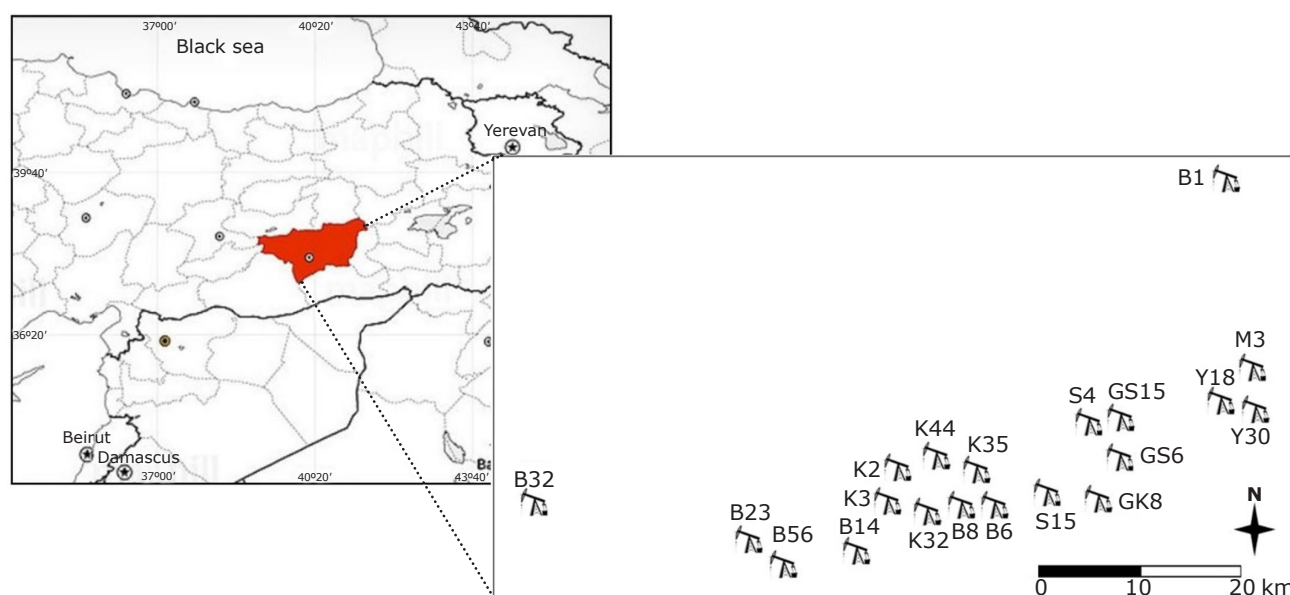


Fig. 1. Sampling locations in Diyarbakır region. Produced water samples were collected from 20 different oil wells © Maphill / Creative Commons Attribution-NoDerivatives (CC BY-ND)

72°C for 40 s and 80°C for 25 s; and a final 72°C for 10 min. In the qPCR method, after each cycle, a signal was formed. By observing the signals for each sample, PCR products could be detected. The reaction was terminated when the desired amount of product was reached. At the second step, a conventional PCR approach was applied to the qPCR products. Reaction mixture in a final volume of 25 µl contained 0.2 µl of each primer, 12.5 µl *Taq* PCR Master Mix (Qiagen, Germany), 9.6 µl RNase-Free Water (Qiagen, Germany) and 0.5 µl DNA template. The PCR was performed in TGradient thermocycler (Biometra, Germany) using the following conditions: 5 min at 95°C; 12 cycles of 95°C for 30 s, 57°C for 40 s and 72°C for 40 s; and a final 74°C for 30 min.

## 2.4 Denaturing Gradient Gel Electrophoresis

The DCode™ system (Bio-Rad Laboratories, USA) was used for DGGE analysis. 25 µl of each PCR product (200–300 ng) were loaded onto 6% polyacrylamide gels (w/v) containing gradients of 20% to 70% denaturants (urea/formamide). The gels were run for 16 h at 100 V and 60°C in 1× Tris-acetate-EDTA buffer. After completion of electrophoresis, the gels were stained with SYBR® Gold Nucleic Acid Gel Stain (Invitrogen™, Thermo Fisher Scientific, USA) for 20 min, visualised and photographed. Selected predominant DGGE bands were excised, eluted in 40 µl of 1× Tris buffer (pH 8) for 2 d at 4°C and re-amplified with 25 cycles as described above. Reaction mixture in a final volume of 25 µl contained 0.125 µl of primer 341F, 0.125 µl of primer 907R, 12.5 µl of *Taq* PCR Master Mix, 9.75 µl of ultra-pure water and 0.5 µl of template. The PCR products were quantified on a 1.5% (w/v) agarose gel and then sequenced by Macrogen Inc (Seoul, South Korea).

## 2.5 Comparative Sequence Analysis

The resulting sequences were first aligned and edited using CodonCode Aligner software (CodonCode Corp, USA). Then they were compared to sequences stored in the database GenBank® using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST®) (28, 29). All obtained partial 16S rRNA gene sequences were deposited in GenBank® database under the following accession numbers: KF720792 - KF720796, KF720798, KF720801 - KF720802, KF720804, KF720806 - KF720808, KF720810 - KF720811, KF720814, KF720818,

KF720820, KF720823, KF720825 - KF720826, KF720828, KF720830 - KF720832, KF720839, KF720844, KF720852, KF720855, KF720858, KF720872, KF720877, KF720882 - KF720884, KF720886 - KF720889, KF720891, KF720893 - KF720894, KF720896 and KF720903.

## 3. Results

### 3.1 Molecular Analysis of Bacterial Communities

Bacterial DNA isolation could only be achieved for 16 (B1, B8, B6, B14, B23, B32, B56, GS6, GK8, K35, K44, M3, S4, S15, Y18, Y30) of the 20 produced water samples. Because the water phase could not be separated from the oil phase for the other four produced water samples, DNA could not be extracted from these samples. The extracted DNA was used as template DNA for the amplification of 16S rRNA gene fragment. Unfortunately, direct PCR with bacterial primers did not yield a product from any of the produced water samples. For this reason, a two-step PCR was applied: the first step was a qPCR to increase the concentration of genetic material to measurable amounts (30), while the second step was a normal PCR to obtain enough material for DGGE analysis. For produced water samples, a total of 113 DGGE gel bands were analysed, but only 69 bands yielded sequences of satisfactory quality (Figure 2).

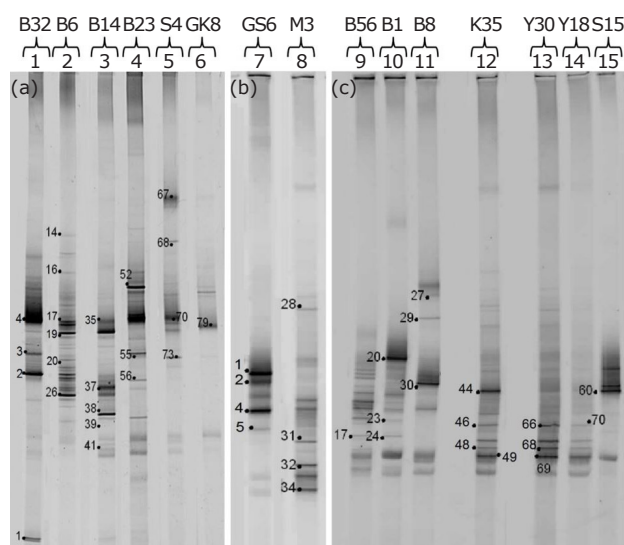


Fig. 2. DGGE profiles of 16S rRNA gene fragments amplified from produced water samples. See legend to Figure 1. (a) 1, B32; 2, B6; 3, B14; 4, B23; 5, S4, 6, GK8; (b) 7, GS6; 8, M3; (c) 9, B56; 10, B1; 11, B8; 12, K35; 13, Y30; 14, Y14; 15, S15

Comparative sequence analysis of the DGGE bands indicated that 50% of the bacterial sequences belonged to 'unclassified bacteria'. Among the classified bacteria, members of the phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria*, and the classes *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Sphingobacteriia*, *Bacilli* and *Actinobacteria* were identified (**Figure 3**).

### 3.1.1 *Proteobacteria*

*Proteobacteria* was the dominant phylum, comprising 29.2% of the total sequences retrieved from the produced water samples (**Figure 3**). The sequences B6\_19 and B14\_37 shared 100% and 99% identity with uncultured bacteria (EU044497 and JF421153, respectively) (**Table I**). The sequence B32\_3 was distantly (94%) related to a moderately thermophilic bacterium *Phenylobacterium lituiforme*, a member of *Alphaproteobacteria* (31). Within *Betaproteobacteria*, the sequence represented by B32\_55 was identified (98%) as *Aquicola* sp. THE-49 (JN128637), isolated from water reservoir (published only in GenBank®). The produced water contained different members of the class *Gammaproteobacteria*. DGGE bands B32\_4, B14\_35, S4\_70, GS6\_2 and GK8\_79 were affiliated (100%, 94%, 99%, 93%, 99%, respectively) to *Pseudomonas stutzeri* (**Table I**), a non-fluorescent denitrifying bacterium (32). The sequence from band B1\_20 showed a 100% similarity to *Acinetobacter* sp. VKPM 2838 (**Table I**). The genus

*Acinetobacter* comprises important soil organisms where they contribute to the mineralisation of aromatic compounds and they are suited to exploitation for biotechnological purposes, such as biodegradation (33). B8\_30 was related (96%) to *Marinobacter* sp. Trimyema-2, a thermophilic strain that was isolated from the hydrothermally heated sea floor at Vulcano Island, Italy (34). Members of the genus *Marinobacter* were also identified in the production water retrieved from a Dutch oil field (35). The sequence from B32\_2 was distantly related (93%) to *Thermithiobacillus* sp. ParkerM (HM173631) that is moderately thermophilic and obligately chemolithoautotrophic on reduced inorganic sulfur compounds (36). Another member of the class *Gammaproteobacteria* was close to the sequence of uncultured hydrocarbon seep bacterium (91% similarity) (AF154088) (**Table I**).

### 3.1.2 *Bacteroidetes*

8.3% of the sequences detected among the produced water samples fell into *Bacteroidetes* (**Figure 3**). The sequence of band B6\_14 was affiliated to unclassified *Chitinophagaceae*. It shared 99% identity with *Chitinophagaceae* bacterium F1 (AB535716), isolated from compost (**Table I**). DGGE bands B6\_16, S4\_67 and B8\_27 were identified (92% to 99% sequence identity) as uncultured *Bacteroidetes* bacteria (**Table I**). The sequences from S4\_67 and B8\_27 were related to uncultured bacteria that were taught as members of biocorroding microbiota colonising on steel surfaces immersed in coastal seawater (37).

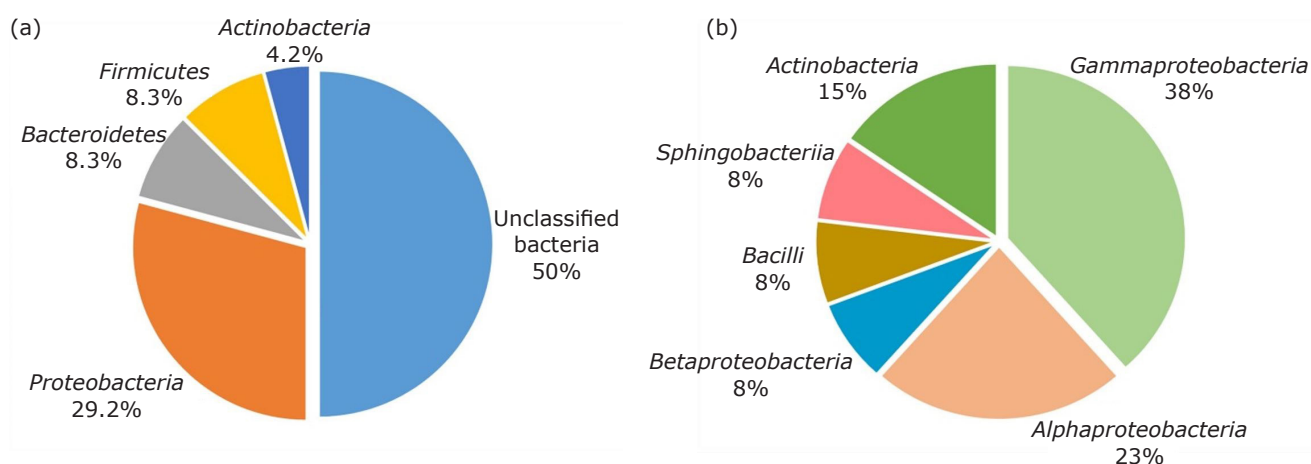


Fig. 3. Phylogenetic distribution of the 16S rRNA sequences of produced water samples from the Diyarbakir oil wells at: (a) the phylum level; and (b) the class level

**Table I Phylogenetic Affiliations of Bacterial Sequences Retrieved from Produced Water Samples Based on 16S rRNA Analysis and GenBank® Accession Numbers Assigned to these Sequences**

Well no.	DGGE band	Accession number	Closest BLAST® match	BLAST® accession number	Similarity, %	Phylum	Class	Isolation source
<b>B1</b>	B1_20	KF720883	<i>Acinetobacter</i> sp. VKPM 2838	JF891390	100	Proteobacteria	Gamma proteobacteria	—
	B1_23	KF720891	<i>Aeribacillus pallidus</i> strain MCM B-886	JN701188	89	Firmicutes	Bacilli	Petroleum reservoir
	B1_24	KF720893	Uncultured Firmicutes bacterium	HM041942	98	Firmicutes	—	Produced fluid
<b>B6</b>	B6_14	KF720818	<i>Chitinophagaceae</i> bacterium F1	AB535716	99	Bacteroidetes	Sphingobacteria	Compost
	B6_16	KF720830	Uncultured Bacteroidetes bacterium	FR871413	92	Bacteroidetes	—	Total copepod extracts
	B6_17	KF720793	Uncultured bacterium	GQ259593	98	—	—	Bioreactor
	B6_19	KF720802	Uncultured <i>Sphingomonas</i> sp.	EU044497	100	Proteobacteria	Alpha proteobacteria	Soil
	B6_20	KF720808	Uncultured Firmicutes bacterium	EU194836	96	Firmicutes	—	Charleston Harbor sediment
	B6_26	KF720798	<i>Coriobacteriaceae</i> bacterium enrichment culture clone B3113	HQ133029	100	Actinobacteria	Actinobacteria	Crude oil contaminated soil
<b>B8</b>	B8_27	KF720882	Uncultured Bacteroidetes bacterium	EF491430	99	Bacteroidetes	—	Steel surfaces immersed in marine water
	B8_29	KF720887	Uncultured bacterium	FJ628289	96	—	—	Brackish water from anoxic fjord Nitinat Lake at depth of 50 m
	B8_30	KF720888	<i>Marinobacter</i> sp. Trimyema-2	AJ292528	96	Proteobacteria	Gamma proteobacteria	The hydrothermally heated sea floor

(Continued)

Well no.	DGGE band	Accession number	Closest BLAST® match	BLAST® accession number	Similarity, %	Phylum	Class	Isolation source
<b>B14</b>	B14_35	KF720804	<i>Pseudomonas stutzeri</i>	HQ189755	94	<i>Proteobacteria</i>	<i>Gamma proteobacteria</i>	Water/soil mix pile of samples from oil wells
	B14_37	KF720814	Uncultured <i>Caenispirillum</i> sp. clone Ppss_Ma27	JF421153	99	<i>Proteobacteria</i>	<i>Alpha proteobacteria</i>	Petroleum-contaminated saline-alkali soil with phytoremediation
	B14_38	KF720820	Uncultured bacterium	FN429535	98	—	—	Wastewater of oil refinery treatment plant
	B14_39	KF720825	<i>Georgenia daeguensis</i>	HQ246163	100	<i>Actinobacteria</i>	<i>Actinobacteria</i>	Activated sludge from industrial wastewater treatment
<b>B23</b>	B14_41	KF720794	Uncultured bacterium	GQ457025	96	—	—	Rhizosphere
	B23_52	KF720810	Uncultured bacterium	FN401244	99	—	—	Domestic toilet biofilm
	B23_55	KF720826	<i>Aquicola</i> sp. THE-49	JN128637	98	<i>Proteobacteria</i>	<i>Beta proteobacteria</i>	Water reservoir
	B23_56	KF720831	Uncultured bacterium	HM921144	99	—	—	Groundwater from drinking water treatment plant
<b>B32</b>	B32_1	KF720792	Uncultured soil bacterium	AY221598	99	—	—	Metal and hydrocarbon contaminated soil
	B32_2	KF720796	<i>Thermithiobacillus</i> sp. ParkerM	HM173631	93	<i>Proteobacteria</i>	<i>Gamma proteobacteria</i>	—
	B32_3	KF720801	<i>Phenyllobacterium lituiforme</i>	AY534887	94	<i>Proteobacteria</i>	<i>Alpha proteobacteria</i>	Subsurface aquifer
	B32_4	KF720807	<i>Pseudomonas stutzeri</i>	FJ345693	100	<i>Proteobacteria</i>	<i>Gamma proteobacteria</i>	Area contaminated by crude oil and chemicals
<b>B56</b>	B56_17	KF720903	Uncultured <i>Firmicutes</i> bacterium	HM041942	97	<i>Firmicutes</i>	—	Produced fluid

(Continued)

Well no.	DGGE band	Accession number	Closest BLAST® match	BLAST® accession number	Similarity, %	Phylum	Class	Isolation source
<b>GK8</b>	GK8_79	KF720828	<i>Pseudomonas stutzeri</i>	JF727663	99	<i>Proteobacteria</i>	<i>Gamma proteobacteria</i>	Petroleum-contaminated saline-alkali soils
	GS6_1	KF720832	Uncultured bacterium	JN030519	99	—	—	Fissure water collected from a borehole
<b>GS6</b>	GS6_2	KF720839	<i>Pseudomonas stutzeri</i>	JN228329	93	<i>Proteobacteria</i>	<i>Gamma proteobacteria</i>	—
	GS6_4	KF720852	Uncultured bacterium	JF497820	90	—	—	Activated sludge
	GS6_5	KF720858	Uncultured marine bacterium	FM211087	90	—	—	Microcosm experiment
	M3_28	KF720855	Uncultured bacterium PHOS-HE31	AF314430	99	—	—	Batch reactor
<b>M3</b>	M3_31	KF720872	Uncultured bacterium	HM921144	98	—	—	Groundwater from drinking water treatment plant
	M3_32	KF720877	Uncultured bacterium	HQ538639	99	—	—	Bulking activated sludge
	M3_34	KF720844	Uncultured bacterium	AB231448	99	—	—	Enhanced biological phosphorus removal (EBPR) sludge
	K35_44	KF720884	Uncultured hydrocarbon seep bacterium BPC028	AF154088	91	<i>Proteobacteria</i>	<i>Gamma proteobacteria</i>	Hydrocarbon seep sediment
<b>K35</b>	K35_46	KF720889	Uncultured bacterium	HM921144	98	—	—	Groundwater from drinking water treatment plant
	K35_48	KF720894	Uncultured bacterium	FJ623379	97	—	—	Batch reactor
	K35_49	KF720896	Uncultured bacterium	AB231448	99	—	—	EBPR sludge

(Continued)

Well no.	DGGE band	Accession number	Closest BLAST® match	BLAST® accession number	Similarity, %	Phylum	Class	Isolation source
<b>S4</b>	S4_67	KF720806	Uncultured <i>Bacteroidetes</i> bacterium	EF491430	92	<i>Bacteroidetes</i>	—	Steel surfaces immersed in marine water
	S4_68	KF720811	Uncultured bacterium	FJ628289	96	—	—	Brackish water from anoxic fjord
	S4_70	KF720823	<i>Pseudomonas stutzeri</i>	JN228329	99	<i>Proteobacteria</i>	<i>Gamma proteobacteria</i>	—
	S4_73	KF720795	Uncultured bacterium	JF514265	100	—	—	Sea
<b>S15</b>	S15_60	KF720886	Uncultured hydrocarbon seep bacterium BPC028	AF154088	91	<i>Proteobacteria</i>	<i>Gamma proteobacteria</i>	Hydrocarbon seep sediment
	Y18_70	KF720889	Uncultured bacterium	HM921144	98	—	—	Groundwater from drinking water treatment plant
<b>Y30</b>	Y30_66	KF720889	Uncultured bacterium	HM921144	98	—	—	Groundwater from drinking water treatment plant
	Y30_68	KF720894	Uncultured bacterium	FJ623379	97	—	—	Batch reactor
	Y30_69	KF720896	Uncultured bacterium	AB231448	99	—	—	EBPR sludge

### 3.1.3 Firmicutes

Sequences belonging to members of *Firmicutes* accounted for 8.3% of the bacteria in the produced water (Figure 3). DGGE band B1\_23 was distantly related (89%) to *Aeribacillus pallidus* strain MCM B-886 (JN701188), isolated from petroleum reservoir (published only in GenBank®) (Table I). In addition, different strains of *Aeribacillus pallidus* (with sequence similarity values from 98% to 99.6%) were isolated previously from various geothermal sites of Turkey (38). DGGE band B6\_20 was distantly related (96%) to an uncultured *Firmicutes* bacterium, isolated from marine sediment in Charleston, South Carolina, USA (39). B56\_17 and B1\_24 were affiliated (97% and 98%, respectively) to an uncultured *Firmicutes* bacterium (Table I), detected in produced fluid from non-water-flooded high-temperature reservoir of the Niibori oilfield, Japan (40).

### 3.1.4 Actinobacteria

The phylum *Actinobacteria* comprised 4.2% of the bacterial community recovered from the produced water (Figure 3). DGGE band B6\_26 displayed 100% sequence similarity to *Coriobacteriaceae* bacterium enrichment culture clone B3113 (HQ133029) isolated from crude oil contaminated soil of Shengli oil fields, China (41). The sequence B14\_39 was closely related (100% similarity) to an aerobic bacterial strain *Georgenia daeguensis* 2C6-43, isolated from an activated sludge sample collected from an industrial wastewater treatment plant in Daegu, South Korea (42). Although little is known about the presence of *G. daeguensis* in oil associated environments, it was reported that different strains of *G. daeguensis* were isolated from hydrocarbon contaminated soil of an industrial zone and oil-saturated soil under laboratory conditions (43–45).

## 4. Discussion

In order to increase our knowledge about microbial diversity, culture-dependent

and molecular-based approaches are used for describing the diversity of microbes. Molecular-based approaches such as PCR-DGGE methodology, which is a useful tool for monitoring the genetic diversity of complex microbial populations (26), provide valuable information about the microbial community structure and dynamics in nature. For these reasons, PCR-DGGE fingerprinting analysis of environmental samples was used in this study.

The choice of appropriate primers for PCR amplification is a crucial step to accurately characterise the microbial communities. In this study, primer pair (341F-GC/907R), targeting the V3-V5 region of the 16S rRNA gene fragment, was selected due to its suitability for DGGE analysis of bacterial populations in environmental samples (26). This primer pair designed by Muyzer *et al.* (27, 28) has been used predominantly for microbial community analysis (26).

The DNA yield obtained from produced water samples was very low. It is known that crude oil samples contain low amounts of biomass which makes DNA isolation difficult to achieve (46). In this study, the permit included taking up to 500 ml of oil/water mixture from each sampling point so that only *ca.* 25 ml of each produced water sample could be obtained. In this scope, the low sample volumes of produced water separated from the oil/water mixture may be a reason for the low amount of DNA. It was reported in other studies that higher sample volumes (100–4000 ml) of produced water were used for DNA isolation (35, 47–50). The low DNA yield affected the efficiency of the PCR technique and for this reason, a two-step PCR was applied to the produced water samples. Thus, a sufficient amount of PCR product for DGGE for the produced water samples could be obtained.

Bacterial communities associated with the produced waters was analysed by the PCR-DGGE approach. Although numerous bands were visible on the DGGE gel, only dominant bands could be excised and sequenced. Most of the sequences retrieved from produced water samples were related to unclassified bacteria. Different studies on oil reservoir microbiota have also shown that oil fields harbour new and still unidentified microbial species. For example, Lenchi *et al.* described microbial communities in production and injection waters from the Algerian oil fields. In their study, they detected that a large number of unclassified bacterial and archaeal sequences were found in the water samples (51). Furthermore, uncultured bacteria such as uncultured *Sphingomonas* sp. and uncultured *Caenispirillum* sp. clone

Ppss\_Ma27 were detected in our study. This result is consistent with the fact that the vast majority of microorganisms are uncultured and do not grow under laboratory conditions as stated by Lewis *et al.* (52). In order to isolate more microbes, an appropriate identification laboratory protocol should be followed. At this point, different strategies such as mimicking natural conditions *via* decreased nutrient, extended incubation times, the modification of isolating media formulations and different incubation parameters (for example, temperature) were suggested for the cultivation of microorganisms (53). For instance, pollutant degrader *Sphingomonas*, which seemed to be previously uncultured by nutrient-rich methods, could be isolated from crude oil contaminated soil by using an *in situ* method that mimics the original environment (54). In addition, culture-dependent investigation should also be supported by molecular techniques.

Based on the sequences, organisms related to known mesophilic bacteria were predominant in the produced water samples. In addition, some organisms related to thermophilic bacteria (*Aeribacillus pallidus*, *Marinobacter* sp. Trimyema-2, *Phenyllobacterium lituiforme* and *Thermithiobacillus* sp.) were also identified. Bacteria having different metabolic capabilities (denitrifying, biodegrading and sulfur removing bacteria) were also detected. In addition, bacteria which may cause biocorrosion on steel surfaces were detected.

The dominant bacterial phylum was the *Proteobacteria*. The members of this phylum were also frequently found in many other studies on microbial diversity of oil field produced waters (55–58). Moreover, it was stated that *Proteobacteria* are ubiquitous in oil reservoirs over all temperature ranges (59).

In this study, among the detected genera in produced water samples that potentially contain hydrocarbon degrading bacteria were *Aeribacillus*, *Acinetobacter*, *Sphingomonas*, *Marinobacter* and *Phenyllobacterium*. It has been known for years that the species belonging to these genera are capable of degrading hydrocarbons (6, 17, 60, 61). In addition, *G. daeguensis*, a hydrocarbonoclastic bacterium, was detected in produced water sample with a 100% sequence similarity. *G. daeguensis* has also been demonstrated as a potential microbe for bioremediation due to its hydrocarbon degradation ability (44). Further investigations are needed because our current knowledge of the metabolic capability of *G. daeguensis* is limited. Moreover, sulfur-oxidising *Thermithiobacillus* sp.

was also identified in produced water sample. Sulfur-oxidising bacteria, which oxidise the sulfur compounds produced by the activity of sulfate-reducing bacteria in oil reservoirs, may play a key role in the oil industry because they can be utilised to resolve processing problems such as reservoir souring (11).

*Pseudomonas* was the dominant genus detected among the produced water samples. *Pseudomonas stutzeri* was the species identified in five produced water samples. *P. stutzeri* was previously isolated not only from formation water, produced from the petroleum wells in Adıyaman (62), but also oil-contaminated soils in Batman petroleum refinery, Turkey (63). These two areas are close to the Diyarbakır region from where the samples in this study were collected and these findings show that *P. stutzeri* is distributed widely in south-eastern Turkey. In other different geographical areas, this species was also isolated from oil-associated environments, such as oil field production water (64), oil sludge (65) and oil contaminated soil (66). However, although *P. stutzeri* is often isolated from oil reservoirs, the origin of *P. stutzeri* in oil reservoirs is a debatable issue. Because oil reservoirs have low redox potentials and contain little oxygen, anaerobic microorganisms are considered as truly indigenous to oil reservoirs (67). In this regard, it is believed that *P. stutzeri*, most of whose strains are aerobes, is an exogenous organism inoculated into oil reservoirs during the oil production processes. Even if strains of *P. stutzeri* are introduced into oil reservoirs with injected fluids, they should adapt to the physicochemical characteristics of the reservoir to survive. At this point, it has been proposed that extreme reservoir conditions may act as special factors for the evolution of *P. stutzeri*, thereby forming mutant strains (68). Furthermore, *P. stutzeri*, being found in a wide variety of habitats, is known for its diverse metabolism. Some strains of *P. stutzeri* are capable of denitrification, degradation of aromatic compounds and nitrogen fixation (32). These metabolic features make *P. stutzeri* highly attractive for biotechnological processes, such as reservoir souring control (69), microbial enhanced oil recovery (64) and bioremediation of oil-polluted environments (65).

In undisturbed oil reservoirs, microorganisms are found in different phases such as reservoir fluid containing crude oil and formation water, and rock surfaces. While planktonic microbes thrive in the water phase, sessile microbes may attach to

oil or rock surfaces (59). In addition, biofilm may form on the metal surfaces of the pipes in the oil-producing wells (70). Oil microbiome studies focus mainly on the analysis of the water phase due to its easy sampling. However, it should be noted that the water phase itself contains only a minor portion of the microbes found in the oil reservoir (59). On the other hand, the sampling of sessile microbes is likely to be more challenging (70).

## 5. Conclusion

This study reported for the first time the bacterial community composition of produced water from Diyarbakır oil reservoirs as obtained by DGGE analysis of PCR-amplified 16S rRNA gene fragments. DGGE analysis of produced water samples demonstrated that the majority of the bacterial sequences belonged to unclassified bacteria, indicating that oil reservoirs harbour still undescribed microbial species. Among the classified bacteria, the members of *Proteobacteria* were more abundant. *Pseudomonas* was the dominant genus detected in the produced water. Although the members of *Pseudomonas* were known as exogenous organisms inoculated into oil reservoirs, *Pseudomonas stutzeri* was found in five produced water samples. Bacteria having different metabolic capabilities (denitrifying, biodegrading and sulfur removing bacteria) were also detected. It can be stated that the metabolic capacities of these bacteria make them potential candidates for utilising in biodegradation, bioremediation, the improvement of oil quality and oil recovery processes. The knowledge of the bacterial community composition in oil reservoirs of the Diyarbakır region obtained in this study will be of great interest for both scientific research and applications in the oil industry. To build on the data presented in this study, metagenomic analyses should be performed to explore the undescribed microbes.

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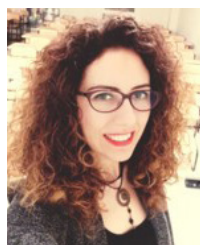
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