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Microbial Diversity of Non-flooded High Temperature Petroleum Reservoir in South of Iran

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Abstract

Introduction: Although bacteria and archaea are able to grow and adapted to the petrol reservoirs during several years, there are no results from microbial diversity of oilfields with high temperature in Iran. Hence, the present study tried to identify microbial community in non-water flooding Zeilaei (ZZ) oil reservoir.

Materials and methods: In this study, for the first time, non-water flooded high temperature Zeilaei oilfield was analyzed for its microbial community based on next generation sequencing of 16S rRNA genes.

Results: The results obtained from this study indicated that the most abundant bacterial community belonged to phylum of *Firmicutes* (*Bacilli*) and *Thermotoga,* while other phyla (*Proteobacteria*, *Actinobacteria* and *Synergistetes*) were much less abundant. *Bacillus subtilis*, *B. licheniformis*, *Petrotoga mobilis*, *P. miotherma, Fervidobacterium pennivorans*, and *Thermotoga subterranea* were observed with high frequency. In addition, the most abundant archaea were *Methanothermobacter thermautotrophicus*.

Discussion and conclusion: Although there are many reports on the microbial community of oil filed reservoirs, this is the first report of large quantities of *Bacillus* spp. from a high temperature oil reservoir.

Key words: Microbial Diversity, 16S rRNA, Next Generation Sequencing, Non-Water Flooded, High Temperature Oilfield

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Introduction

The severe conditions within the oil reservoir make it possible for certain microorganisms to adapt to these conditions. These bacteria have been adapted to reservoirs conditions during many years. Various groups of microorganisms isolated from oil reservoirs with diverse physiological and metabolic characteristics and phylogenetic affiliations including sulfate reducing bacteria (SRB), fermentative bacteria, nitrate or manganese and iron reducers, sulphidogens, acetogens and methanogens. These microorganisms influence the quality of crude oil and reservoir conditions during metabolism activities (1-3). Therefore, in recent years, many studies have been carried out to identify varieties of microbial populations inhabiting oil reservoirs around the world (4). High-temperature oilfields are always considered more than other oil fields (5-8), for extending new technologies, microbial enhanced oil and energy recovery, control of souring, bioremediation and further understanding of biogeochemical procedures and life in extreme environments (9).

By molecular techniques and non-dependent culturing methods, a much larger range of different thermophilic microorganisms were detected from high temperature oilfields. Most of Archaea belong to CO_2-reducing methanogens including *Methanobacteria*, *Methanococci* and *Thermococcales* and Bacterial sequences affiliated with *Firmicutes* and *Thermotoga* in high-temperature oil reservoirs (2, 5-8). Nevertheless, some moderate and non-thermophilic microorganisms were identified in various quantities in these oilfields. It has been hypothesized that the mesophilic microorganisms may originate from contaminant microorganisms that are capable to colonize the upper cooler parts of the oil well (9-11). Contamination of oilfields happens during drilling, sampling and enhanced oil recovery (EOR), especially during the water flooding processes (1, 9, 11, 12). It is difficult to distinguish indigenous microorganisms from allochthonous ones in these oil reservoirs.

In this study, for the first time, the microbial diversity of a non-water flooding oilfield with high temperature in southern Iran was evaluated using next generation sequencing of 16S rRNA gene analysis.

Materials and Methods

Reservoir Conditions: All inoculum sources originated from non-water flooding Zilaei (ZZ) oil reservoir which is located at the northeastern of Ahwaz, Iran, with a length of 3 km and a width of 8 km. According to internal report of National Iranian South Oil Company (NISOC, 2014), the study area was carbonate oil bed, having the salinity of 194000 ppm, with bottom-hole temperature of 85 - 90 °C, at a depth of approximately 3700 m above sea level. The reservoir contained light crude oil, holding an API gravity of 37 °C and pH of 8.0 (13).

Sampling: Five liters ZZ produced water sample were obtained from its oil-water processing site, during the period of July to December 2016. The bottles were filled completely and immediately taken to the laboratory for extraction of DNA (14).

DNA Extraction: All samples were filtered directly using 0.2-µm Startolab 150 V filter (Sartorius Biotech) and placed in 5 ml Homogenizer buffer (100 mM Tris–HCl (pH 8.2); 100 mM EDTA (pH 8); 1.5 M NaCl). The mixture was incubated under shaking conditions (150

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rpm) for 12 h at room temperature for re-suspended microbial cells. The metagenomic DNA extraction was performed in a harsh manner which combined several lysis methods together. For achieving better result in DNA extraction, the treatment of the glass bead was used along with lysis buffer. The lysis buffer was formulated according to the method developed by Siddhapura et al. (15). The re-suspended microbial cells were applied to the enzymatic buffer (Tris-HCl pH 8; 20 mM, EDTA pH 8; 10 mM and Triton X-100 1.2%) with 20 mg/ml lysozyme and incubated overnight under shaking conditions. Before the addition of lysis buffer, the tubes were vigorously mixed with 1g glass beads by a vortex and subjected to intermittent freeze thaw in the liquid N_2 treatment. The next steps for the chemical lysis and purification were continued according to Siddhapura et al (15). The concentrations of double-stranded DNA in the extracts were determined using the Quant-iT dsDNA Assay Kit and the Qubit fluorometer (Invitrogen, USA) .

16S rRNA Gene Amplicon Library and High-throughput Sequencing: The bacterial diversity was studied by pyrosequencing the amplified V1–V3 domain of the 16S rRNA gene amplifying a fragment of 520 bp by the 27F: 5'-AGAGTTTGATCCTGGCTCAG -3' and 534R: 5'- ATTACCGCGGCTGCTGG -3' primers (14). Ligated 454-adaptors were included in the forward primer, followed by a 10 bp Multiplex Identifier (MID). PCR amplification was done by 1X enzyme buffer, 0.2 mM dNTPs mixture (Fermentas), 1 U High-Fidelity DNA Polymerase (Fermentas), 0.5 µM of each primers (forward and reverse), 1.5 mM $MgCl_2$ and 2 µL of the DNA sample. The archaeal community analysis was performed by pyrosequencing the amplified 457 bp domain of the 16S rRNA gene amplifying by the 349F: 5'-GYGCASCAGKCGMGAAW -3' and 806R: 5'-GGACTACVSGGGTATCTAAT -3' primers with PCR reaction conditions as same as above (14, 16). The PCR incubation for bacterial and archaeal amplicon libraries products was performed according to Pournia and colleagues (14). PCR products were purified and quantified by the Qubit flourometer (Invitorgen, USA). The amplicon pool was used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Macrogen) according to the manufacturer's instructions using Titanium chemistry.

Bioinformatics Analysis: Raw reads were firstly filtered according to the 454 amplicon processing pipeline. Sequences were investigated by QIIME 1.6.0 software (17). Raw reads were demultiplexed and further filtered through the split library of QIIME. For 16S rRNA gene reads and the analysis were carried out as follows: sequences that passed the quality filter were denoised and singletons were excluded. OTUs defined by a 97% of similarity were picked using the uclust method and the representative sequences, selected as the most abundant in each cluster, were subjected to the RDPII classifier to get the taxonomy assignment and the relative abundance of each OTU using the Greengenes database (17).

Results

Rarefaction Curve: In this research, 6.88 µg archeal and 22.3 µg bacterial DNA per µL of the PCR product were obtained from the produced water of Zilaei oil field. During their sequencing, 6350 archeal and 14100 bacterial sequences were identified. The rarefaction curve is shown in Figure 1.

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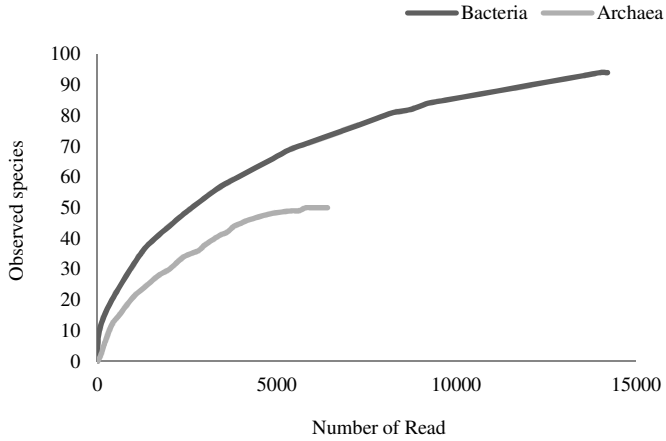

Fig. 1- Rarefaction Analysis of 16S rRNA Archaea and Bacteria Genes

Archaeal Community in Crude Oil: The 16S rRNA sequencing of archaeal genes showed that there are 7 different archeal species which belonged to the phylum of *Euryarchaeota* and one uncultured archaeon (Fig. 2). The family of *Methanobacteriaceae*, species of *Methanothermobacter thermautotrophicus* was observed with the frequency of 26.6% while *Methanothermobacter thermophilus*, *Methanotorris formicicus*, *Methanococcus aeolicus*, *Halosimplex carlsbadense*, *Haloplanus natans* and *Picrophilus oshimae* were characterized by less than 1% predominance (Table 1).

Table 1- Phylogenetic Affiliations and Relative Abundance of Archaeal Clone Library

Archaea/ Domain: Class	Family / Genus Species	Frequency (%Coverage)
Archaea / Unclassified	Unclassified; *Uncultured archaeon*	0.62
Euryarchaeota: Halobacteria	*Halobacteriaceae*; *Haloplanus natans*	0.62
Euryarchaeota; Halobacteria	*Halobacteriaceae*; *Halosimplex carlsbadense*	0.62
Euryarchaeota; Methanococci	*Methanococcaceae*; *Methanococcus aeolicus*	0.62
Euryarchaeota; Methanococci	*Methanocaldococcaceae*; *Methanotorris formicicus*	0.62
Euryarchaeota; Methanobacteria	*Methanobacteriaceae*; *Methanothermobacter thermautotrophicus*	26.66
Euryarchaeota; Methanobacteria	*Methanobacteriaceae*; *Methanothermobacter thermophilus*	0.62
Euryarchaeota; Thermoplasmata	*Picrophilaceae*; *Picrophilus oshimae*	0.62

Bacterial Community in Crude Oil: Phylogenetic association of bacterial 16S rRNA genes sequences regain from ZZ produced water oilfield included members of *Firmicutes*; *Bacilli* (41.63%), *Thermotogae* (24.69%), *Firmicutes*; *Clostridia* (0.53%), *Proteobacteria* (0.43%), *Actinobacteria* (0.1%), *Synergistetes* (0.04%), *Bacteroidetes* (0.02%), and unclassified *Bacteria,* MPZ 101 (1.56%) as shown in Figure 2.

Sequence alignment studies showed that the major group of bacteria belonged to the family of *Bacillaceae*, genera of *B. subtilis* and *B. licheniformis*. While other main bacterial groups included the family of *Thermotogaceae*, genera of *Petrotoga mobilis*, *Fervidobacterium pennivorans*, *Petrotoga miotherma* and *Thermotoga subterranea* were observed. In addition, one unclassified genus has been identified with the frequency of 1.56%. The other recognized bacteria in this oil field were abundantly less than 0.5% (Table 2).

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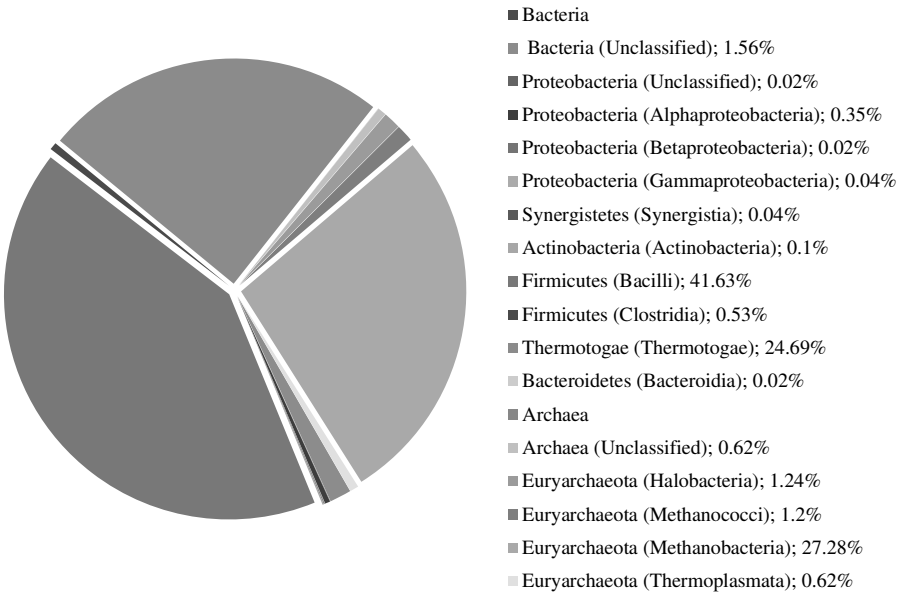

Fig. 2- Relative Abundance of Bacterial and Archaeal Groups within Clone Libraries

Discussion and Conclusion

Archaeal sequences with a close relationship to the family members of *Methanobacteriaceae*, genus of *Methanothermobacter*, had the greatest frequency in the samples taken from the ZZ reservoir (Table 1). The *M. thermautotrophicus* is a thermophilic, chemoautotroph, non-motive, obligatory anaerobic, which consumes H_2/CO_2 for methanogenesis (18). This methanogens and other species of *Methanothermobacter* have been reported many times from various oilfields at different temperatures by Orphan in South Elwood oil field, California (7), in Linch, Kalol and Nandasan oil wells, India (19), high temperature shengli oil reservoir, China (20-22), high temperature not water flooded Niburi oil field in Japan (23), high-temperature horizons of the Dagang and Kongdian oilfields, China (24-26), high temperature gas-water producing Niigata wells, Japan (27) and high temperature, water-flooded Huabei Petroleum (28).

Resource studies show that no comprehensive study has been carried out to identify methanogens in Iranian oilfields. However, Pournia et al. (14) by molecular methods identified several different methanogens from two non-water flooded oil reservoirs (LA and HK, located south of Iran) with an average temperature of 54 and 45 °C, respectively. The genus *Methanofollis* was observed with high presence, while genera of *Methanothermobacter*, *Methanobrevibacter, Methanomethylovorans* and *Methanothermococcus* were characterized with a lower abundance.

The major group of bacteria belonged to the division of *Firmicutes* (*Bacilli*) and *Thermotogae* (Fig. 2). Phylum of *Firmicutes* and *Thermotogae* are commonly more abundant taxa in non-water flooded oilfield, while in the flooded oil reservoirs, *Proteobacteria* is usually seen predominantly (7, 9, 23, 29). However, in this reservoir, only *B. subtilis* and *B. licheniformis* belonged to the phylum of *Firmicutes* which was present in large quantities, and the rest are much less abundant.

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Table 2- Phylogenetic Affiliations and Relative Abundance of Bacterial Clone Library

Bacteria/ Domain; Class	Family; Genus Species	Frequency (%Coverage)
Bacteria / Unclassified	Unclassified; *Uncultured bacterium (MPZ 101)*	1.56
Proteobacteria; Unclassified	Unclassified; *Uncultured proteobacterium*	0.02
Proteobacteria; Alphaproteobacteria	Unclassified; *Uncultured alphaproteobacterium*	0.06
Proteobacteria; Alphaproteobacteria	*Sphingomonadaceae*; *Sphingomonas sp. EK-1*	0.24
Proteobacteria; Alphaproteobacteria	*Rhizobiaceae*; *Sinorhizobium sp. R-25067*	0.02
Proteobacteria; Alphaproteobacteria	*Methylobacteriaceae*; *Methylobacterium rhodesianum*	0.03
Proteobacteria; Betaproteobacteria	Unclassified; *Uncultured betaproteobacterium*	0.02
Proteobacteria; Gammaproteobacteria	*Enterobacteriaceae*; *Enterobacter aerogenes, E. cloacae*	0.04
Synergistetes; Synergistia	*Synergistaceae*; *Aminobacterium colombiense*	0.02
Synergistetes; Unclassified	Unclassified; *Synergistetes bacterium SGP1*	0.02
Actinobacteria; Actinobacteria	*Propionibacteriaceae*; *Propionibacterium acnes*	0.06
Actinobacteria; Actinobacteria	*Actinomycetaceae*; *Actinomyces viscosus*	0.02
Actinobacteria; Actinobacteria	*Corynebacteriaceae*; *Corynebacterium matruchotii*	0.02
Firmicutes; Bacilli	*Bacillaceae*; *Bacillus subtilis*	35.53
Firmicutes; Bacilli	*Bacillaceae*; *Bacillus licheniformis*	4.42
Firmicutes; Bacilli	*Bacillaceae*; *Bacillus sp. (MPZ 103)*	0.45
Firmicutes; Bacilli	*Bacillaceae*; *Bacillus amyloliquefaciens, B. thermoamylovorans, B. pumilus, B. mojavensis, B. methanolicus, B. weihenstephanensis, B. hwajinpoensis, B. vallismortis, B. clausii, B. sp. 3EC2B1, B. sp. KHg1, B. sp. RH219*	0.63
Firmicutes; Bacilli	*Bacillaceae*; *Virgibacillus necropolis*	0.02
Firmicutes; Bacilli	*Bacillaceae*; *Halobacillus karajensis, H. trueperi*	0.04
Firmicutes; Bacilli	*Paenibacillaceae*; *Paenibacillus polymyxa*	0.02
Firmicutes; Bacilli	*Thermoactinomycetaceae*; *Laceyella sacchari*	0.02
Firmicutes; Bacilli	*Staphylococcaceae*; *Staphylococcus epidermidis*	0.18
Firmicutes; Bacilli	*Streptococcaceae Lactococcus lactis*	0.2
Firmicutes; Bacilli	*Lactobacillaceae*; *Lactobacillus curvatus, L. sp. (MPZ 102)*	0.06
Firmicutes; Bacilli	*Streptococcaceae*; *Streptococcus parauberis, St. gordonii, St. gallolyticus*	0.06
Firmicutes; Clostridia	*Clostridiaceae*; *Clostridium sp. Kas107-2, Cl. sp. MK11*	0.24
Firmicutes; Clostridia	*Lachnospiraceae*; *Butyrivibrio fibrisolvens*	0.23
Firmicutes; Clostridia	*Peptococcaceae*; *Desulfosporosinus sp. DB*	0.02
Firmicutes; Clostridia	*Thermoanaerobacteraceae*; *Moorella thermoacetica*	0.02
Firmicutes: Clostridia	*Ruminococcaceae*; *Ruminococcus albus*	0.02
Thermotogae; Thermotogae	*Thermotogaceae*; *Fervidobacterium pennivorans*	5.25
Thermotogae; Thermotogae	*Thermotogaceae*; *Thermotoga subterranea*	2.84
Thermotogae; Thermotogae	*Thermotogaceae*; *Petrotoga mobilis*	14.05
Thermotogae; Thermotogae	*Thermotogaceae*; *Petrotoga miotherma*	2.55
Bacteroidetes; Bacteroidia	*Porphyromonadaceae*; *Tannerella forsythia*	0.02

A variety of species related to the genus of *Bacillus* have been recognized from different oil reservoirs such as in high-temperature water-flooded reservoir, China (5), Caratinga and Barracuda high temperature oil fields in the Rio de Janeiro (10), in the LA and HA oilfields, Iran (14), oily sludge and petroleum muck, India (30, 31), high temperature Shengli oilfield, China (32), and Potiguar oilfield, Brazil (33). Furthermore, Cunha and colleagues (34) recovered *Bacillus* sp. (*B. cereus* and *B. licheniformis*) from core samples of a high temperature Virgin oil reservoir (Brazil) and suggested that these bacteria were autochthonous in such environments. It seems that the existence of the spore and use of other compounds like nitrate as the final receptor of the electron during respiration, help them to survive in harsh conditions and penetrate deeply into petroleum reservoirs (35). This genus was

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biotechnologically useful through much diversified metabolic pathway. Potentials of production of some alcohols, organic acids, gas, biopolymer and biosurfactants make them the ideal bacteria to be used in bioremediation of oil pollution and the MEOR technology (13, 32, 35). To the best of authors' knowledge, *Bacillus* sp. has not been reported in large quantities from high temperature oil reservoirs.

In contrast, several genera of the phylum of *Thermotoga* have been reported frequently from heated geothermal environments and different high temperature oilfields (36). *Thermotogaceae* currently comprises of the genera, *Thermotoga*, *Thermosipho*, *Fervidobacterium*, *Geotoga*, *Petrotoga*, *Marinitoga*, *Kosmotoga*, *Thermococcoides* and *Oceanotoga* (36). All members have a characteristic outer sheath like structure (toga) and some species are able to reduce sulfur compounds and produce H_2S (36, 37). The *Petrotoga* species have been reported only in oil reservoirs, but *Fervidobacterium* and *Thermotoga* are also isolated from the hot springs. Grassia et al. (38) isolated two bacteria that resembled *F. nodosum* from high temperature oil fields in Venezuela and Australia. The *F. pennivorans* was isolated from a hot springs in Portugal (36, 37, 39). However, to the best of authors' knowledge, this is the first report of the *F. pennivorans* from an oil field.

The abundance of other bacteria in this oil field was very low (less than 0.5%). It seems that lack of water flooding, high temperatures and salinity have caused relatively limited microbial diversity. The frequency of *Proteobacteria* was only about 0.43%, while these bacteria are often more abundant in flooded high-temperature oil fields (7, 9, 23, 29).

Comparing the results with its adjacent oilfields in southern Iran (LA and HK) showed that in the non-water flooded LA oilfield, *Thermotogae* (*Petrotoga*), *Firmicutes* (*Bacillus*) and *Synergistes* (*Thermovirga*) were more abundant. Nevertheless, microbial diversity of long-duration gas injection HK oil reservoir, were *Synergistetes* (*Anaerobaculum*), *Proteobacteria* (*Rhodocyclaceae*) and *Firmicutes* (*Bacillus*), respectively (14). However, in two water flooded oil fields in Oman, Middle East (hole temperature is about 64 °C), the major bacteria identified belonged to the *Thermotogae*, *Proteobacteria* and *Firmicutes* (40). It seems that EOR processes increase the microbial diversity and the frequency of *Proteobacteria* and *Synergistetes*, in these adjoining oilfields (14).

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بررسی تنوع میکروبی میدان نفتی سیلاب زنی نشده با دمای بالا در جنوب ایران

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چکیده

مقدمه: با وجود توانایی رشد و سازگاری باکتری‌ها و ارکی‌ها درون میدان‌های نفتی طی سالیان دراز، تاکنون بررسی جامعی برای شناسایی تنوع میکروبی میادین نفتی با دمایی بالا در ایران صورت نگرفته است. از این رو در این مطالعه تنوع میکروبی میدان نفتی سیلاب زنی نشده ذیلایی (ZZ) با دمایی بالا در جنوب ایران مورد شناسایی قرار گرفت.

مواد و روش‌ها: در این بررسی برای اولین مرتبه تنوع جمعیت های مختلف میکروبی میدان نفتی سیلاب زنی نشده ذیلایی با دمایی بالا به روش توالی یابی نسل نوین ژن های S rRNA۱۶ مورد ارزیابی قرار گرفت.

نتایج: نتایج بدست آمده در این تحقیق نشان می دهد که فراوان ترین باکتری های شناسایی شده متعلق به شاخه Firmicutes (Bacilli) و Thermotoga می‌باشند. سایر شاخه‌های باکتریایی (Proteobacteria , Actinobacteria و Synergistetes) در مقادیر بسیار کم شناسایی گردیدند. باکتری‌های Bacillus subtilis، B. licheniformis، Petrotoga mobilis، P. miotherma، Fervidobacterium pennivorans و Thermotoga subterranea با فراوانی بالا مشاهده شدند. از سویی دیگر فراوان‌ترین آرکی شناسایی شده نیز متعلق به Methanothermobacter thermautotrophicus بود.

بحث و نتیجه‌گیری: با وجود گزارش های زیادی از محققین مختلف در رابطه با بررسی میادین نفتی، برای اولین مرتبه وجود مقادیر زیاد گونه‌های مختلف باسیلوس درون یک میدان نفتی با دمایی بالا گزارش می گردد.

واژه‌های کلیدی: تنوع میکروبی، S rRNA۱۶، توالی یابی نسل جدید، سیلاب زنی نشده، میدان نفتی با دمای بالا

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