

Interactive comment on “Molecular analysis of the microbial community structures in water-flooding petroleum reservoirs with different temperatures” by L.-Y. Wang et al.

Dear Dr. Kirsten Küsel,

We would like to thank you and the reviewers for your time and efforts to review our manuscript.

We also thank the reviewers for their valuable comments and suggestions. The two reviewer’s comments were considered carefully and we have addressed the issues raised accordingly. Detailed answers to the reviewers’ comments are given below in the section Authors’ Responses to Reviewers Comments and Suggestions.

Thank you for your time and efforts.

Sincerely yours,

Bo-Zhong Mu

Professor

The corresponding author:

Prof Bo-Zhong Mu

State Key Laboratory of Bioreactor Engineering and Institute of Applied Chemistry

East China University of Science and Technology

130 Meilong Road, Shanghai 200237, P. R. China

E-mail: bzmu@ecust.edu.cn

Authors' Responses to Reviewers Comments and Suggestions

Anonymous Referee 1

Received and published: 14 June 2012

1. General comments: The article deals with a characterization of the microbial communities in water-flooding petroleum reservoirs with different temperatures. The community structure and diversity of Bacteria and Archaea in six reservoirs were elucidated via sequence analysis of 16S rRNA genes from clone libraries. The paper does not really have new findings. The solely DNA-based results do not provide information on the in situ activity of the studied microorganisms. Therefore, the study cannot draw real conclusions about the potential role of these communities. The introduction drives attention to the considerable potential of microorganisms in such systems in energy flow and nutrient cycling. Therefore, an RNA-based approach would have been more suitable to elucidate the active fractions of the studied communities. In addition, attempts to cultivate members of specific functional groups (e.g. methane producers, sulfate-reducers or yet not known thermophilic archaea) would have been useful.

Authors' response: We fully agree with the comment of this reviewer. Though RNA-based approach would have been more suitable to elucidate the active fractions of the studied communities, traditional PCR-based amplification of 16S rDNA remain the most rapid and convenient way to characterize the microbial community in petroleum reservoirs samples such as those described in our manuscript. To avoid misunderstanding in the interpretation of our data, the term "activities" has been omitted in the revised manuscript. Ongoing investigations of specific functional microbes that include anaerobic hydrocarbon-utilizing bacteria, methane producers, sulfate- and nitrate-reducers are being conducted via culture-dependent approaches. In addition, RNA-based approach will be adopted to investigate the activity of specific functional microorganisms in the enrichment cultures. The present work mainly focuses on the composition and distribution of microbial communities associated with different temperature horizons petroleum reservoirs.

2. The Abstract is too descriptive. Summarizing the results is not sufficient; an abstract has to clearly state the new findings of the study as well as the conclusions drawn by the authors.

Authors' response: We have modified the abstract as suggested. The new abstract is as shown below,

Abstract

Analysis of microbial communities from six water-flooding petroleum reservoirs at temperatures from 21 to 63 °C by 16S rRNA gene clone libraries indicates the presence of physiologically diverse and temperature-dependent microorganisms in these subterrestrial ecosystems. In samples originating from high-temperature petroleum reservoirs, most of the archaeal sequences belong to thermophiles affiliated with members of the genera *Thermococcus*, *Methanothermobacter* and the order *Thermoplasmatales*, whereas bacterial sequences predominantly belong to the phyla *Firmicutes*, *Thermotogae* and *Thermodesulfobacteria*. In contrast to high-temperature petroleum reservoirs, microorganisms belonging to the *Proteobacteria*, *Methanobacteriales* and *Methanomicrobiales* were the most encountered in samples collected from low-temperature petroleum reservoirs. Canonical correspondence analysis (CCA) revealed that temperature, mineralization, ionic type as well as volatile fatty acids showed correlation with the microbial community structures, in particular members of the *Firmicutes* and the genus *Methanothermobacter* showed positive correlation with temperature and the concentration of acetate. Overall, these data indicate the large occurrence of hydrogenotrophic methanogens in petroleum reservoirs and imply that acetate metabolism via syntrophic oxidation may represent the main methanogenic pathway in high-temperature petroleum reservoirs.

3. The Introduction gives an overview of studies in the field of oil reservoir microbiology. These investigations applied several (not only DNA-based molecular biological) approaches. On the contrary, the last paragraph with the aims of this study makes the reader insecure if techniques applied in this study are properly chosen to answer the questions linked to the potential role of bacteria and archaea in the petroleum reservoirs.

Authors' response: We totally agree with this reviewer that investigations cited in the introduction of our manuscript have applied several approaches (not only DNA-based molecular biological method). These investigations characterizing microorganisms of petroleum reservoirs provided a background for our research presented in this manuscript. The application of solely DNA-based techniques was only to provide a general survey of the microbial community structures associated with different temperatures petroleum reservoirs. As stated in comment 1 above, we are conducting some culture-based investigations on specific functional microbial groups.

4. In the Results section it does not become clear why the physicochemical parameters were grouped before analyzing the relationships between environmental factors and microbial community structures. The following sentence (Results, section 3.3) “The physicochemical

parameters of petroleum reservoirs were divided to three groups to better analyze the relationships.” does not really clarify the reason.

Authors’ response: Considering too many environmental factors, we have to carry out some artificial classification for these environmental factors.

5. The Discussion interprets the results linked to sequence similarities, showing that members of the studied communities were mostly related to sequences originating from similar environments, referring to the adaptation of these microorganisms to their habitat. Furthermore, some discussion aims to support this findings based on the results of correlation analyses on environmental factors and microbial communities. However, the discussion stays speculative as the DNA-based sequence information does not allow insights into the diversity and structure of metabolically active members of the investigated microbial communities.

Authors’ response: It is exact that DNA-based sequence information does not allow insights into the diversity and structure of metabolically active members of the investigated microbial communities. However, petroleum reservoir sampling is a very tough task and samples transportation back to laboratory needs time. In the samples treatment process, many microbial members will lose their metabolic activity due to the degradation of RNA. So, RNA-based analysis would probably result in inadequate understanding of the microorganisms from petroleum reservoirs. On the contrary, DNA-based analysis will better access the population information (not effected by microbial activity). Although DNA-based sequence information does not show their metabolic activity, we can speculative or extrapolate on their function when the identified microbes are present in samples collected from petroleum reservoir according to their mostly related sequences originating from similar environments. On this basis, we have decreased the tone on the “activity” in the revised version of the manuscript.

Anonymous Referee 2

Received and published: 17 June 2012

1. General comments: This paper (bg-2012-148) carefully studied about the bacterial and archaeal community structures in six water-flooding petroleum reservoirs with different temperatures (20 to 63 degree C). The microbial communities shifted from high-temperature (e.g. archaea: *Thermococcus*, *Methanothermobacter*, *Thermoplasmatales*, bacteria: *Firmicutes*, *Thermotogae*, *Thermodesulfobacteria*) to low-temperature (e.g. archaea: *Methanobacterium*, *Methanoculleus*, *Methanocalculus*, bacteria: *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*), which was a valuable information for microbial petroleum researchers. Canonical

correspondence analysis (CCA) revealed that temperature showed correlation with the microbial community structures. Furthermore, the physical, chemical, and geochemical characteristics (e.g. mineralization, ionic type, volatile fatty acids) of reservoirs also correlated with microbial community structures, and the syntrophic acetate oxidation demonstrated as the main methanogenic pathway in high-temperature petroleum reservoirs.

Authors' response: Thank you for your excellent comments.

2. The location of samples and characteristics of sampling spots (petroleum reservoirs) are critical to the results and conclusions. Information about the name of samples and physiochemical parameter were mainly listed in Table 1; however, there was little information about the sampling position. In order to help readers figure out the outline of samples and connect the results with the characteristics of samples more precisely, a brief statement or supplement information is suggested to be added in the section of 2.1 Collection of sample and nucleic acid extraction.

Authors' response: Thank you for pointing this to us. A statement for samples information has been added in the revised version of the manuscript. The content is shown below:

All microbial nucleic acid samples originated from oil reservoirs production waters sampled from six water-flooding oilfields in China, namely Zhan 3 block of Shengli oilfield, Baolige oilfield (Ba 18 and Ba 51 block), the Menggulin oilfield, No.7 and No. 6 blocks of Xinjiang Kelamayi oilfield. The Zhan 3 block of Shengli oilfield is located in Shandong province of China. This oilfield has been water-flooded for over 20 years. The depths of the sampling horizons are about 1,300 m with a temperature of 63 °C. The porosity of the reservoir was 30%, and air permeability was 0.8 μm^2 . The viscosity of the crude oil was 1720 mPas. The Menggulin (MGL) sandstone block in the MGL oilfield as well as the Ba19 fault block in the Baolige oilfield (Huabei Oil Field) are all located in the central part of Inner Mongolia, China. The distance between the two blocks is approximately 50 km. MGL oilfield has been water-flooded since 1989. Baolige oilfield has been water-flooded since 2001. The depths of the two blocks horizons ranged from 800 m to 1,500 m, with a temperature of 37~58 °C. The porosities of the reservoirs ranged from 17 to 25%. The No.7 and No. 6 blocks are located in Kelamayi oilfield of Xinjiang. The two oilfields are located in the Zhungeer Basin of northwestern China. The No.7 block has been water-flooded for over 40 years and the No.6 block for over 30 years. The depths of the two blocks horizons ranged from 480 m to 1,088 m, with a

typical low temperature of 21~32 °C. The temperatures and the mineralization of the six sampled petroleum reservoirs range from 21 to 63°C and 1301 to 11196 mg/L respectively and the pH of these production waters were neutral or slightly alkaline. The characterization of the petroleum reservoirs water sampled are listed in Table 1. In order to characterize the microbial community from the different temperature petroleum reservoir, these samples were grouped into two classes: high-temperature (45 ~ 63°C) and low-temperature (21 ~37°C).

3. Authors stated the presence of physiologically diverse and temperature-dependent microorganisms in these subterrestrial ecosystems. Samples S1 to S6 were from different production oil wells and the depth of sampling spots were different from 480 to 1490 meters. From Table 1, there was a high correlation between the depth and temperature. The temperature increased with the depth of sampling spots. Furthermore, authors agreed that the microbial population differs from one oil reservoir to another because of the variations in their physical, chemical, and geochemical entities (Discussion, line 3-4), therefore, it is necessary to sample a single oil reservoir with different depths. Based on the new results obtained from the same oil reservoir, the discussion between the relationship of temperature and community shift will be convincing.

Authors' response: We thank this reviewer for his good suggestions. In general, the oil layer is quite centralized in thickness, the thickness of oil layer is no more than 200 m and the difference in temperature is less than 5°C. Therefore, it is very difficult to sample different temperature from a single oil reservoir.

4. This article focused on the effect of temperature to microbial community. The results showed clear difference of main bacteria composition between low-temperature and high-temperature. There were few discussions about the comparison between the results of this study with other related researches. Authors should strengthen this part with statements and a table, and try to find out the highlight of this study.

Authors' response: A table and some statements have been added in the revised version of the manuscript. This information is shown below in Table 2:

Table 2 16S rRNA gene sequencing surveys conducted in different temperature petroleum reservoirs

This article

Other researches

Sample	Temp (°C)	Lineages detected	Oil reservoir site	Temp (°C)	Lineages detected	Reference
S1	63	<i>γ-Proteobacteria</i> , <i>Thermococcus</i> , <i>Thermogymnomonas</i> , <i>Halogeotricum</i> , <i>Methanosaeta</i>	Hubei oil field, China	75	<i>α, β, γ, ε-Proteobacteria</i> , <i>Firmicutes</i> , <i>Actinobacteria</i> , <i>Thermotogales</i> , <i>Nitrospira</i> , <i>Methanobacteriales</i> , <i>Methanococcales</i> , <i>Methanomicrobiales</i> , <i>Methanosarcinales</i>	Li et al., 2006, 2007
S2	58	<i>Firmicutes</i> , <i>Thermotogae</i> , <i>α, β, γ, ε-Proteobacteria</i> , <i>Bacteroidetes</i> <i>Methanothermobacter</i>	Troll oil formation, North sea	70	<i>Firmicutes</i> , <i>γ, δ-Proteobacteria</i> , <i>Thermotogales</i> , <i>Spirochetes</i> , <i>Bacteroidetes</i> , <i>Methanococcus</i> , <i>Methanolobus</i> , <i>Thermococcus</i>	Dahle et al., 2008
S3	45	<i>Firmicutes</i> , <i>β, γ</i> , <i>δ-Proteobacteria</i> , <i>Bacteroidetes</i> , <i>Thermodesulfobacteria</i> , <i>Methanomethylovorans</i> , <i>Methanothermobacter</i> ,	Multiple oil fields, California	70 ~ 75	<i>α, β, γ, δ-Proteobacteria</i> , <i>Bacteroidetes</i> , <i>Firmicutes</i> , <i>Methanomicrobiales</i> , <i>Methanosarcinales</i> , <i>Thermococcales</i>	Orphan et al., 2000
S4	37	<i>α, β, γ-Proteobacteria</i> , <i>Firmicutes</i> , <i>Chloroflexi</i> , <i>Actinobacteria</i> , <i>Methanolinea</i> , <i>Methanocella</i>	Qinghuang offshore oil field, China	65	<i>Firmicutes</i> , <i>Nitrospira</i> , <i>Thermotogae</i> , <i>α, β, γ</i> , <i>ε-Proteobacteria</i> , <i>Methanobacteriales</i> , <i>Methanococcales</i> , <i>Crenarchaeota</i>	Li et al., 2007
S5	32	<i>α, β, γ, ε-Proteobacteria</i> , <i>Firmicutes</i> , <i>Bacteroidetes</i> , <i>Actinobacteria</i> <i>Methanobacterium</i> , <i>Methanoculleus</i>	Schrader Bluff Formation of Alaska	27	<i>Proteobacteria</i> , <i>Firmicutes</i> , <i>Syntrophs*</i> , <i>WS6</i> , <i>Spirochaetes</i> , <i>Deferribacteres</i> , <i>Bacteroidetes</i> , <i>Chloroflexi</i> , <i>Thermotogae</i> , <i>Actinobacteria</i> , OP11, OP9, <i>Thermodesulfobacteria</i> , <i>Methanosaeta</i> <i>Methanoplanus</i> , <i>Methanolobus</i> , <i>Methanocalculus</i> , <i>Methanoculleus</i>	Pham et al., 2009
S6	21	<i>α, β, γ-Proteobacteria</i> , <i>Bacteroidetes</i> , <i>Firmicutes</i> ,	Pelikan lake oil field	18 ~ 20	<i>ε-Proteobacteria</i> <i>Methanomicrobiales</i> <i>Methanosarcinales</i>	Grabowski et al., 2005

In order to compare the results of this study with other related researches, the microbial community based on 16S rRNA gene sequencing surveys conducted on different temperature petroleum reservoirs from related references and this study were listed in Table 2. Obviously, in this study most of the thermophilic microorganisms including *Thermococcus*, *Thermogymnomonas*, *Methanothermobacter*, *Firmicutes* and *Thermotogae* were dominant, which are in line with other related high-temperature oil reservoirs researches such as the survey of the continental Huabei oilfield in China, the Troll oil formation in the North Sea and an offshore oilfield in Qinghuang,

China. In the low petroleum reservoir, the bacterial sequences affiliated with the phylum *Proteobacteria* were dominant; the same phenomenon has also been observed in other low temperature reservoir studies in Schrader Bluff Formation of Alaska and Pelikan lake oil field.

5. After obtaining some new results related to suggestions 2 and 3, it is possible to make substantial revision to the section of Conclusions.

Authors' response: It is difficult to sample different temperature from a single oil reservoir, so only a part of the content was added according to the second suggestion. Please see the Authors response 4. The conclusion (shown below) has also been reviewed according to some modifications made in the manuscript.

Conclusions

Overall, physiologically diverse and temperature-dependent microbial communities inhabit petroleum reservoirs. Thermophilic archaea including members of the *Thermococcus*, *Methanothermobacter* and *Thermoplasmatales* as well as bacterial sequences belonging to the phylum *Firmicutes*, *Thermotogae* and *Thermodesulfobacteria* are widely spread in high-temperature petroleum reservoirs. In contrast, archaeal sequences belonging to the genera *Methanobacterium*, *Methanoculleus* and *Methanocalculus*, and bacterial sequences closely related to members of the phylum *Proteobacteria* appear to be dominant in low-temperature petroleum reservoirs. These observations agree with several investigations indicating the occurrence of similar microorganisms in related subterranean ecosystems, especially oil reservoirs. Canonical correspondence analysis (CCA) and principal coordinates analysis (PCoA) showed a consistency between phylogenetic data and physico-chemical parameters for the sampled environments. On the basis of the data obtained, methanogenesis via syntrophic acetate oxidation is expected to be the dominant pathway in high-temperature petroleum reservoirs.

6. Some of the references cited in this article are too old and only about 30% of cited references are within 5 years. Since energy related researches are widely studied, authors should cite newer references.

Authors' response: Most of the “old” references are papers that reported for the first time the physiological and biochemical characteristic of microorganisms isolated from oil reservoirs fluids. Respective 16S rRNA gene sequences of those isolated microorganisms are mostly related to the petroleum reservoir microorganisms identified in the present work. So, it is necessary to keep those references as cited in the manuscript. In addition, we have added new references in the introduction section as suggested by this reviewer.

References

- Gray, N. D., Sherry, A., Grant, R. J., Rowan, A. K., Hubert, C. R. J., Callbeck, C. M., Aitken, C. M., Jones, D. M., Adams, J. J., Larter, S. R., and Head, I. M.: The quantitative significance of *Syntrophaceae* and syntrophic partnerships in methanogenic degradation of crude oil alkanes, *Environ. Microbiol.*, 13, 2957-2975, 2011
- Guan, J., Xia, L-P., Wang, L-Y., Liu, J-F., Gu, J-D., & Mu, B-Z. Diversity and distribution of sulfate-reducing bacteria in four petroleum reservoirs detected by using 16S rRNA and *dsrAB* genes. *Int. Biodeterior. Biodegrad.*, 1-9, 2012
- Li, W., Wang, L-Y., Duan, R-Y., Liu, J-F., Gu, J-D., and Mu, B-Z.: Microbial community characteristics of petroleum reservoir production water amended with *n*-alkanes and incubated under nitrate-, sulfate-reducing and methanogenic conditions, *Int. Biodeterior. Biodegrad.*, 69, 87-96, 2012.
- Pham, V. D., Hnatow, L. L., Zhang, S., Fallon, R. D., Jackson, S. S., Tomb, J-F., Delong, E. F., and Keeler, S. J.: Characterizing microbial diversity in production water from an Alaskan mesothermic petroleum reservoir with two independent molecular methods, *Environ. Microbiol.*, 11, 176-187, 2009.
- Zhou, L., Li, K-P., Mbadinga, S-M., Yang, S-Z., and Mu, B-Z.: Analyses of *n*-alkanes degrading community dynamics of a high-temperature methanogenic consortium enriched from production water of a petroleum reservoir by a combination of molecular techniques, *Ecotoxicology*, 21, 1680-1691, 2012