Identify the core bacterial microbiome of hydrocarbon degradation and a shift of dominant methanogenesis pathways in oil and aqueous phases of petroleum reservoirs of different temperatures from China

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Abstract

Microorganisms in petroleum reservoirs play significant roles in hydrocarbon degradation and the terminal electron accepting process of methanogenesis, they also contribute to microbial enhanced oil recovery (MEOR) worldwide with great economic and environmental benefits. Here, a molecular investigation, using the 16S rRNA and *mcrA* gene profiles based on MiSeq sequencing and clone library construction methods, was conducted on oil and water (aqueous) phases of samples of high (82-88°C), moderate (45-63°C) and low temperatures (21-32°C) from seven petroleum reservoirs in China. A core bacterial microbiome with a small proportion of shared operational taxonomic unit (OTU), but a high proportion of sequences among all reservoirs was discovered, including aerobic degraders, sulfate/nitrate-reducing bacteria, fermentative bacteria and sulfur-oxidizing bacteria distributed mainly in *Proteobacteria*, *Bacteroidetes*, *Deferribacteres*, *Deinococcus-Thermus*, *Firmicutes*, *Spirochaetae,* and *Thermotogae*. Their prevalence in the previously reported petroleum reservoirs and successive enrichment cultures suggests their common roles and functions involved in aliphatic and aromatic hydrocarbon degradation. The methanogenic process generally shifts from the dominant hydrogenotrophic pathway in aqueous phase to acetoclastic pathway in oil phase in high-temperature reservoirs, but the opposite was true for low-temperature reservoirs. No difference was detected between two phases in moderate temperature reservoirs. Physicochemical factors, including pH, temperature, phase conditions, and nitrate, Mn^{2+}, and Mg^{2+} concentrations are the main ones correlated to the microbial compositional and functional profiles significantly. LEfSe analysis shows distribution differences of microbial groups towards pH, temperature, and oil/aqueous phases. Tax4Fun functional profiling indicates major functional metabolism differences between the two phases, including amino acids, hydrocarbons in oil phase, and carbohydrates in aqueous phase.

Keywords: Reservoir; Core microbiome; Oil and aqueous phases; Temperature; Methanogenesis; Hydrocarbon

1 Introduction

Microbial enhanced oil recovery (MEOR) is one of the most feasible and profitable technologies for extracting residual oil from low-productivity reservoirs by utilizing microorganisms for hydrocarbon transformation via fermentation and methanogenesis as the major functional players in MEOR applications (Magot et al., 2000; Mbadinga et al., 2011; Mesle et al., 2013). Implementation of MEOR could also attenuate side-effects by injection of nitrate into oil wells to stimulate the propagation of nitrate-reducing bacteria (NRB) and inhibit the growth of sulfate-reducing bacteria (SRB), subsequently reducing sulfidogenic negative effects on corrosion and oil quality deterioration (Gao et al., 2013). Currently, investigations on compositional and functional profiles of petroleum reservoir microbiome have attracted attention from both scientists and engineers because the information will improve the current understanding on microbial diversity and function in oil reservoir systems and implement MEOR through manipulating microbial community composition and activities (Magot et al., 2000; Mesle et al., 2013; Youssef et al., 2009).

Petroleum reservoirs are often characterized as high temperature, and high pressure subterranean ecosystems, but low-temperature reservoirs also exist around the world (Grabowski et al., 2005; Li et al., 2013; Voordouw et al., 1996; Li et al., 2014). The physicochemical and geographic differences among petroleum reservoirs together with exogenous injection of microorganisms and nutrients during MEOR operation could affect the composition and functional groups of the indigenous microbial community. Many previously discovered bacterial and archaeal groups have important functional roles, e.g.,

sulfate-reducing, nitrate/nitrite-reducing and fermentative bacteria, thermophilic archaeal hydrocarbon degraders and methanogens as well as exogenous aerobic hydrocarbon degraders in the microbial community (Gao et al., 2015a; Gao et al., 2015b; Lenchi et al., 2013; Li et al., 2012a; Orphan et al., 2003; Wang et al., 2012; Grabowski et al., 2005; Pham et al., 2009). The common and specific roles and functions of these microorganisms in petroleum reservoirs are of great value to be comprehensively examined.

Methanogens cooperate with hydrocarbon degraders and fermentative bacteria synergistically to make the overall degradation processes thermodynamically favorable under largely anaerobic conditions (Mbadinga et al., 2011). Fermentative products from hydrocarbon degradation provide the essential growth substrates for methanogens with two major pathways, namely hydrogenotrophic and acetoclastic methanogenesis, and in many cases the syntrophic acetate oxidization pathway coupling with hydrogenotrophic methanogenesis is prevalent in both *in situ* and enrichment incubation *ex situ* (Mesle et al., 2013; Liang et al., 2015; Wang et al., 2012; Lee et al., 2015; Mayumi et al., 2011). However, very little is known on the influencing factors that shape the methanogenesis pathways and methanogen community structure and composition.

Petroleum reservoir is a complex system consisting of porous sandstones with oil, water and air. The microorganisms attached to the oil phase of petroleum fluids are largely neglected in most of the previous investigations due to technical difficulties in DNA extraction and sequencing (Kryachko et al. 2012). Oil-attached microorganisms influence the oil-water interface properties by the production of biosurfactants and metabolites or forming adsorbed colloidal particles to enhance oil recovery performance (Kobayashi et al., 2012; Kryachko et al., 2012; Wang et al., 2014). The distribution, function, and contribution of these microorganisms to MEOR success could be very different from those of the aqueous phase

(Kobayashi et al. 2012). Oil-degrading microorganisms, including thermophilic hydrocarbon-degrading archaea and methanogens, play different roles in the MEOR process (Mbadinga et al., 2011). Their compositional patterns and functional profiles towards temperature and oil/aqueous phase, are of great value to understand the mechanism of MEOR operation. Knowledges on the major microbial drivers, their potential functions, distribution characteristics and changing patterns towards environmental parameters should be one of the research directions for better understanding the MEOR process. In this study, the objectives were to study the compositional and functional differences of petroleum reservoir inhabiting microorganisms under different temperatures (high: 82-88°C, moderate: 45-63°C and low: 21-32°C); the methanogenesis pathways in oil and aqueous phases of these samples; and the influence of physicochemical factors on microbial community composition.

2 Materials and Methods

2.1 Characterization of geographic properties of sampling reservoirs

Petroleum production fluid samples were collected from seven areas across China, covering oilfields of different geographical locations and temperatures. The reservoirs and crude oil properties together with the aqueous phase chemical characteristics of this study are listed in Table 1. Detail reservoir properties are described in the Supplementary Materials.

2.2 Sample collection, ion concentration measuring, and DNA extraction

Each sample containing a mixture of crude oil and water was collected on site into a sterilized container after flushing each wellhead for at least 3-5 min. The containers were screwcapped to avoid air invasion and transported to the nearby laboratory immediately for further processing. The mixture was gently heated to 50°C to make it semi-fluid and then separated into oil and aqueous phases in a separatory funnel. The heating was operated as short as

116 possible according to the solidification degree of individual petroleum fluid samples, in order
117 to reduce the lysis effect of microbial cells within. Ion concentrations of the aqueous phase
118 were measured using Dionex 600 ion chromatography (Triad Scientific, Inc., Manasquan, NJ,
119 USA) following the manufacturer's instructions.
120
121 To obtain aqueous phase DNA, the aqueous phase of a sample after oil and water separation
122 was first filtered through a 0.22 polycarbonate membrane filter and a slice of the membrane
123 was used to extract DNA by AxyPrep™ Bacterial Genomic DNA Miniprep Kit according to
124 manufacturer's instruction (Axygen Biosciences, USA). For the oil phase, three volumes of
125 isooctane (2, 2, 4-trimethylpentane) were used to dissolve the crude oil, and then centrifuged
126 at 5000 rpm for 30 min to concentrate non-dissolved particulate and microbial cells for at
127 least three times to obtain enough materials. Repetitive DNA extractions were then conducted
128 on these materials and combined to meet the quantity requirement for the downstream quality
129 control. Finally, all DNA samples from aqueous and oil phases were measured by Nanodrop
130 for concentration and checked for DNA integrity by electrophoresis.

131 **2.3 Clone library construction and analysis**

132 The methyl-CoA enzyme-encoding gene (*mcrA*) primer pair, ME3MF&ME3MF-
133 e/ME2r'(Narihiro and Sekiguchi, 2011) and the 16S rRNA gene primer pair targeting
134 methanogens, MetA86f/ARC915r (Wright and Pimm, 2003; Narihiro and Sekiguchi, 2011;
135 Yu et al., 2008), were used to generate gene clone libraries for each sample (aqueous and oil
136 phases separately) according to the method established previously (Zhou et al., 2015) (Table
137 2).
138
139 FunGene pipeline chimera check was applied to check *mcrA* gene sequences using the
140 UCHIME *de novo* mode (Edgar et al., 2011). USEARCH software was used to check

chimeras of methanogenic 16S rRNA gene sequences using QIIME compatible SILVA 119 release SSURef database ("rdp_gold fasta") file as a reference. Then, for *mcrA* gene sequences, *de novo* OTU picking method was applied by QIIME at a cutoff value of 0.05 (Caporaso et al., 2010). Representative OTU sequences were aligned and inserted into the *mcrA* gene ARB database by maximum parsimony method without changing the initial tree topology (Angel et al., 2012; Ludwig et al., 2004). The phylogenetic affiliation was assigned, and taxonomic composition results were processed by QIIME accordingly (Figure S1). For methanogenic 16S rRNA gene sequence clustering and diversity analysis, the same method was applied via QIIME as described in the followings.

2.4 MiSeq sequencing and QIIME based analysis

The prokaryotic universal primer pair 515F/909R (Caporaso et al., 2012; Wang and Qian, 2009) and archaeal universal primer pair Arch347F/Arch806R (Takai and Horikoshi, 2000) were used to amplify samples of this study (both with barcodes attached to the forward primers, Table 2). Two PCR reactions were conducted and then combined to yield enough PCR products to compromise variations between different batches. Then, pooled PCR products of each sample with approximately 100 ng or 200 ng DNA were subjected to one MiSeq run. Sequencing samples were prepared using TruSeq DNA Kit according to the manufacturer's instruction. The library was uploaded to an Illumina MiSeq platform for sequencing with reagent kit v2 (2× 250 bp) or v3 (2× 300 bp) as described in the manufacturer's manual.

After merging paire-end reads from raw sequencing data by FLASH-1.2.8, fastx-toolkit was applied to split merged reads from one run into individual samples according to the primer barcodes (Magoc and Salzberg, 2011). Then, all sequences were split into each library with the name of each sample attached according to the barcode map using QIIME command

"split_libraries" (Caporaso et al., 2010). The criterion for filtering out underqualified sequences was "-s 15 -k -a 6 -r -l 150 -b 12 -M 5 -e 0". Chimera checking was conducted by USEARCH software using QIIME compatible SILVA 119 release SSURef database ("rdp_gold" fasta) file as the reference (Edgar, 2010). Clustering, picking OTU, taxonomy assignment, aligning, filtering alignments and phylogenetic tree construction, taxonomic composition summarizing, alpha and beta diversity analyses were conducted step-by-step by the QIIME pipeline with QIIME compatible SILVA 119 SSURef database as the reference (Caporaso et al., 2010). In clustering, "pick_open_reference_otus.py" command was used to conduct OTU dividing and BLAST method was used to assign taxonomy to input sequences. This subsampled open reference OTU picking method was the performed-optimized and optimal strategy suggested by the developers (Rideout et al., 2014). After OTU table "biom" files were generated, in order to get the bacterial community composition information from prokaryotic 16S rRNA gene primer amplified libraries, "filter_taxa_from_otu_table.py" command was introduced to only retain bacterial OTUs in the "biom" file. Similarly, exclusive archaeal and methanogenic OTU table files could also be processed from archaeal 16S rRNA gene primer amplified libraries. Summary information of each sample OTU abundance could be calculated by "biom summarize-table" command, and then the lowest number among all samples was chosen as the subsampling size to make each library acquire an even size using "multiple_rarefaction_even_depth.py" command. The taxonomic compositional table was drawn according to the subsampled biom file. Since there was no lanemask file available in this SILVA compatible 119 release SSURef database, alignment filtering method was performed independently with entropy threshold as 0.1 and gap filter threshold as 0.9 after obtaining aligned sequences by PyNAST method. Diversity parameters of each library could be generated by alpha diversity calling, with rarefaction curve, Good's coverage value, Shannon, Chao1, Simpson and PD whole tree indices calculated. Beta diversity, which delineates the dissimilarity relationship among samples, was generated and

visualized through unweighted & weighted UniFrac matrix and non-phylogenetic Bray-Curtis matrix method. The pairwise-shared OTU numbers were calculated from "biom" by the command "shared_phylotypes.py". Core microbiome (shared OTU table in a specific sample category) was identified by "compute_core_microbiome.py" command.

2.5 Diversity and statistical analysis

The statistical significance of community composition of samples among different categories was valued by anosim and adonis method, implemented in "compare_categories" command in QIIME. Mantel Test was used to compare the distance matrix of physicochemical parameters and UniFrac/Bray-Cutis distance matrix from beta diversity analysis by QIIME. For aqueous phase samples, both of the *in situ* physicochemical parameters and ion concentrations were used in the analysis; while only *in situ* physicochemical parameters were included for oil phase samples. The compositional bar chart and bubble chart were modified and illustrated from taxonomic summary results. Tax4Fun was used to predict the functional capabilities based on abundance profiles of microbial 16S rRNA gene datasets (Asshauer et al., 2015). LDA Effect Size (LEfSe) analysis was applied to illustrate the biomarker species with high statistical significance in different sample categories and the functional profiles statistically distributed in different sample categories (Segata et al., 2011).

2.6 Quantitative PCR on *mcrA* gene abundance

The quantitative PCR measurement was conducted using iTaq™ Universal SYBR® Green Supermix Kit (BIO-RAD). The qPCR mixture contained in 15 μl: 7.5 μl of supermix, 16 μg of BSA (Roche), and 1 μM final concentration of primer pair [ME3MF&ME3MF-e (250:1) and ME2r']. Annealing temperature was set to be the same as the clone library PCR setting, and the rest thermocycling settings were according to the manufacturer's instructions. Randomly picked one pMD18-T plasmid with *mcrA* gene inserted was used to make the

standard curve. The DNA template concentration was adjusted to 0-40 ng/μl. Results, which deviated significantly from values in the replicate groups, were omitted and undetermined results (under the detection limit) were also deleted. The property of final adjusted standard curve is $r^2 = 0.995$ and Eff% = 83.32.

2.7 Sequencing result deposition

For clone library sequences, KT314862-KT315353, were assigned to methanogenic 16S rRNA gene sequences; KT314340-KT314835 were assigned to *mcrA* gene sequences. For high throughput sequencing data based on Illumina MiSeq platform, PRJEB9855 was assigned to prokaryotic 16S rRNA gene sequencing data; PRJEB10996 was assigned to archaeal 16S rRNA gene sequencing data.

3 Results and Discussions

3.1 Common OTU among different categories and core bacterial microbiome

Community composition results showed that 21 bacterial phyla were obtained with an average abundance of more than 0.1% (Figure 1a), and three major archaeal phyla were *Thaumarchaeota*, *Euryarchaeota,* and *Crenarchaeota* (Figure 1b). Pairwise-shared OTU numbers of all samples indicated that, irrespective of combinations between aqueous and oil phase samples, the average numbers of shared bacterial OTUs ranged from 199.9 to 292.4, accounting for 26.6-36.2% of the total OTU numbers within individual samples; average numbers of shared archaeal OTUs ranged from 1.8 to 11.9, accounting for 8.5-23.4% of total OTU numbers within individual samples (Table S4). Core bacterial OTU numbers among aqueous, oil and all samples were 73, 57 and 46, which accounted for 7.1-10.1%, 5.9-10.3% and 4.5-8.3% of OTU numbers in individual samples. Core archaeal OTU number among

aqueous samples was only 3, accounting for 3.9-8.1% of OTU numbers in individual samples, and no archaeal OTU was shared among oil phase samples.

However, by investigating taxonomic profiles of core bacterial OTUs, the shared OTUs were 49, 41 and 34 genera in aqueous, oil and all samples, corresponding to 65.5%, 59.9% and 58.8% of average sequences in the total bacterial community, respectively (Tables S5 and S6). Most of the core bacterial OTUs belonged to the most abundant 36 genera, of which numbers of shared genera among aqueous, oil and all samples were 28, 23 and 23, respectively (Figure 2, and Tables S5 and S6).

There was no significant difference of shared bacterial OTU numbers within and between aqueous and oil phase samples, suggesting a core microbiome was shared among all components. The core OTUs covered around 2/3 of the total bacterial sequences, even though the percentages of core/total OTU number for individual samples were 4.5% to 10.3%. The core microbiome shared among all petroleum reservoirs could be the key participants mediating critical microbial processes, such as activation, degradation, fermentation, oil-emulsification and methane generation (Yamane et al., 2008; Wang et al., 2014; Pham et al., 2009; Orphan et al., 2000; Magot et al., 2000). This spectrum of core microbiome shares common functional roles in facilitating MEOR and is modified by the *in situ* physicochemical conditions of different reservoirs (Figure 2 and Table S5). It is important to connect the major microbial players, including their community compositions and specific functional capacities, to the interpretation of MEOR processes in the petroleum reservoirs. Meanwhile, the core microbiome serves as a good study target for primarily simplifying microbial participants and their roles in the petroleum reservoirs, and is useful for modeling and monitoring the MEOR processes for petroleum reservoirs from different locations. Moreover, substantial portions of aerobic bacteria being discovered in the core bacterial

264 microbiome across reservoirs imply that exogenous bacteria introduced into reservoirs by
265 water flushing can be also represented in the core composition and play important roles in
266 enhancing oil recovery (Gao et al., 2015b; Youssef et al., 2009).
267
268 Bacterial community distribution in aqueous phase showed correlation with temperature by
269 Mantel Test using unweighted UniFrac matrix method, and temperature also significantly
270 affected the dissimilar distance matrix of PCoA coordinates of the bacterial community by
271 both adnois and anosim statistical analyses (Tables S3, S7, and S8). However, temperature
272 changed the proportions of the taxa of the core microbiome significantly, but not the
273 presence/absence (Tables S3, S7, and S8). Consequently, the common genera discovered
274 from this study show that despite geographic and physicochemical differences, there is a core
275 microbiome with small OTU numbers but large sequence proportions in these petroleum
276 reservoirs across China which are possibly responsible for mediating hydrocarbon
277 degradation processes. Prevalent core genera discovered in this study also appear to be the
278 most abundant components as previously reported in petroleum reservoirs within and beyond
279 China (Gao et al., 2015a; Gao et al., 2015b; Li et al., 2012a; Orphan et al., 2003; Orphan et
280 al., 2000; Tang et al., 2012; Yamane et al., 2008; Dahle et al., 2008; Li et al., 2015; Liang et
281 al., 2015; Li et al., 2014) and enrichment cultures from petroleum reservoirs worldwide (Gray
282 et al., 2011; Li et al., 2012b; Liang et al., 2015; Wang et al., 2011).

283 **3.2 Shift of major methanogenesis pathways between oil and aqueous phases**

284 The methanogenic process generally shifts from the dominant hydrogenotrophic pathway in
285 aqueous phase to acetoclastic pathway in oil phase in high-temperature reservoirs. The
286 opposite is true for low-temperature samples, but no difference was detected between the two
287 phases in moderate temperature reservoirs (Figure 3). The different patterns of shifting of the
288 major methanogenesis mechanisms in aqueous and oil phases were evident in different

289 temperature petroleum reservoirs of this study. Quantitative measurements of *mcrA* gene in
290 both aqueous and oil phases of all samples are summarized in Supplementary Material.
291
292 The underlying methanogenesis mechanism could change substantially in reservoirs with
293 distinctive geochemical properties (Magot et al., 2000). The dominance of methanogenic
294 process mediated by methylotrophic methanogens is rarely observed in petroleum reservoirs.
295 It is claimed that there is a very low possibility that methyl-compounds could be generated
296 during the degradation of kerogen (Mesle et al., 2013). However, the dominance of obligate
297 methylotrophic *Methanolobus* was found in all water, rock and coal samples in a coalbed
298 methane reservoir within Eastern Ordos Basin of China (Guo et al., 2012). It could be
299 deduced that the prevalence of methylotrophic methanogenesis under certain conditions is
300 directly fueled by the existence and availability of methyl-containing substances. Since that
301 bacterial relative abundances in petroleum reservoir samples are always higher than those of
302 methanogenic archaeal community, the methanogenesis process might not be the dominant
303 process among all microbial processes. For instance, in P1 and P5 aqueous samples, sulfate
304 concentrations are considerably high (Table 1), geochemical conditions are more favorable
305 for sulfate reduction than methanogenesis. A large proportion of Firmicutes and
306 Deferribacteres in P5A are potentially responsible for the activities of sulfate reduction
307 according to their relative fractions (Figure 1) and functional capacities (Figure 2). It is
308 suggested the methylated compounds could be produced by the degradation of glycine
309 betaine (an osmoprotective agent), that are accumulated or generated by halophiles in saline
310 petroleum (Ollivier and Alazard, 2010). Subsequently, it fuels the growth of methylotrophic
311 methanogens in certain petroleum environments. Although, no molecular or chemical
312 evidence was reported for this process in petroleum environments, a pioneer study on
313 hydraulic fracturing in shales has detected glycine betaine as the major known
314 osmoprotectant in the produced fluids, and the combined metagenomic and metabolite

findings suggests the similar glycine betaine utilization pathways in fractured shales (Daly et al., 2016).

Numerous studies *in situ* or in enrichment incubation microcosms on aqueous phase of reservoir fluids claimed that syntrophic acetate oxidation associated with hydrogenotrophic methanogenesis is the major hydrocarbon degradation pathway (Wang et al., 2012; Mayumi et al., 2011; Lee et al., 2015; Mbadinga et al., 2012; Gray et al., 2011). The dominance of hydrogenotrophic methanogenesis in subsurface ecosystem could result from the external hydrogen originated from maturation of organic matters and/or mineral hydrolysis (Head et al., 2003), and the synergistic effect in association with acetate oxidizers, whereby acetate was firstly oxidized to H_2 and CO_2, then utilized by methanogenesis (Liu and Whitman, 2008). Additionally, a stable isotope labeling experiment on oil-degrading microcosm showed that despite coexistence of acetoclastic methanogenesis and acetate syntrophic oxidization in the initial state, the latter process prevailed over the former one when introducing low initial acetate concentration (Gray et al., 2011). The above evidence suggests that acetate syntrophic oxidization could exceed acetoclastic methanogenesis and contribute substrates H_2 and CO_2 to potentially favor hydrogenotrophic methanogenesis process.

It is still difficult to determine whether the temperature has directly or indirectly been involved in the alteration of the methanogenesis pathways. Reservoir fluid constituents may affect methanogenic degradation because crude oil and creosote inhibit acetoclastic methanogenesis (Warren et al., 2004) and volatile hydrocarbons (nC$_5$-nC$_{10}$) inhibit methanogenic degradation rate without changing the abundances of both hydrogenotrophic and acetoclastic methanogens (Sherry et al., 2014). Since most currently available community studies are based on the microbial assemblages within injection or production water, new understanding on the local microbiome distribution and changes shall focus on oil or

hydrophobic fraction of the reservoir fluids (Kobayashi et al., 2012; Tang et al., 2012; Lenchi et al., 2013). Consequently, the alteration pattern of major methanogenesis in the aqueous and oil phases under different temperature conditions could be further delineated. A combination of methods, including synthesis and quantification of degradation intermediate (Bian et al., 2015), stable isotope labeling on tracing substrate transformation (Gray et al., 2011) and molecular analysis of the metabolically active microorganisms can advance the studies on anaerobic degradation and methanogenesis processes in reservoir systems.

3.3 Physicochemical influence and Taxa & Function profiles

Temperature is an important physical factor shaping the community structure of bacterial (anosim & adonis $P < 0.01$) and methanogenic communities (anosim $P < 0.05$ & adonis $P < 0.01$) of the samples in this study (Figure 4 and Table S7). Furthermore, a significant difference of taxa abundance among the three temperature categories for both bacterial and archaeal communities was evident by LEfSe analysis (Figure 5). For bacterial community, sample group (aqueous or oil phases from the same sample group), temperature (anosim & adonis $P < 0.01$), and pH (adonis $P < 0.05$) showed significant effects on separating samples into different categories. For archaeal community, significant differences among sample categories were detected with sample group and temperature (both adonis $P < 0.05$), pH and phase (anosim & adonis $P < 0.05$). For methanogenic archaeal community, significant differences among sample categories were detected with sample group and temperature (sample group: anosim & adonis $P < 0.05$; temperature: anosim $P < 0.05$ & adonis $P < 0.01$) (Table S7).

Nitrate concentration dissimilarity matrix was significantly associated with all unweighted/weighted UniFrac and Bray-Cutis matrices (all $P < 0.05$) for aqueous bacterial community (Table S8). Meanwhile, methanogenic archaeal community in oil phase was

significantly affected by reservoir depth, temperature, pH and water flooding operation years based on association analysis of weighted UniFrac matrix but not unweighted UniFrac matrix (all $P < 0.05$) (Table S8), indicating that it was the abundance difference of certain taxa affecting the compositional pattern. More detailed relationships between physicochemical factors and bacterial/archaeal/methanogenic communities are summarized in Tables S7 and S8.

Nitrate dissimilarity matrix was significantly correlated with dissimilarity matrices of all aqueous phase bacterial communities using both unweight/weight UniFrac and Bray-Curtis matrices (all $P < 0.05$) (Table S8). Nitrate is an important chemical used in the injection water to inhibit corrosion and maintain crude quality (Gao et al., 2013). Nitrate stimulates the growth of nitrate-reducing bacteria and inhibits the growth of SRB (Nemati et al., 2001; Gao et al., 2013). Consequently, nitrate injection shapes the microbial communities in petroleum reservoirs. Mn^{2+} and Mg^{2+} were shown to be strongly associated with bacterial community of aqueous phase based on Bray-Curtis matrix (both $P < 0.05$) (Table S8). Metal ions can be electron acceptor for direct or indirect hydrocarbon degradation under anaerobic conditions (Mbadinga et al., 2011). Metal reducers could also utilize electrons from syntrophic partners to further facilitate direct aromatic hydrocarbon degradation (Kunapuli et al., 2007).

Differences in taxa and function profiles in oil and aqueous phases were analyzed based on LEfSe and Tax4Fun (Figures S2 and S3, and Table S9). For bacterial communities, amino acid metabolism and xenobiotics biodegradation and metabolism were distributed more in oil phase, while carbohydrate metabolism was distributed more in aqueous phase. For archaeal communities, ubiquinone and other terpenoid quinone biosynthesis and butanoate and tryptophan metabolism together with other glycan degradation pathways were distributed more in oil phase, while folding, sorting and degradation as well as several infectious

diseases related pathways, such as *Salmonella* infection, tuberculosis, were distributed more in the aqueous phase. Since FTU values of archaeal communities (fraction of taxa that could be mapped to existing KEGG pathway) were unevenly distributed from sample to sample, the reliability of these functional predictions is in question (Table S9). Meanwhile, the database of Tax4Fun is far from completeness, due to the enormous amount of uncultured microorganism and their unknown genomes. To date, there are still very limited studies on investigating inhabiting preference of microbiome in petroleum reservoirs. Meanwhile, their functional contributions to hydrocarbon degradation and methanogenesis in both aqueous and oil phases remain elusive, which calls for further efforts on this point (Kryachko et al., 2012; Wang et al., 2014; Kobayashi et al., 2012).

4 Conclusions

A core bacterial microbiome containing a small proportion of OTUs but a relatively large proportion of sequences mediating hydrocarbon degradation and fermentation was revealed by analysis of oil reservoirs of different temperatures. The core and common bacterial microbiome of major biodegrading functions were shared among geographically and physicochemically different reservoirs. The different shifting patterns of the dominant methanogenesis pathway in aqueous and oil phase within samples of different temperature were featured. Factors of pH, temperature, phase conditions and nitrate, Mn^{2+}, and Mg^{2+} concentrations shaped the microbial compositional and functional profiles significantly. Moreover, biomarker groups of bacteria and archaea associated with different pH, temperature and phase conditions indicate major differences in function of amino acid metabolism, xenobiotics metabolism enriched in the oil phase, and carbohydrate metabolism enriched in the aqueous phase.

Author contributions. ZZ, BL, L-YW, B-ZM, HS, and J-DG conceived the project and designed experiments. ZZ, BL, and L-YW conducted the sampling, chemical and molecular experiments. J-FL, B-ZM, and J-DG managed sample collection, supervised on data interpretation. ZZ performed the original data analysis and drafted the original manuscript. All members contributed to refining the manuscript and approved the final version.

Competing interests. The authors declare no conflict of interest.

Acknowledgments. Dr. Xiangzhen Li's group at Chengdu Institute of Biology, Chinese Academy of Sciences was thanked for MiSeq sequencing efforts and related technical support. Ms. Kelly Lau was thanked for her supportive technician work. We thank the support from local administrative and technical staff in Shengli, Daqing, Huabei, Xinjiang Karamay, and Jiangsu Oilfields. This projected was supported by NSFC/RGC (grant no. N HKU718/11, J-DG) and NSFC (grant no. 41373070 and 41530318, B-ZM) and a postgraduate scholarship from HKU (ZZ).

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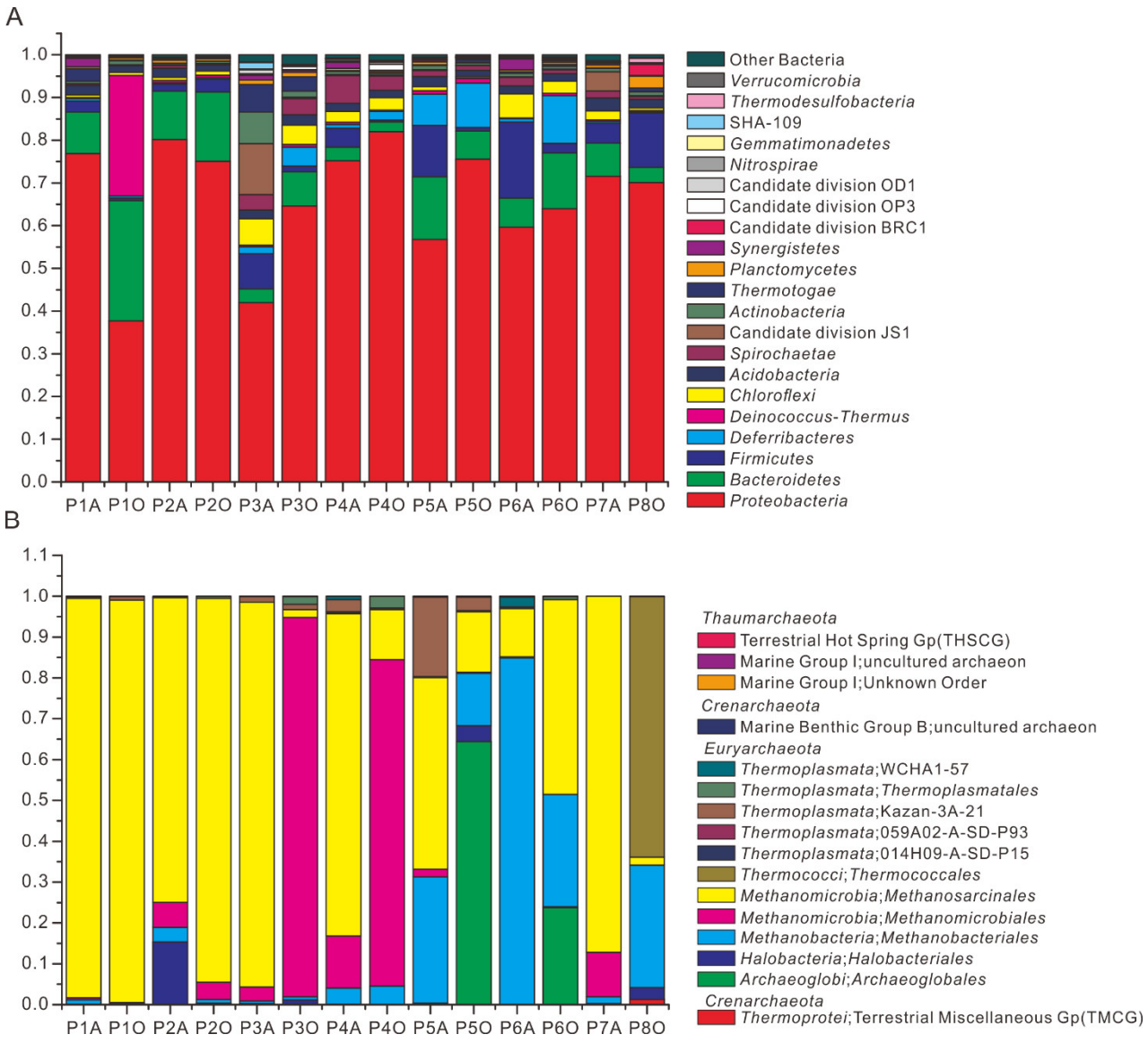

Figure 1. Relative abundance of bacteria (A) and archaea (B) from 14 aqueous and oil phase samples. Bacterial community was taxonomically assigned at the level of phylum. Those phyla with an average abundance of all samples below 0.1% were combined into "Other Bacteria" category. The archaeal community was taxonomically assigned at the level of class.

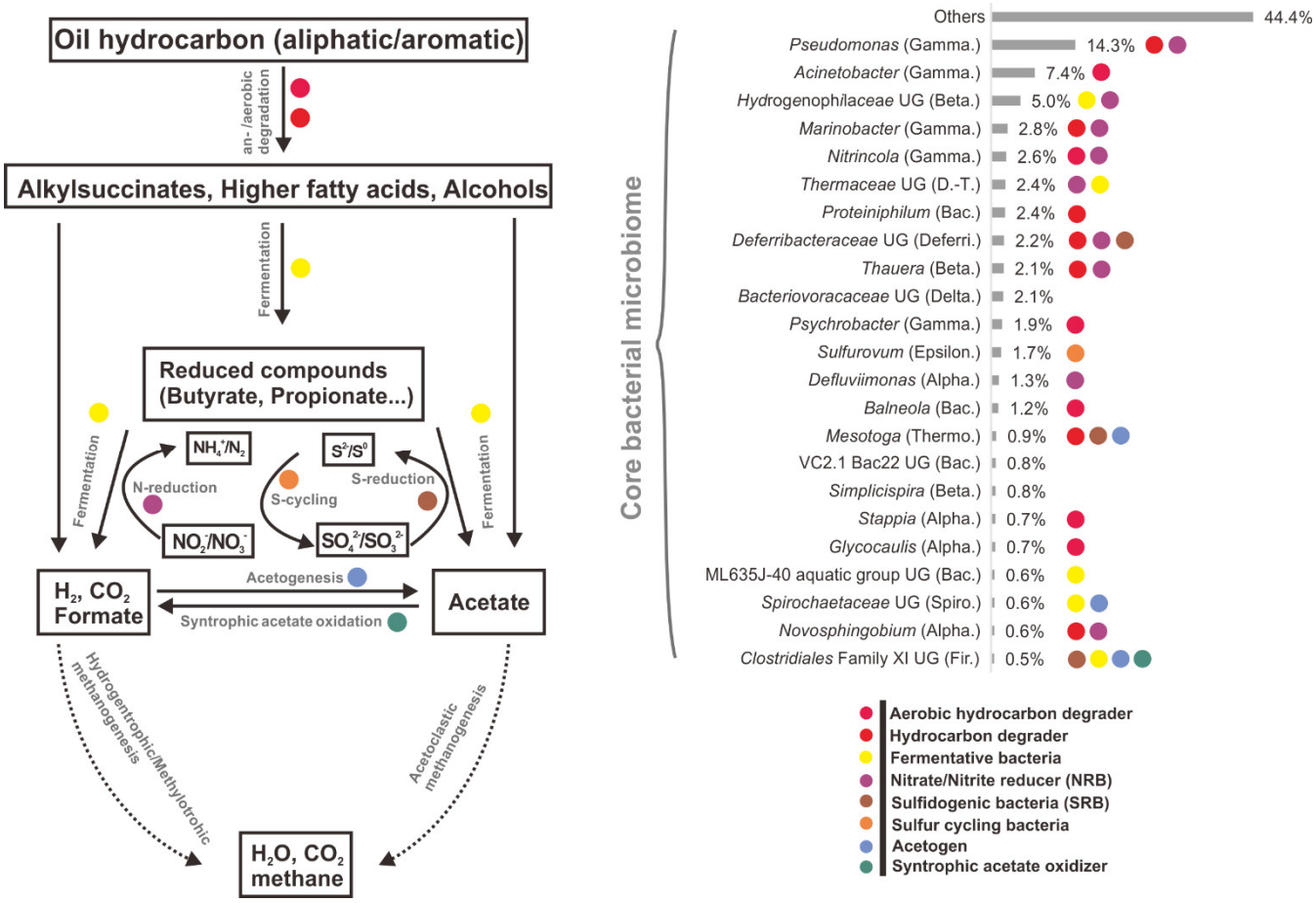

Figure 2. Average abundances and functional roles of core bacterial microbiome in all petroleum samples (including aqueous and oil phase samples). Core microbial OTUs (23 OTUs) were affiliated to the most abundant genera. The functional roles were assigned according to reference publications (Supplementary Material). Degradation pathway was modified from Mbadinga et al., 2011. Abbreviations: Alpha., Beta., Gamma., Delta., Epsilon (*Alpha-*, *Beta-*, *Gamma-*, *Delta-, Epsilonproteobacteria*), D.-T. (*Deinococcus-Thermus*), Bac. (*Bacteroidetes*), Deferri. (*Deferribacteres*), Delta. (*Deltaproteobacteria*), Thermo. (*Thermotogae*), Spiro. (*Spirochaetae*) and Fir. (*Firmicutes*). UG stands for uncultured genus.

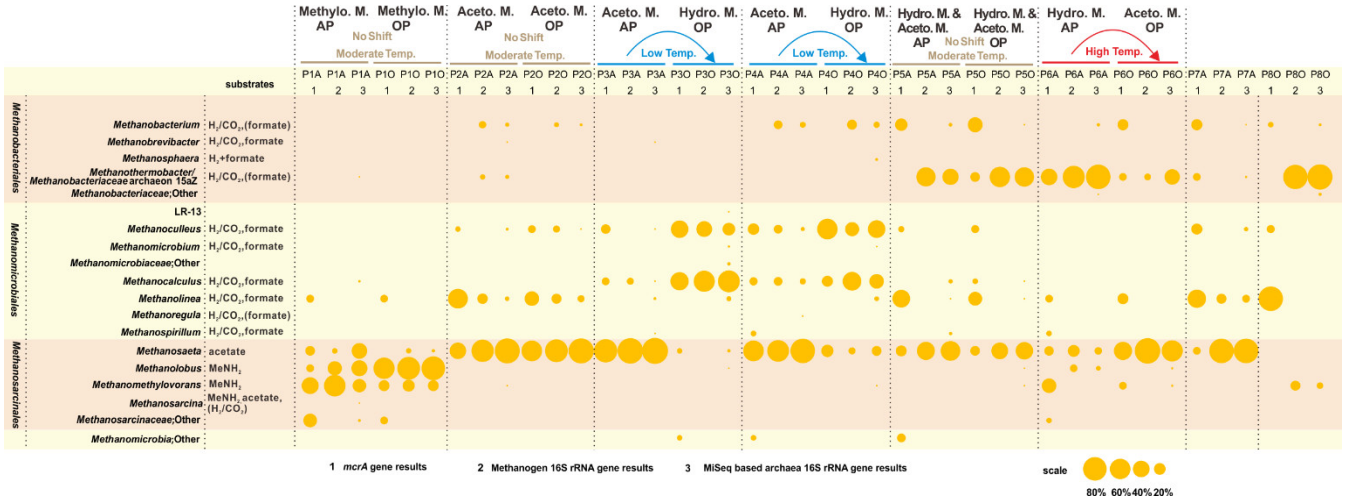

Figure 3. Bubble chart of the proportional composition of methanogens based on MiSeq sequencing results of archaeal 16S rRNA genes and clone sequencing results of *mcrA* gene and methanogenic 16S rRNA gene. The major substrate utilization properties were originated from Liu et al., 2008. $MeNH_2$ is methylamine and substrates in parentheses refer to being utilized by some but not all species. *Methanothermobacter thermautotrophicus* strain Delta H is the phylogenetically closest cultured clone to *Methanobacteriaceae* archaeon 15aZ. Thus, the combination of *Methanothermobacter* and *Methanobacteriaceae* archaeon 15aZ was used. Methanogenesis shift was based on the transition of major methanogenesis pathway. Abbreviations: Aceto. M., Hydro. M. and Methylo. M. (Acetoclastic methanogenesis, Hydrogenotrophic methanogenesis, and Methylotrophic methanogenesis).

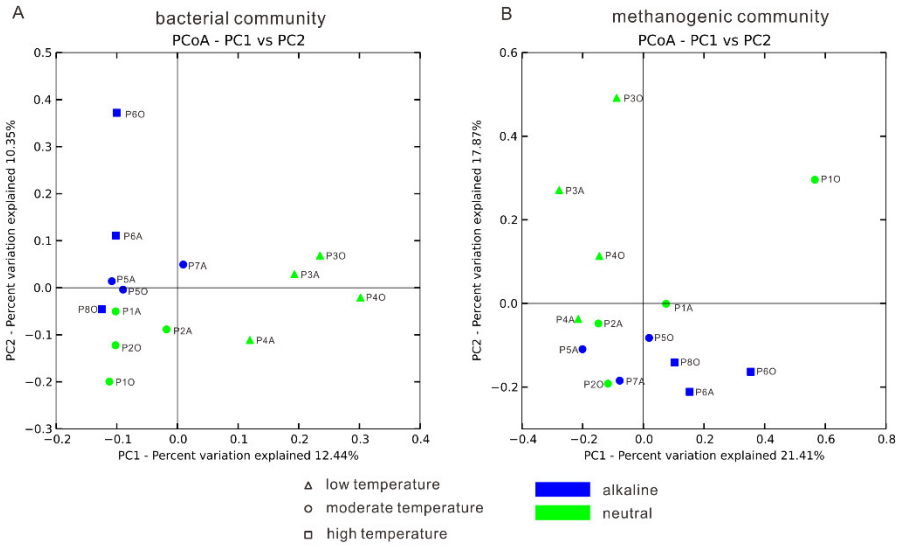

Figure 4. Principal Coordinate Analysis plot figures based on unweighted UniFrac matrices. Bacterial (A) and methanogenic (B) communities of 14 samples were separately analyzed to delineate the dissimilarity distances between each sample based on phylogenetic classification. The sample dots were categorized in terms of temperature (dot shape) and pH condition (color).

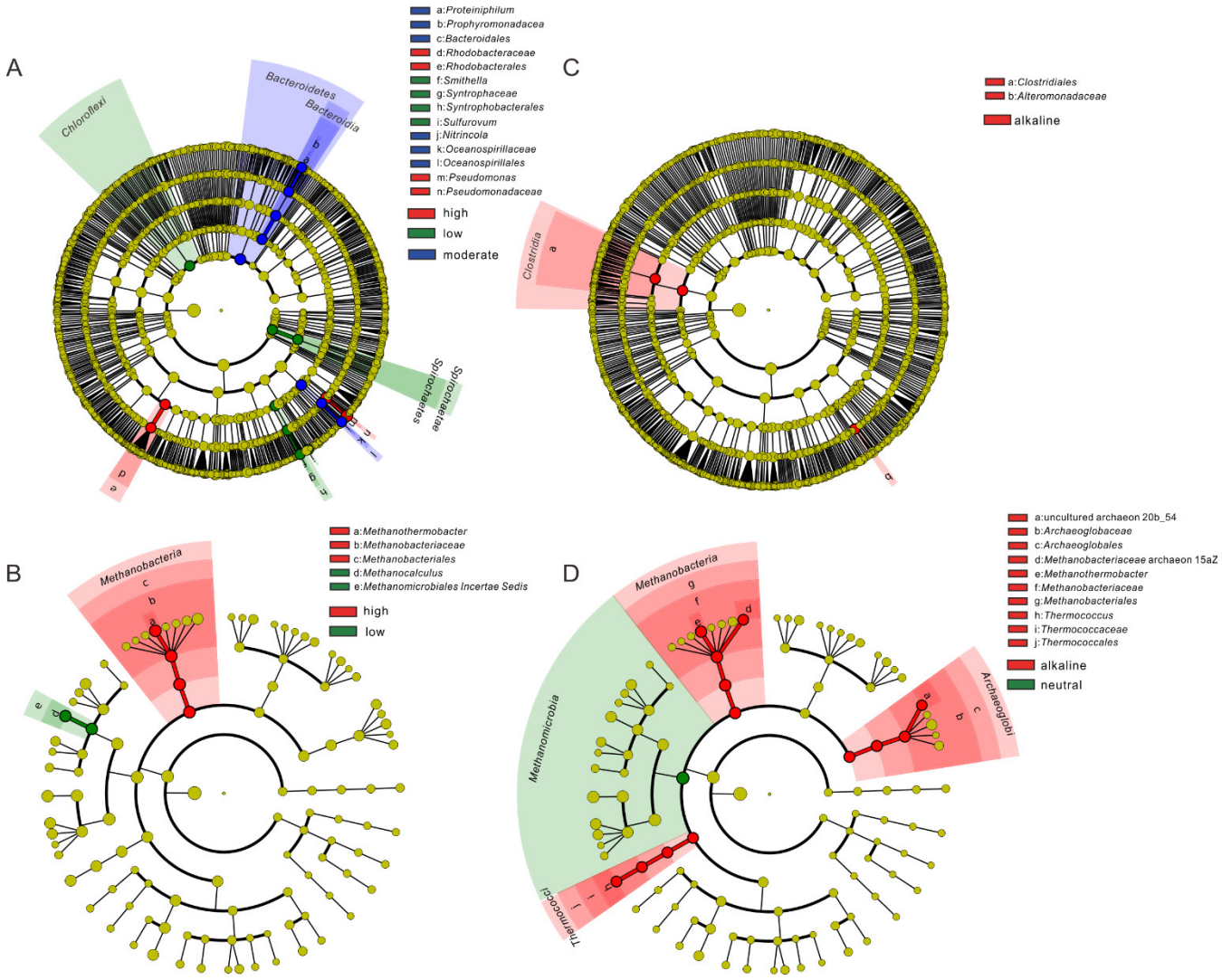

Figure 5. Cladogram based on LEfSe analysis results on bacteria (A, C) and archaea (B, D) in terms of temperature (A, B) and pH (C, D) categories. The taxonomic trees were generated from phylum to genus (inside to outside) in the hierarchical structure. Biomarker taxonomic levels were labeled in color which had at least logarithmic LDA score of 3.5. Pre-sample normalization was used to format the relative abundance data. All-against-all strategy was used in the multi-class analysis step.

Table 1. Reservoir geophysical properties and physicochemical properties of aqueous phase samples

Location name	Shengli-Zhan3-26	Huabei-B51	Xinjiang (Karamay)-Liuzhong	Xinjiang (Karamay)-Qixi	Jiangsu-Wei5	Jiangsu-Gao6	Daqing (Xingbei)-Xing4	Jiangsu-Qinying
Sample name	P1	P2	P3	P4	P5	P6	P7	P8
Depth (m)	~1300	~1101	~480	~1088	1018	1970	800-1200	2280-2524
Temp (°C)	63	45	21	32	53.5	82	49	88
pH	7.1	7.2	7.0	7.1	8.62	8.88	8.5	8.2
Effective porosity (%)	30	22.2	20.5	17.4	21.7	17.4	28.3	16.5
Average permeability ($\times 10^{-3}\mu m^2$)	800	12.6	466	274	91.8	94.5	481	22
Oil viscosity (mPa.s)	1720	402	417	44.8	292	8.4-83.9	13.86	98-188
Water flooding operation (years)	22	4	38	46	13	18	30	0
Cl^- (mg l^{-1})	3850	819	3864	2000	7563.01	1155.95	659.31	-
SO_4^{2-} (mg l^{-1})	2244	32.4	124.8	7.7	1921.06	156.81	333.29	-
PO_4^{3-} (mg l^{-1})	0.1	Nd	Nd	Nd	139.77	6.29	Nd	-
NO^{3-} (mg l^{-1})	Nd	Nd	34.1	1.4	Nd	0.44	0.45	-
Na^+ (mg l^{-1})	3313	1064	4196	5399	7363.52	1593.55	1363.43	-
K^+ (mg l^{-1})	94.2	22.3	35.1	45.6	9.36	3.26	3.55	-
Ca^{2+} (mg l^{-1})	195.6	53.0	103.3	128.2	17.42	7.24	41.94	-
Mg^{2+} (mg l^{-1})	46.1	17.6	44.7	64.0	10.27	2.25	11.02	-
Mn^{2+} (mg l^{-1})	0.3	0.1	0.3	0.4	Nd	Nd	Nd	-
Acetate (mg l^{-1})	32	57.9	344	6.97	44.07	285.66	Nd	-

"Nd" means not detected. No aqueous phase was obtained from Jiangsu-Qinying reservoir sample.

Table 2. PCR primers and PCR settings

	Primers	Sequences	Primer final concentration	Template DNA quantity	References
mcrA gene primer	ME3MF&ME3MF-e	ME3MF: ATGTCNGGTGGHGTMGGSTTYAC, ME3MF-e: ATGAGCGGTGGTGTCGGTTTCAC; concentration ratio = 250:1	1 μM	10-20 ng	(Narihiro and Sekiguchi, 2011)
	ME2r'	TCATBGCRTAGTTDGGRTAGT	1 μM	10-20 ng	
PCR setting	95°C2 min; 35 × (95°C 30 s, 59.5°C 30 s and 72°C 40 s); 72°C 5 min and 4°C 2 min.				
methanogenic 16S rRNA gene primer	MetA86f	GCTCAGTAACACGTGG	300 nM	10-20 ng	(Wright and Pimm, 2003; Narihiro and Sekiguchi, 2011; Yu et al., 2008)
	ARC915r	GTGCTCCCCCGCCAATTCCT	300 nM	10-20 ng	
PCR setting	95°C 5 min; 35 × (95°C for 30 s, 57°C for 30 s, and 72°C for 1 min); 72°C 10 min, 4°C 2 min.				
Prokaryotic universal primer	515F	GTGCCAGCMGCCGCGGTAA (12 nt barcode added)	1 μM	10 ng	(Caporaso et al., 2012; Wang and Qian, 2009)
	909R	GGACTACHVGGGTWTCTAAT	1 μM	10 ng	
PCR setting	94°C 3 min; 30 × (94°C 40 s, 56°C 60 s and 72°C 60 s); 72°C for 10 min, and 4°C for 2 min				
Archaeal universal primer	Arch347F	GYGCASCAGKCGMGAAW (12 nt barcode added)	1 μM	10 ng	(Takai and Horikoshi, 2000)
	Arch806R	GGACTACVSGGGTATCTAAT	1 μM	10 ng	
PCR setting	95°C for 5 min; 32 × (95°C 30 s, 50°C 30 s and 72°C 40 s); 72°C 10 min, and 4°C 2 min				