Distinct microbial composition and functions in an underground high-temperature hot spring at different depths

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Abstract:

The microbial diversity and functions of three high-temperature neutral hot springs water samples at different depths (0 m, 19 m and 58 m) were investigated based on 16S rRNA gene sequencing and a functional gene array (GeoChip 5.0). The results revealed that the bacterial communities were distinct at different depths in the hot springs. Additionally, in response to the depths, bacterial/archaeal community compositions exhibited shifts over the depth profiles. Aquificae, Alpha-proteobacteria, and Deinococcus-Thermus were the dominating phyla at 0 m, 19 m, and 58 m, respectively. Hydrogenobacter, Sphingobium, and Thermus were the most abundant genera at 0 m, 19 m, and 58 m, respectively. The phylum Thaumarcheota was the most abundant member of the archaeal community in the samples at different hot spring depths. Functional results of the microbial communities indicated that microbial metabolic functions were mainly related to sulfur, nitrogen cycling, and hydrogen oxidation. In summary, our
results demonstrated that distinct microbial communities and functions were found at different depths of hot springs in a very limited area. These findings will provide new insights into the deep-subsurface biosphere associated with terrestrial hot springs.

Keywords: Hot springs, Microbial diversity, Functions, Underground, High temperature

Introduction
Extreme environments on Earth refer to those with diverse harsh environmental conditions. These conditions include acid, alkaline, high salinity, high and low temperatures, high metal concentrations, high radiation, and high pressures (Mirete et al., 2016). Hot springs, as an extreme environment, harbor many thermophilic and hyperthermophilic microbes with optimal growth temperatures > 55 °C and > 80 °C, respectively. The initial studies of hot springs related to microbes were focused on the isolation and characterization of strains using traditional culture-dependent approaches (Marsh and Larsen, 1953). However, since ~99% of the microorganisms are uncultivable on the earth (Amann et al., 1995), cultivation-independent molecular methods were developed to overcome the uncultivable issue, giving support to research focused on the microbial diversity using a high-throughput sequencing approach based on 16S rRNA genes. This method has been extensively used to uncover microbial communities
and their compositions in different hot springs around the world, providing a comprehensive realization of microbial diversity in hot spring environments (Huang et al., 2013; Bowen et al., 2013; Kambura et al., 2016). According to previous studies, hot spring environments are generally observed to be much less diverse than common habitats such as wetland sediments and marine surface water. Nonetheless, considering the possibility that hot spring environments may have existed on our planet for more than billions of years (Gold, 1992), some distinct microorganisms could adapt to the conditions via unique physical, chemical, and geographical characteristics. Many microbiologists are attracted by these exclusive traits and to reveal the microbial communities of hot springs around the world, such as in USA (Bowen et al., 2013), Iceland (Menzel et al., 2015), Russia (Rozanov et al., 2014), Kenya (Kambura et al., 2016), India (Saxena et al., 2017) and China (Wang et al., 2013; Li et al., 2015; Chen et al., 2016). However, most hot spring samples are taken from the surface layer, as either water, mat or sediments; thus, very little is known about the microbial diversity and functions under the subsurface. Therefore, knowledge regarding microbial functions and diversity from depths within hot springs, which provide valuable information about deep-subsurface biospheres on land, is still lacking. Considering the differences in surface and deep environments, such as oxygen, light, and organic and inorganic substances, the microbial composition and functions should be different between the surface and deep water layers in hot springs.
Functional gene arrays (FGAs) target genes involved in various functional processes and are valuable for evaluating the functional composition and structure of microbial communities (Zhou et al., 2015). GeoChip, a generic FGA targeting hundreds of functional gene categories that are involved in important biogeochemical, ecological, and environmental studies, has been successfully applied to different environmental samples (Colin et al., 2017; Ma et al., 2017).

Niujie town, located in the Eryuan county of Dali city, Yunnan province, China, is one of the most important places along the Tea-horse Caravan road between Yunnan and Tibet. Tectonically, it is situated at the collision boundary between the Indian and Eurasian plates and belongs to the eastern end of the Tibet-Yunnan geothermal zone (Kearey and Wei, 1993). To gain insights into the microbial diversity and potential functions of microbial communities in hot spring waters at different depths, we performed 16S rRNA gene sequencing and functional gene array (GeoChip 5.0) (Shi et al., 2019) analysis on hot spring waters at three different depths (0 m, 19 m and 58 m). We addressed the following questions in this study: (1) are the microbial communities at different depths in a hot spring taxonomically and functionally different due to the depths and (2) how is the community functional potential altered by the depth, specifically those functions involved in the cycling of key natural elements/compounds (e.g., nitrogen, methane and sulfur).

**Materials and methods**
Site description and sampling

The study area is located in Niujie town, Eryuan county, Dali city, Yunnan province, China (Fig. 1). Almost all families in this town have a hot spring well, and the hot spring wells are directly connected with hot springs at different depths by pipeline. Due to the various depths of well drilling, the hot spring waters from different families represent hot spring waters from different depths. Three hot spring water samples with different depths were taken from three hot spring wells in a small area. The distance between each sampling site is less than 50 m. The temperature was measured by a DeltaTrak Waterproof Lollipop Min/Max Autocal Thermometer (Model 11050, Pleasanton, CA, USA), and the pH was measured by an HQd Portable Meter pH (Model HQ40d, Loveland, CO, USA). The depth information of the different hot spring wells was provided by the villagers of each family, and this depth information was from the drilling company after the specific wells were drilled. Equal volumes of hot spring water (80 L) were collected from 0 m, 19 m and 58 m at each hot spring wells and then filtered through 0.22-μm polyethersulfone membrane filters (Millipore, MA, USA). The filters were maintained in a box full of dry ice, transferred to the lab and then stored at −80 °C until DNA extraction.

DNA extraction and GeoChip 5.0 analysis

To obtain three duplicate samples from each hot spring well at depths of 0 m, 19 m and 58 m, each of the 0.22-μm filter membranes used to collect
Microorganisms from different hot spring wells were divided into three parts using sterile scissors and forceps on a super clean bench. DNA was then extracted from the filters with MoBio PowerSoil DNA Isolation Kits (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions.

For each sample, 20 ng of DNA was taken to perform whole community genome amplification with the GE Healthcare Life Sciences illustra TempliPhi Amplification kit (GE Healthcare, Piscataway, NJ) (Wu et al., 2006). One microgram of amplified DNA from each sample was labeled with fluorescent Cy-3 dye (GE Healthcare, CA, USA) by random priming as described previously (Bai et al., 2013). After purification using a QIA quick Purification kit (Qiagen, CA, USA), the DNA was dried in a SpeedVac (Thermo Savant, NY, USA) and rehydrated with 13 µl of DNase/RNase-free distilled water. A total of 42 µl of buffer containing 1 × HI-RPM hybridization buffer, 1 × aCGH blocking agent, 0.05 µg/µl Cot-1 DNA, 10 pM universal standard, and 10% formamide (final concentrations) was added to each sample. After mixing completely, the solution was incubated at 95 °C for 3 min and then incubated at 37 °C for 30 min. The prepared samples were hybridized with GeoChip 5.0 arrays (180 K) at 67 °C for 24 h. Scanned images of the hybridized GeoChips were converted and extracted using the Agilent Feature Extraction 11.5 software (Agilent Technologies, Inc., CA, USA). The extracted information from the hybridized GeoChips was analyzed through the microarray analysis pipeline on the web site (http://ieg.ou.edu/microarray/) as previously described (Zhao et al., 2014). To call probes positive, we used a floating SNR so
that the hyperthermophile probes accounted for 5% of the positive signals. We then removed probes considered to be negative if the signal was <1500 or <1.3 times the background.

16S rRNA gene amplification and Illumina Sequencing

To determine the diversity and composition of the bacterial and archaeal communities in each of the 12 samples, the 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CNN GGG TAT CTA AT-3′) primer set was used to amplify the V4 region of the bacterial 16S rRNA gene. The Arch519F (5′-CAG CCG CCG CGG TAA-3′) and Arch915R (5′-GTG CTC CCC CGC CAA TTC CT-3′) primer set was used to amplify the V4 region of the archaeal 16S rRNA gene. All PCRs were carried out in 30 μl reaction with 15 μl of Phusion High-Fidelity PCR Master Mix (New England Biolabs, MA, USA), 0.2 μM of forward and reverse primers, and approximately 10 ng of DNA. Thermal cycling consisted of an initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 60 s, and a final elongation at 72 °C for 5 min. The PCR products were analyzed on a 2% agarose gel, and the target DNA was purified with the Gene JET Gel Extraction Kit (Thermo Scientific). Sequencing libraries were generated using the NEBNext Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs). The libraries were sequenced on an Illumina MiSeq platform 2500 and 250-bp paired-end reads were generated at Novogene (Beijing, China). The
sequencing reads were submitted to the Short Read Archive database at NCBI under accession no. SRP120991 for bacterial sequences and accession no. SRP121000 for archaeal sequences.

Data processing and predictive functional profiling of microbial communities

The sequences were split to samples according to their barcodes allowing for one mismatch. Pairs of reads of sufficient length were merged with at least 30 bp using the FLASH program (Magoč and Salzberg, 2011). The threshold, including a quality score > 20 and window size of 5, was used to remove the low-quality sequences via the Btrim program (Kong, 2011), and any sequences containing N's or ambiguous bases were discarded. Only sequences from 245 bp to 260 bp in length for bacterium or 370 bp to 400 bp in length for archaea were treated as targeted sequences. The UPARSE program (Edgar, 2013) was used to remove chimeras and cluster sequences into 97% identical operational taxonomy units (OTUs) with singletons; the bacterium and archaea OTU tables were randomly resampled for the normalization of different sample reads. A representative sequence from each OTU was selected for taxonomic annotation by comparison to the full SILVA 128 database (Quast et al., 2013). The Functional Annotation of Prokaryotic Taxa (FAPROTAX) (Louca et al., 2016) was used to convert the taxonomic microbial community profiles into putative functional profiles based on the taxa identified in the sample; FAPROTAX defines functional groups in terms of taxa (e.g., species or genera) affiliated with each functional
group. These affiliations are mostly based on peer-reviewed literature, such as announcements of cultured representatives.

Ecological and statistical analysis

The diversity indices (Shannon, Simpson and Observed Richness) for each sample were calculated by the vegan package in R software version 3.1.3 (R Development Core Team, 2012). Chao1 values were calculated using the Mothur program (Schloss et al., 2009). The principal coordinate analysis (PCoA) was generated using PyNAST (Caporaso et al., 2010), the FastTree program (Price et al., 2009), and the UniFrac matrix (Lozupone and Knight, 2005; Lozupone et al., 2006; Lozupone et al., 2007) from step-by-step analysis. The detrended correspondence analysis (DCA) was generated by the vegan package in R. The statistical analysis was conducted by one-way analysis of variance (ANOVA) and Tukey’s test. A significance level of p<0.05 was adopted for all comparisons (He and Wang, 2011).

Results

Sampling

Three hot springs from Niujie town were selected based on their different depths. The temperatures ranged from 79 °C to 82.5 °C, and the pH ranged from 6.64 to 6.67. According to the temperatures and pH, there were no significant differences between the samples. The environmental parameters data were
collected before sampling and are summarized in Table 1.

Fig 1. The geographical map showing the hot springs sampling locations in Nuijie Town, Eryuan county, Dali city, Yunnan province, China.

Table 1. Sampling site parameters in this study.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Latitude °N</th>
<th>Longitude °E</th>
<th>depth (m)</th>
<th>Temperature °C</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 m-1</td>
<td>26°14'58.4514&quot;</td>
<td>99° 59' 32.604&quot;</td>
<td>0</td>
<td>79.0</td>
<td>6.64</td>
</tr>
<tr>
<td>0 m-2</td>
<td>26°14'58.4514&quot;</td>
<td>99° 59' 32.604&quot;</td>
<td>0</td>
<td>79.0</td>
<td>6.64</td>
</tr>
<tr>
<td>0 m-3</td>
<td>26°14'58.4514&quot;</td>
<td>99° 59' 32.604&quot;</td>
<td>0</td>
<td>79.0</td>
<td>6.64</td>
</tr>
<tr>
<td>19 m-1</td>
<td>26°14'58.3794&quot;</td>
<td>99° 59' 29.58&quot;</td>
<td>19</td>
<td>82.5</td>
<td>6.64</td>
</tr>
<tr>
<td>19 m-2</td>
<td>26°14'58.3794&quot;</td>
<td>99° 59' 29.58&quot;</td>
<td>19</td>
<td>82.5</td>
<td>6.64</td>
</tr>
<tr>
<td>19 m-3</td>
<td>26°14'58.3794&quot;</td>
<td>99° 59' 29.58&quot;</td>
<td>19</td>
<td>82.5</td>
<td>6.64</td>
</tr>
<tr>
<td>58 m-1</td>
<td>26°15'0.324&quot;</td>
<td>99° 59' 27.132&quot;</td>
<td>58</td>
<td>82.5</td>
<td>6.67</td>
</tr>
<tr>
<td>58 m-2</td>
<td>26°15'0.324&quot;</td>
<td>99° 59' 27.132&quot;</td>
<td>58</td>
<td>82.5</td>
<td>6.67</td>
</tr>
<tr>
<td>58 m-3</td>
<td>26°15'0.324&quot;</td>
<td>99° 59' 27.132&quot;</td>
<td>58</td>
<td>82.5</td>
<td>6.67</td>
</tr>
</tbody>
</table>

Microbial diversity and community taxonomic composition

To determine the microbial diversity of the hot spring at different depths, 16S rRNA genes were amplified and sequenced. After quality control, a total of
534875 sequences for bacterium and 111989 sequences for archaea were clustered into 9 hot spring samples, and operational taxonomy unit tables were generated for bacterium and archaea, respectively. For the microbial diversity, the composition and structure of each sample could be compared; random resampling was conducted for further analyses. The alpha diversity of the microbial communities from different hot spring depths were calculated. The Shannon and Inverse Simpson indexes indicated that the highest $\alpha$-diversity was observed in the 19 m samples for both bacterial and archaeal communities (Fig. 2).

The microbial community taxonomic composition was revealed at the phylum/class and genus levels with a similarity of 97% for OTU classification. After quality control and random resampling of the 9 samples, the sequence reads were clustered into 4164 OTUs for bacteria at a 97% similarity level. The bacterial groups at 0 m with the highest relative abundances at the phylum level were members of Aquificae, Gamma-proteobacteria, and Deinococcus-Thermus. For the 19 m sample, the dominant taxa were Alpha-proteobacteria, Gamma-proteobacteria, and Firmicutes. The bacterial groups Deinococcus-Thermus, Firmicutes, and Gamma-proteobacteria dominated in the 58 m samples (Fig. 3A). At the genus level, the OTUs were distributed, with the most abundant belonging to Hydrogenobacter and Thermus in hot spring samples at 0 m, while Sphingobium and Bacillus dominated in the hot spring samples at 19 m. In the hot spring samples at 58 m, the most abundant belonged to Thermus.
(37.6% - 59.3%) and Bacillus (Fig. 3B). For the archaeal communities, after quality control and random resampling for the twelve samples, the sequence reads were clustered into 43 OTUs for archaea at a 97% similarity level. Thaumarchaeota was the most abundant phylum across all samples (Fig. 4A). At the genus level, OTUs were distributed with the most abundant belonging to the Uncultured Desulfurococcales archaeon in hot spring samples at 0 m and 58 m. In contrast, the most abundant belonged to Candidatus Nitrososphaera and Ignisphaera in hot spring samples at 19 m (Fig. 4B).
Fig 2. Comparison of the alpha diversity indexes, Shannon index and Inverse Simpson index (A: Bacterial communities; B: Archaeal communities). The value is the mean of the indices within each group. Error bars represent the standard error (SE). *p < 0.05; **p < 0.01; and ***p < 0.001 based on Student’s t-test.
Fig 3. Stacked bar chart showing the relative abundance of the bacterial community composition at the phyla and classes of Proteobacteria level (A), and the genera level (B).

Fig 4. Stacked bar chart showing the relative abundance of the archaeal community composition at the phyla level (A), and the genera level (B).

Fig 5. Principal coordinate analysis (PCoA) of bacterial communities from hot springs at different depths (A). The results are based on weighted the UniFrac distances of the detected OTUs, and Detrended correspondence analysis (DCA) of bacterial communities from hot springs at different depths (B). The results are based on the detected OTUs.
Fig 6. Principal coordinate analysis (PCoA) of archaeal communities from hot springs at different depths (A). The results based on the weighted UniFrac distances of the detected OTUs, and Detrended correspondence analysis (DCA) of archaeal communities from hot springs at different depths (B). The results are based on the detected OTUs.

Fig 7. The 50 most abundant bacterial community OTUs from hot springs at different depths. Bacterial abundance was scaled with a log transformation in the heatmap.
Fig 8. The 20 most abundant archaeal community OTUs from hot springs at different depths. Archaeal abundance was scaled with a log transformation in the heatmap.

Microbial community structure of hot springs at different depths

To examine the microbial community structure of the hot spring at different depths, β-diversity-based statistical tools were applied, such as principal coordinate analysis (PCoA) and detrended correspondence analysis (DCA). Both PCoA and DCA showed that the bacterial community structures were distinctly separate from each group (Fig. 5), suggesting that there were differences in bacterial community structures of the hot spring at different depths. However, the archaeal community structure at 0 m and 58 m were similar, though they differed from the structure at 19 m (Fig. 6). A heatmap based on the 50 most abundant bacterial community OTUs and 20 most abundant archaeal community OTUs indicated different depths of hot springs could harbor distinct microbial communities (Fig. 7, 8).

Predictive functional profiling of bacterial and archaeal communities

According to the FAPROTAX results based on the bacterial communities,
the bacterium at 0 m are mainly involved in hydrogen, sulfur and thiosulfate oxidation and nitrate reduction. The most frequent predicted function at 19 m and 58 m was chemoheterotrophy (Fig. 9). The FAPROTAX results based on the archaeal communities showed that all the archaea are involved in ammonia oxidation and nitrification (Fig. 10).

Fig 9. Stacked bar chart showing the mean relative abundance of the predicted metabolic potential of bacterium from hot springs at different depths, as predicted by FAPROTAX.
Fig 10. Stacked bar chart showing the mean of the relative abundance of the predicted metabolic potential of archaea from hot springs at different depths, as predicted by FAPROTAX.

Functional genes involved in the nitrogen, methane and sulfur cycle

Key functional genes for ammonification, nitrification, assimilatory N reduction, anammox, denitrification, and nitrogen fixation were detected in all samples. The functional genes involved in the nitrogen cycle at 58 m were the lowest among all samples (Fig. 11B). The heatmap results of functional genes involved in the nitrogen cycle showed that the functional structures of the microbial communities were similar at 19 m and 58 m, but differed from that at 0 m (Fig. 11A). The signal intensity of genes involved in the methane cycle indicated that the metabolic potential for methane production or methanogenesis was very similar at all three hot springs depths (Fig. 12). For the functional genes involved in sulfur and sulfate metabolism, there were no significant differences between the samples at 0 m, 19 m and 58 m (Fig. 13B), though the functional gene structures of the sulfur and sulfate cycles showed some structural
Fig. 11. The normalized signal intensity of the detected key genes involved in the nitrogen cycle (A). The signal intensity for each functional gene category is the average of the total signal intensity from all the replicates, and the heatmap of the functional genes involved in the nitrogen cycle at different hot springs depths (B).

Fig. 12. The normalized signal intensity of the detected key genes involved in the methane cycle (A). The signal intensity for each functional gene category is the average of the total signal intensity from all the replicates, and the heatmap of the functional genes involved in the methane cycle at different hot springs depths (B).
Fig. 13. The normalized signal intensity of the detected key genes involved in the sulfur cycle (A). The signal intensity for each functional gene category is the average of the total signal intensity from all the replicates, and the heatmap of functional genes involved in the sulfur cycle at different hot springs depths (B).

Discussion

To better understand the diversity of life on Earth, especially the evolution and potential origin of life (Des Marais and Walter, 2019), intensive study of intraterrestrial microbes from harsh condition environments and the mechanism of how microorganisms tolerate extreme environmental conditions should not be ignored (Fredrickson and Balkwill, 2006). The diversity of archaea and bacteria in hot springs, an extreme environment, has been investigated extensively. However, most of the research has been focused on the surface of hot springs (Wang et al., 2013; Li et al., 2015; Chen et al., 2016; Bowen De León et al., 2013; Menzel et al., 2015; Rozanov et al., 2014; Kambura et al., 2016; Saxena et al., 2017; Tang et al., 2018). To date, not many studies have attempted a direct comparison of microbe composition and functions at different depths of hot springs. In this study, we investigated the microbial and functional gene diversity at different depths of
hot springs in Niujie town, Yunnan province, China. The research area was an ideal study site for research on hot springs at different depths. We characterized the bacterial and archaeal communities in neutral (pH 6.64 – 6.72) high-temperature (79 °C - 83 °C) hot springs. Although the environmental parameters were similar, the bacteria datasets demonstrated a general shift from Aquificae at 0 m to Proteobacteria and Firmicutes at 19 m, with an additional shift to Deinococcus-Thermus and Firmicutes at 58 m. At the genus level, the dominant species were different at different depths of hot spring water, with *Hydrogenobacter* being the most dominant among the 0 m samples. By increasing the depth to 19 m, the dominant species observed were *Sphingobium* and *Bacillus*, whereas *Thermus* and *Bacillus* dominated the hot spring at 58 m. DCA and PCoA also showed that the bacterial communities were different at different depths. Previously, Hou et al. showed that *Hydrogenobacter* and Aquificae were the dominant genus and phylum, respectively, in neutral and alkaline high-temperature surface hot springs in Tengchong, Yunnan Province, China. Our bacterial community results at 0 m were consistent with the results of Hou et al. Ferrous iron (Fe²⁺), thiosulfate (S₂O₃²⁻), elemental sulfur (S⁰), hydrogen sulfide (H₂S), and hydrogen (H₂) are very common inorganic electron donors in hydrothermal environments (Amend and Shock,. 2001; Shock et al., 2010). In oxidation-reduction reactions, the oxidation of H₂ is generally coupled with the reduction of oxygen (O₂), nitrate (NO₃⁻), S⁰, sulfate (SO₄²⁻), S₂O₃²⁻, or ferric iron (Fe³⁺) (Shock et al., 2010; Spear et al., 2005). The bacterial community results...
showed that *Hydrogenobacter* from Aquificae can be detected at all three hot springs depths, and functional profiling of the bacterial communities revealed the bacteria in the hot springs are involved in hydrogen, sulfur and thiosulfate oxidation and nitrate reduction, especially at 0 m. This finding supports work focused on inorganic sources of oxidation and microbial metabolism in environments with high temperatures (Lindsay et al., 2018). Surprisingly, *Sphingobium* and *Bacillus* dominated the hot spring at 19 m. As is known, microbes in hot springs can produce thermostable enzymes, which is one reason that thermophilic microbes can tolerate harsh conditions, such as high temperature (Chalopagorn et al., 2014). *Thermus aquaticus* is a classic example that produces Taq DNA polymerase (Chien et al., 1976). Another example is a Bacillus strain isolated from a hot spring in Kalianda Island. Its lipase gene *lip256* was cloned and expressed; Lip256 exhibited high activity at high temperatures, with 40% maximum activity at 80 °C and good stability at temperatures ranging from 50 to 80 °C (Li and Liu. 2017). *Sphingobium*, which is capable of degrading hydrocarbons, are very common microorganisms in oil-contaminated environments (Chaudhary et al., 2017; Park et al., 2019), but they are very rarely found in hot springs environments. This finding was unexpected and this result may be explained by the fact that the hot spring well was just completed; the drilling equipment was still present and may have leaked some oil into the ground. Not only that, the main predicted functions at 19 m were chemoheterotrophy and aromatic compound degradation, which indicated that the bacteria at 19 m were
involved in the hydrocarbon cycle. The phylum Deinococcus-Thermus is divided into the orders Deinococcales and Thermales. *Thermus*, which dominated the samples at 58 m, belongs to the order Thermales. Previous reports found that *Thermus* was only detected in specific areas, such as the Gongxiaoshe hot spring in Ruidian, Yunnan province, China. Another interesting fact is that *Thermus* in China differed from that from a Yellowstone hot springs (Song et al., 2013). The pH and temperature of the Gongxiaoshe hot spring in Ruidian were 7.3 and 73.8 °C, respectively (Hou et al., 2013), which are consistent with previous results for the neutral and alkaline hot springs in Yellowstone where *Thermus*, which generally requires an optimum temperature of approximately 70 °C -75 °C (da Costa et al., 2006), was found. However, in our results, *Thermus* dominated the 82 °C hot spring at the 58 m depth, which may expand our understanding of the growth temperature of *Thermus*.

Previous studies have suggested that archaea are very rare in neutral and alkaline hot springs (Reysenbach et al., 1994; Hugenholtz et al., 1998; Inskeep et al., 2010). However, studies have also shown that bacterium and archaea can ubiquitously coexist in nonacid hot springs (Schouten et al., 2007; Bowen De León et al., 2013). In our studies, Thaumarchaeota was the dominant phylum in the neutral high-temperature hot spring, and the majority of archaeal sequences in this hot spring were related to "Candidatus_Nitrosocaldus", a putative ammonia-oxidizing archaeon (Hou et al., 2013; Bowen De León et al., 2013). Our archaeal community results at 0 m, 19 m, and 58 m were consistent with previous
results. Based on the cultivation and characterization of 'Candidatus Nitrosocaldus yellowstonii', Candidatus Nitrosocaldus was thought to be involved in ammonia oxidation (de la Torre et al., 2008; Nishizawa et al., 2016). Our predictive functional profiling of archaeal communities and functional gene array results indicated the potentially important role for nitrogen cycling in the neutral high-temperature hot spring, both at the surface or at the varying depths.

Prior studies have noted the importance of methanogenesis in the early Archaean era (Ueno et al., 2006). Many methanogens are encountered in thermophilic or hyperthermophilic hydrothermal vents and form the lower roots of the evolutionary tree, providing the hypothesis that life on earth originated in thermal environments with energy conserved by methanogenesis (Russell and Nitschke. 2017). Therefore, methane cycling in the hot spring environments should be noticed. However, in our results, we did not find intense biotic methane metabolic processes, such as methanogenesis or methane oxidation. Most methanogenesis is derived from microorganisms affiliated with Euryarchaeota (McKay et al., 2019), though some microbes from Bathyarchaeota (Evans et al., 2015) and Verstraetearchaeota (Vanwonterghem et al., 2016) were recently found to be involved in methanogenesis. According to our archaeal community results, we only detected a few Methanosarcina and Methanobrevibacter species, which are affiliated with Euryarchaeota, at 19 m and 50 m. Some methane-oxidizing bacterium, such as Methylophilum, Methylocaldum, Methylobacter, Methylothermus, and Methylocystis were found in our bacteria datasets but they
mostly belong to minor groups.

In summary, three different depths in a neutral (pH 6.64 – 6.72) high-temperature (79 °C - 83 °C) hot springs were investigated by 16S rRNA gene high-throughput sequencing and GeoChip functional gene microarray. Our results revealed that the bacterial communities were different at different depths. Our results showed that the microbial diversity and composition shifted at different depths in a very small area and that the microbes at different hot springs depths are mainly involved the following processes: hydrogen, sulfur and thiosulfate oxidation; nitrate reduction; ammonia oxidation; and nitrification. Our study not only provides comprehensive insights into the microbial community at the different depths in hot springs but also provides new insights into the deep-subsurface biosphere associated with terrestrial hot springs.

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