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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 Thaumarchaeota 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

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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 ℃ and > 80 ℃, 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

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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.

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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 Yunan 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

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92 Site description and sampling

93 The study area is located in Niujie town, Eryuan county, Dali city, Yunnan
94 province, China (Fig. 1). Almost all families in this town have a hot spring well,
95 and the hot spring wells are directly connected with hot springs at different depths
96 by pipeline. Due to the various depths of well drilling, the hot spring waters from
97 different families represent hot spring waters from different depths. Three hot
98 spring water samples with different depths were taken from three hot spring wells
99 in a small area. The distance between each sampling site is less than 50 m. The
100 temperature was measured by a DeltaTrak Waterproof Lollipop Min/Max Autocal
101 Thermometer (Model 11050, Pleasanton, CA, USA), and the pH was measured
102 by an HQd Portable Meter pH (Model HQ40d, Loveland, CO, USA). The depth
103 information of the different hot spring wells was provided by the villagers of each
104 family, and this depth information was from the drilling company after the specific
105 wells were drilled. Equal volumes of hot spring water (80 L) were collected from 0
106 m, 19 m and 58 m at each hot spring wells and then filtered through 0.22-μm
107 polyethersulfone membrane filters (Millipore, MA, USA). The filters were
108 maintained in a box full of dry ice, transferred to the lab and then stored at –
109 80 °C until DNA extraction.

110

111 DNA extraction and GeoChip 5.0 analysis

112 To obtain three duplicate samples from each hot spring well at depths of 0 m, 19
113 m and 58 m, each of the 0.22-μm filter membranes used to collect

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114 microorganisms from different hot spring wells were divided into three parts using
115 sterile scissors and forceps on a super clean bench. DNA was then extracted
116 from the filters with MoBio PowerSoil DNA Isolation Kits (Mo Bio Laboratories,
117 Carlsbad, CA, USA) according to the manufacturer's instructions.

118 For each sample, 20 ng of DNA was taken to perform whole community
119 genome amplification with the GE Healthcare Life Sciences illustra TempliPhi
120 Amplification kit (GE Healthcare, Piscataway, NJ) (Wu et al., 2006). One
121 microgram of amplified DNA from each sample was labeled with fluorescent Cy-3
122 dye (GE Healthcare, CA, USA) by random priming as described previously (Bai *et*
123 *al.*, 2013). After purification using a QIA quick Purification kit (Qiagen, CA, USA),
124 the DNA was dried in a SpeedVac (Thermo Savant, NY, USA) and rehydrated
125 with 13 μl of DNase/RNase-free distilled water. A total of 42 μl of buffer containing
126 1 × HI-RPM hybridization buffer, 1 × aCGH blocking agent, 0.05 μg/μl Cot-1 DNA,
127 10 pM universal standard, and 10% formamide (final concentrations) was added
128 to each sample. After mixing completely, the solution was incubated at 95 °C for 3
129 min and then incubated at 37 °C for 30 min. The prepared samples were
130 hybridized with GeoChip 5.0 arrays (180 K) at 67 °C for 24 h. Scanned images of
131 the hybridized GeoChips were converted and extracted using the Agilent Feature
132 Extraction 11.5 software (Agilent Technologies, Inc., CA, USA). The extracted
133 information from the hybridized GeoChips was analyzed through the microarray
134 analysis pipeline on the web site (http://ieg.ou.edu/microarray/) as previously
135 described (Zhao et al., 2014). To call probes positive, we used a floating SNR so

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that the hyperthermophile probes accounted for 5% of the positive signals. We then we 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

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158 sequencing reads were submitted to the Short Read Archive database at NCBI
159 under accession no. SRP120991 for bacterial sequences and accession no.
160 SRP121000 for archaeal sequences.

161

162 Data processing and predictive functional profiling of microbial communities

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

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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

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collected before sampling and are summarized in Table 1.

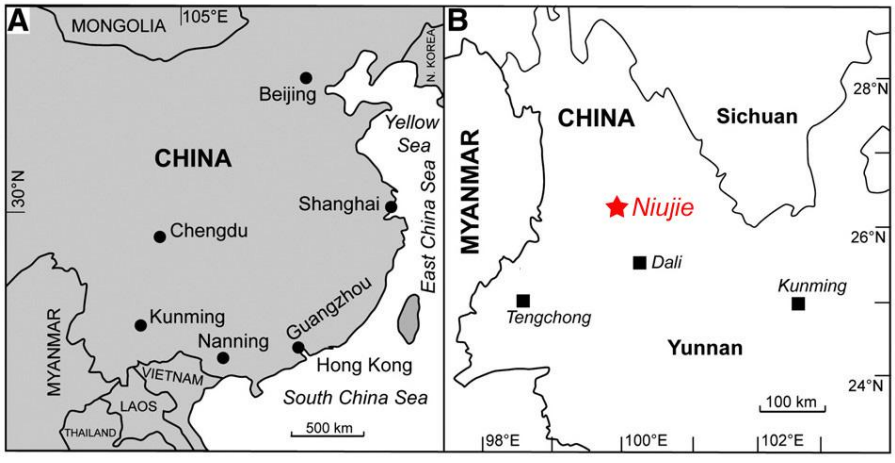

Fig 1. The geographical map showing the hot springs sampling locations in Niujie Town, Eryuan county, Dali city, Yunnan province, China.

Table 1. Sampling site parameters in this study.

Sample ID	Latitude °N	Longitude °E	depth (m)	Temperature °C	pH
0 m-1	26°14'58.4514"	99° 59' 32.604"	0	79.0	6.64
0 m-2	26°14'58.4514"	99° 59' 32.604"	0	79.0	6.64
0 m-3	26°14'58.4514"	99° 59' 32.604"	0	79.0	6.64
19 m-1	26°14'58.3794"	99° 59' 29.58"	19	82.5	6.64
19 m-2	26°14'58.3794"	99° 59' 29.58"	19	82.5	6.64
19 m-3	26°14'58.3794"	99° 59' 29.58"	19	82.5	6.64
58 m-1	26°15'0.324"	99° 59' 27.132"	58	82.5	6.67
58 m-2	26°15'0.324"	99° 59' 27.132"	58	82.5	6.67
58 m-3	26°15'0.324"	99° 59' 27.132"	58	82.5	6.67

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

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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 α-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*

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(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).

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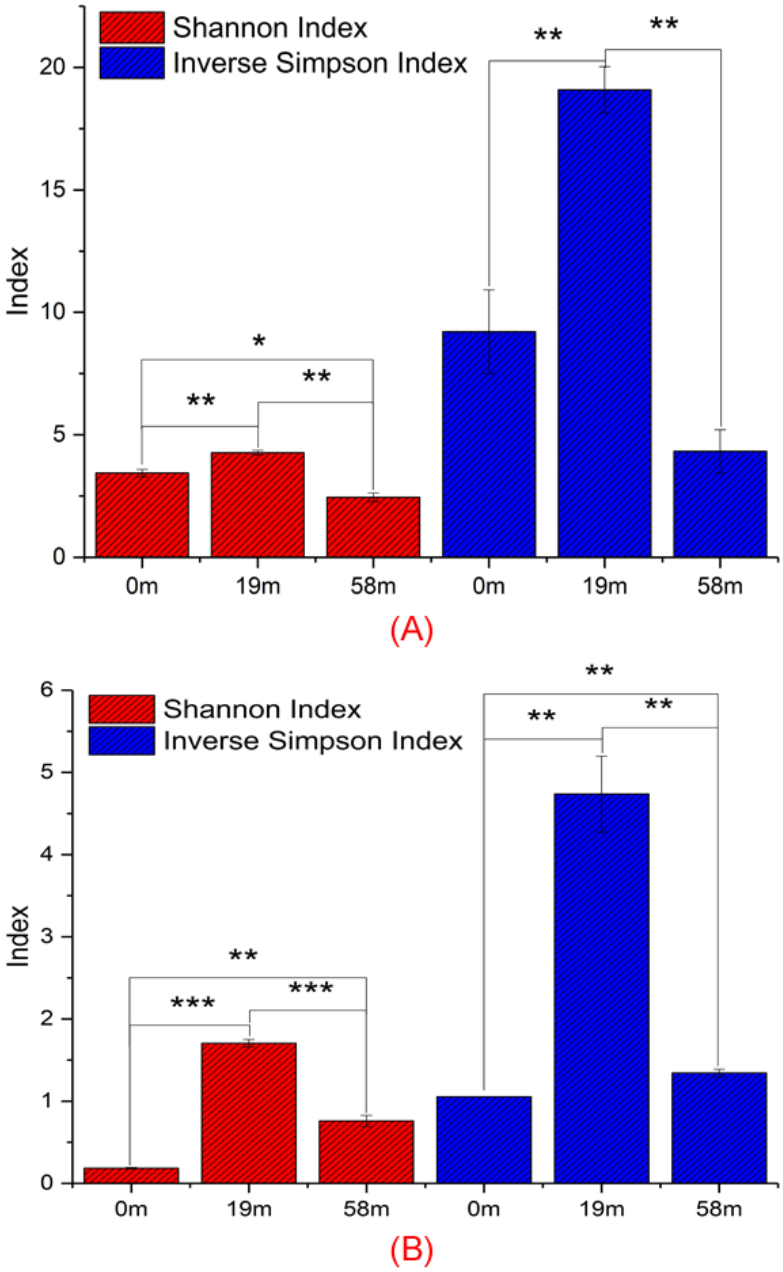

Fig 2. Comparison of the alpha diversity indexes, Shannon index and Inverse Simpson index (A: Bacterial communities; B: Archaeal communities). The value is the mean of the indices within each group. Error bars represent the standard error (SE). *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ based on Student's t-test.

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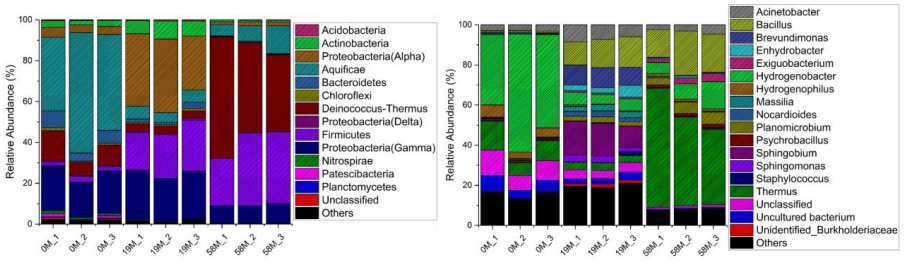

Fig 3. Stacked bar chart showing the relative abundance of the bacterial community composition at the phyla and classes of Proteobacteria level (A), and the genera level (B).

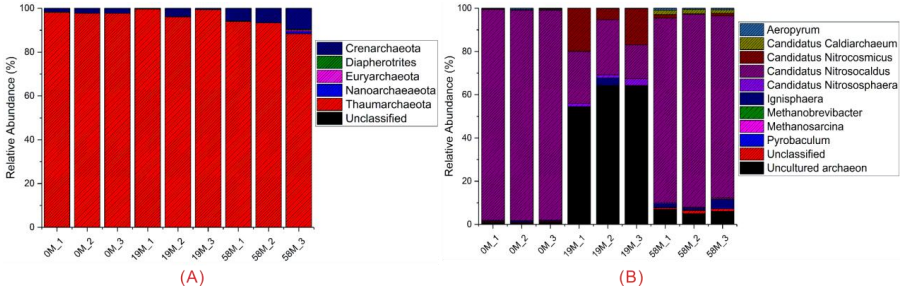

Fig 4. Stacked bar chart showing the relative abundance of the archaeal community composition at the phyla level (A), and the genera level (B).

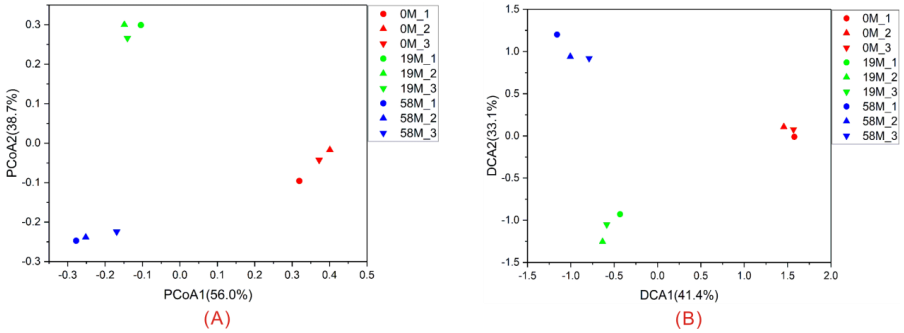

Fig 5. Principal coordinate analysis (PCoA) of bacterial communities from hot springs at different depths (A). The results are based on weighted the UniFrac distances of the detected OTUs, and Detrended correspondence analysis (DCA) of bacterial communities from hot springs at different depths (B). The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

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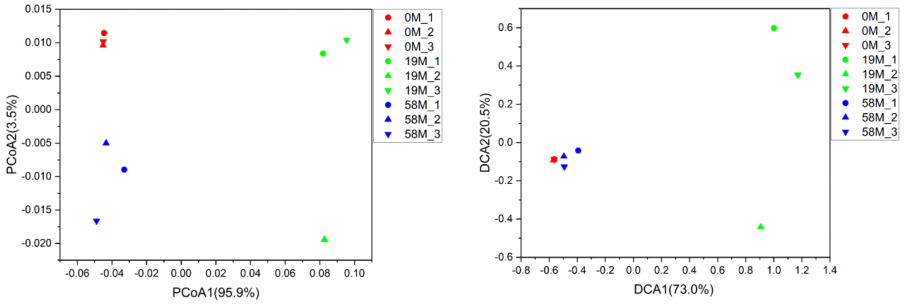

Fig 6. Principal coordinate analysis (PCoA) of archaeal communities from hot springs at different depths (A). The results based on the weighted UniFrac distances of the detected OTUs, and Detrended correspondence analysis (DCA) of archaeal communities from hot springs at different depths (B). The results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

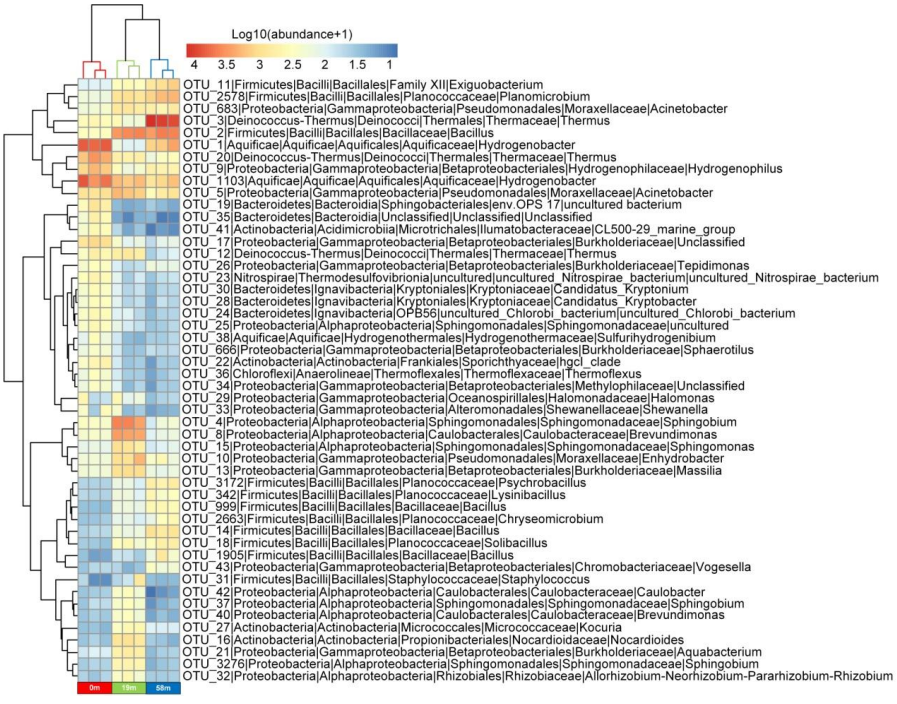

Fig 7. The 50 most abundant bacterial community OTUs from hot springs at different depths. Bacterial abundance was scaled with a log transformation in the heatmap.

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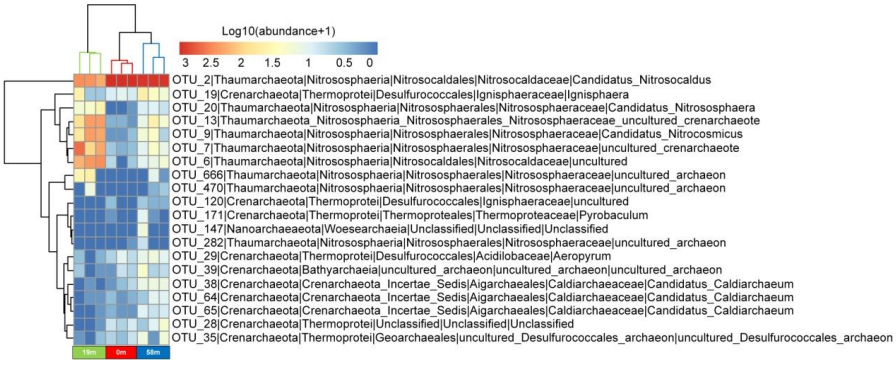

Fig 8. The 20 most abundant archaeal community OTUs from hot springs at different depths. Archaeal abundance was scaled with a log transformation in the heatmap.

Microbial community structure of hot springs at different depths

To examine the microbial community structure of the hot spring at different depths, ß-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,

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294 the bacterium at 0 m are mainly involved in hydrogen, sulfur and thiosulfate
295 oxidation and nitrate reduction. The most frequent predicted function at 19 m and
296 58 m was chemoheterotrophy (Fig. 9). The FAPROTAX results based on the
297 archaeal communities showed that all the archaea are involved in ammonia
298 oxidation and nitrification (Fig. 10).

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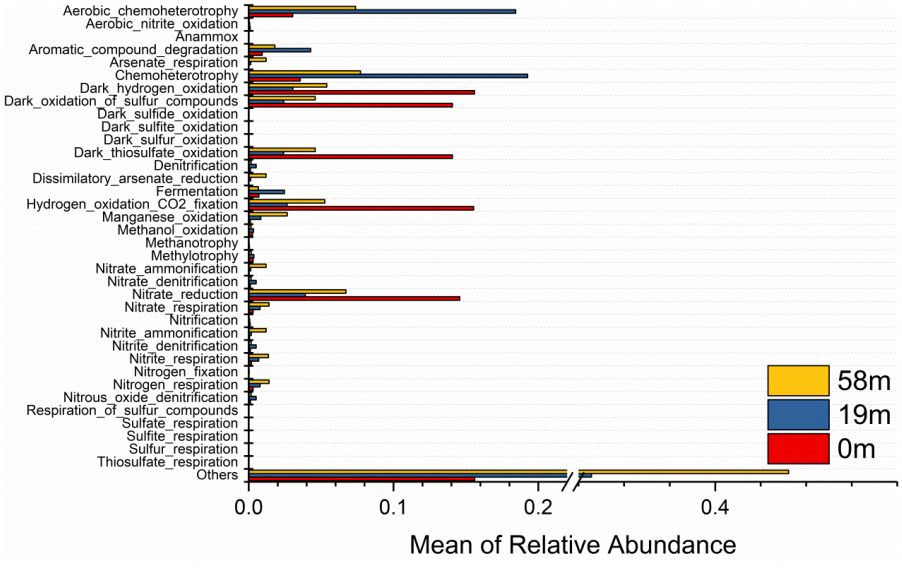

300
301 Fig 9. Stacked bar chart showing the mean relative abundance of the predicted
302 metabolic potential of bacterium from hot springs at different depths, as predicted by
303 FAPROTAX.

304

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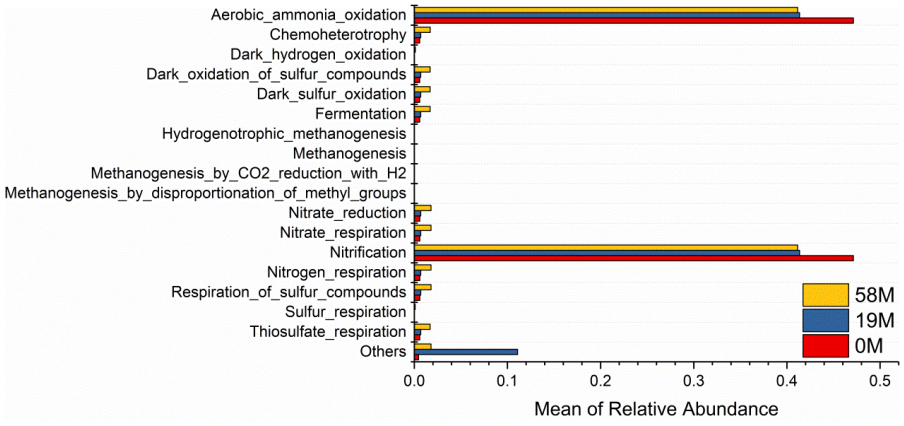

Fig 10. Stacked bar chart showing the mean of the relative abundance of the predicted metabolic potential of archaea from hot springs at different depths, as predicted by FAPROTAX.

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

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323 divergence (Fig. 13A).

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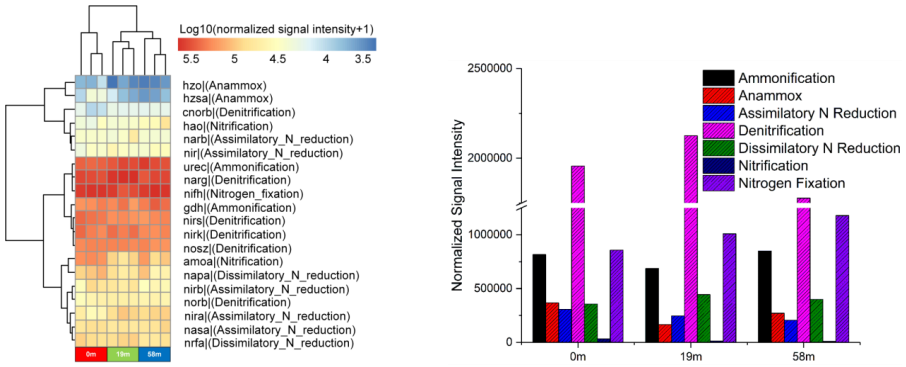

325

326 Fig. 11. The normalized signal intensity of the detected key genes involved in the
327 nitrogen cycle (A). The signal intensity for each functional gene category is the
328 average of the total signal intensity from all the replicates, and the heatmap of the
329 functional genes involved in the nitrogen cycle at different hot springs depths (B).

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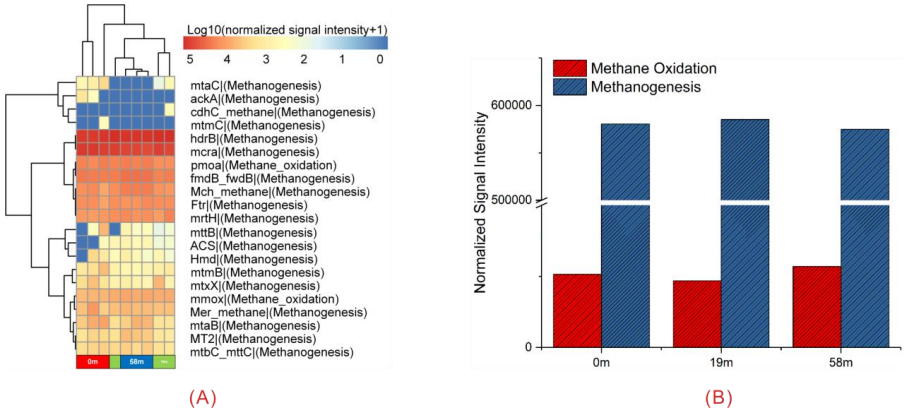

331

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

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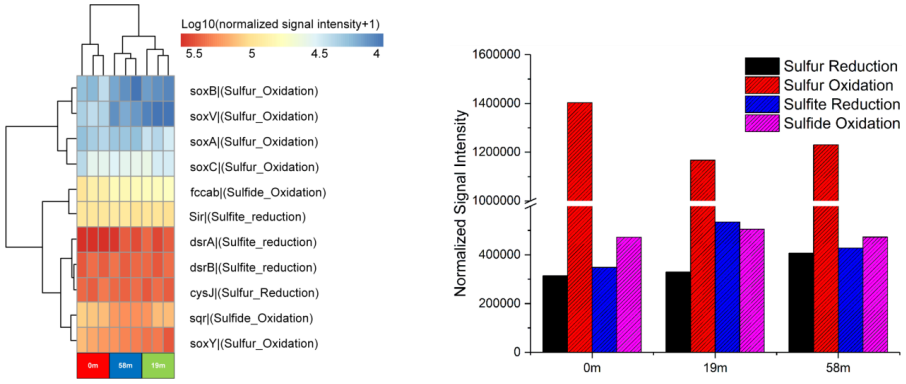

Fig. 13. The normalized signal intensity of the detected key genes involved in the sulfur cycle (A). The signal intensity for each functional gene category is the average of the total signal intensity from all the replicates, and the heatmap of functional genes involved in the sulfur cycle at different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

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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 (Fe^{2+}), thiosulfate ($S_2O_3^{2-}$), elemental sulfur (S^0), hydrogen sulfide (H_2S), and hydrogen (H_2) are very common inorganic electron donors in hydrothermal environments (Amend and Shock., 2001; Shock et al., 2010). In oxidation-reduction reactions, the oxidation of H_2 is generally coupled with the reduction of oxygen (O_2), nitrate (NO_3^-), S^0, sulfate (SO_4^{2-}), $S_2O_3^{2-}$, or ferric iron (Fe^{3+}) (Shock et al., 2010; Spear et al., 2005). The bacterial community results

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379 showed that *Hydrogenobacter* from Aquificae can be detected at all three hot
380 springs depths, and functional profiling of the bacterial communities revealed the
381 bacteria in the hot springs are involved in hydrogen, sulfur and thiosulfate
382 oxidation and nitrate reduction, especially at 0 m. This finding supports work
383 focused on inorganic sources of oxidation and microbial metabolism in
384 environments with high temperatures (Lindsay et al., 2018). Surprisingly,
385 *Sphingobium* and *Bacillus* dominated the hot spring at 19 m. As is known,
386 microbes in hot springs can produce thermostable enzymes, which is one reason
387 that thermophilic microbes can tolerate harsh conditions, such as high
388 temperature (Chalopagorn et al., 2014). *Thermus aquaticus* is a classic example
389 that produces Taq DNA polymerase (Chien et al., 1976). Another example is a
390 Bacillus strain isolated from a hot spring in Kalianda Island. Its lipase gene *lip256*
391 was cloned and expressed; Lip256 exhibited high activity at high temperatures,
392 with 40% maximum activity at 80 °C and good stability at temperatures ranging
393 from 50 to 80 °C (Li and Liu. 2017). *Sphingobium*, which is capable of degrading
394 hydrocarbons, are very common microorganisms in oil-contaminated
395 environments (Chaudhary et al., 2017; Park et al., 2019), but they are very rarely
396 found in hot springs environments. This finding was unexpected and this result
397 may be explained by the fact that the hot spring well was just completed; the
398 drilling equipment was still present and may have leaked some oil into the ground.
399 Not only that, the main predicted functions at 19 m were chemoheterotrophy and
400 aromatic compound degradation, which indicated that the bacteria at 19 m were

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401 involved in the hydrocarbon cycle. The phylum Deinococcus-Thermus is divided
402 into the orders *Deinococcales* and *Thermales*. *Thermus*, which dominated the
403 samples at 58 m, belongs to the order *Thermales*. Previous reports found that
404 *Thermus* was only detected in specific areas, such as the Gongxiaoshe hot spring
405 in Ruidian, Yunnan province, China. Another interesting fact is that *Thermus* in
406 China differed from that from a Yellowstone hot springs (Song et al., 2013). The
407 pH and temperature of the Gongxiaoshe hot spring in Ruidian were 7.3 and
408 73.8 °C, respectively (Hou et al., 2013), which are consistent with previous
409 results for the neutral and alkaline hot springs in Yellowstone where *Thermus*,
410 which generally requires an optimum temperature of approximately 70 °C -75 °C
411 (da Costa et al., 2006), was found. However, in our results, *Thermus* dominated
412 the 82 °C hot spring at the 58 m depth, which may expand our understanding of
413 the growth temperature of *Thermus*.

414 Previous studies have suggested that archaea are very rare in neutral and
415 alkaline hot springs (Reysenbach et al., 1994; Hugenholtz et al., 1998; Inskeep et
416 al., 2010). However, studies have also shown that bacterium and archaea can
417 ubiquitously coexist in nonacid hot springs (Schouten et al., 2007; Bowen De
418 León et al., 2013). In our studies, Thaumarchaeota was the dominant phylum in
419 the neutral high-temperature hot spring, and the majority of archaeal sequences
420 in this hot spring were related to "*Candidatus_Nitrosocaldus*", a putative
421 ammonia-oxidizing archaeon (Hou et al., 2013; Bowen De León et al., 2013). Our
422 archaeal community results at 0 m, 19 m, and 58 m were consistent with previous

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423 results. Based on the cultivation and characterization of '*Candidatus*
424 *Nitrosocaldus yellowstonii*', *Candidatus Nitrosocaldus* was thought to be involved
425 in ammonia oxidation (de la Torre et al., 2008; Nishizawa et al., 2016). Our
426 predictive functional profiling of archaeal communities and functional gene array
427 results indicated the potentially important role for nitrogen cycling in the neutral
428 high-temperature hot spring, both at the surface or at the varying depths.

429 Prior studies have noted the importance of methanogenesis in the early
430 Archaean era (Ueno et al., 2006). Many methanogens are encountered in
431 thermophilic or hyperthermophilic hydrothermal vents and form the lower roots of
432 the evolutionary tree, providing the hypothesis that life on earth originated in
433 thermal environments with energy conserved by methanogenesis (Russell and
434 Nitschke. 2017). Therefore, methane cycling in the hot spring environments
435 should be noticed. However, in our results, we did not find intense biotic methane
436 metabolic processes, such as methanogenesis or methane oxidation. Most
437 methanogenesis is derived from microorganisms affiliated with Euryarchaeota
438 (McKay et al., 2019), though some microbes from Bathyarchaeota (Evans et al.,
439 2015) and Verstraetearchaeota (Vanwonterghem et al., 2016) were recently found
440 to be involved in methanogenesis. According to our archaeal community results,
441 we only detected a few *Methanosarcina* and *Methanobrevibacter* species, which
442 are affiliated with Euryarchaeota, at 19 m and 50 m. Some methane-oxidizing
443 bacterium, such as *Methylomonas*, *Methylocaldum*, *Methylobacter*,
444 *Methylothermus*, and *Methylocystis* were found in our bacteria datasets but they

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445 mostly belong to minor groups.

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

457

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463 References

464

465 Amann, R.I., Ludwig, W., Schleifer, K.H.: Phylogenetic identification and in situ detection of
466 individual microbial cells without cultivation, Microbiol. Rev., 59(1), 143-169, 1995.
467
468 Amend, J.P., Shock, E.L.: Energetics of overall metabolic reactions of thermophilic and
469 hyperthermophilic Archaea and bacteria, FEMS. Microbiol. Rev., 25(2), 175-243, 2001.

https://doi.org/10.5194/bg-2019-406
Preprint. Discussion started: 10 December 2019

Bai, S., Li, J., He, Z., Van, Nostrand, J.D., Tian, Y., Lin, G., Zhou, J., Zheng, T.: GeoChip-based analysis of the functional gene diversity and metabolic potential of soil microbial communities of mangroves, Appl. Microbiol. Biotechnol., 97(15), 7035-7048, 2013.

Bowen De León, K., Gerlach, R., Peyton, B.M., Fields, M.W.: Archaeal and bacterial communities in three alkaline hot springs in Heart Lake Geyser Basin, Yellowstone National Park, Front. Microbiol., 4, 330, 2013.

Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., Knight, R.: PyNAST: a flexible tool for aligning sequences to a template alignment, Bioinformatics., 26(2), 266-267, 2010.

Chalopagorn, P., Charoenpanich, J., Choowongkomon, K.: Genome shuffling enhances lipase production of thermophilic *Geobacillus* sp, Appl. Biochem. Biotechnol., 174(4), 1444-1454, 2014.

Chaudhary, D.K., Jeong, S.W., Kim, J.: *Sphingobium naphthae* sp. nov., with the ability to degrade aliphatic hydrocarbons, isolated from oil-contaminated soil, Int. J. Syst. Evol. Microbiol., 67(8), 2986-2993, 2017.

Chen, S., Peng, X., Xu, H., Ta, K.: Composition of ammonia-oxidizingarchaea and their contribution to nitrification in a high-temperature hotspring, Biogeosciences., 13(1), 2051-2060, 2016.

Chien, A., Edgar, D.B., Trela, J.M.: Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*, J. Bacteriol., 127(3), 1550-1557, 1976.

Colin, Y., O, Nicolitch., J.D., Van, Nostrand., J.Z., Zhou., M.P., Turpault., S, Uroz.: Taxonomic and functional shifts in the beech rhizosphere microbiome across a natural soil toposequence, Sci. Rep., 7, 9604, 2017.

da Costa, M.S., Rainey, F.A., Nobre, M.F.: The genus Thermus and relatives, In book: The Prokaryotes., pp:797–812.doi:10.1007/0-387-30747-8_32, 2006.

de la Torre, J.R., Walker, C.B., Ingalls, A.E., Könneke, M., Stahl, D.A.: Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol, Environ. Microbiol., 10(3), 810-818, 2008.

Des Marais, D.J., Walter, M.R.: Terrestrial Hot Spring Systems: Introduction, Astrobiology., doi: 10.1089/ast.2018, 1976, 2019.

Edgar, R.C.: UPARSE: highly accurate OTU sequences from microbial amplicon reads, Nat. Methods., 10(10), 996-998, 2013.

https://doi.org/10.5194/bg-2019-406
Preprint. Discussion started: 10 December 2019

514
515 Evans, P.N., Parks, D.H., Chadwick, G.L., Robbins, S.J., Orphan, V.J., Golding, S.D., Tyson,
516 G.W.: Methane metabolism in the archaeal phylum Bathyarchaeota revealed by
517 genome-centric metagenomics, Science, 350(6259), 434-438, 2015.
518
519 Fredrickson, J.K., Balkwill, D.L.: Geomicrobiological processes and biodiversity in the deep
520 terrestrial subsurface, Geomicrobiol. J., 23(6), 345–356, 2006.
521
522 Ghosh D, Bal B, Kashyap VK, Pal S (2003) Molecular phylogenetic exploration of bacterial
523 diversity in a Bakreshwar (India) hot spring and culture of *Shewanella*-related thermophiles.
524 Appl Environ Microbiol 69(7):4332-4336.
525
526 Gold, T.: The deep, hot biosphere, Proc. Natl. Acad. Sci. U S A., 89(13), 6045-6049, 1992.
527
528 He, M., Wang, W.X.: Factors affecting the bioaccessibility of methylmercury in several marine
529 fish species, J. Agric. Food. Chem., 59(13), 7155-7162, 2011.
530
531 Huang, Q., Jiang, H., Briggs, B.R., Wang, S., Hou, W., Li, G., Wu, G., Solis, R., Arcilla, C.A.,
532 Abrajano, T., Dong, H.: Archaeal and bacterial diversity in acidic to circumneutral hot springs in
533 the Philippines, FEMS. Microbiol. Ecol., 85(3), 452-464, 2013.
534
535 Hugenholtz, P., Pitulle, C., Hershberger, K.L., Pace, N.R.: Novel division level bacterial diversity
536 in a Yellowstone hot spring, J. Bacteriol., 180(2), 366-376, 1998.
537
538 Hou, W., Wang, S., Dong, H., Jiang, H., Briggs, B.R., Peacock, J.P., Huang, Q., Huang, L., Wu,
539 G., Zhi, X., Li, W., Dodsworth, J.A., Hedlund, B.P., Zhang, C., Hartnett, H.E., Dijkstra, P.,
540 Hungate, B.A.: A comprehensive census of microbial diversity in hot springs of Tengchong,
541 Yunnan Province China using 16S rRNA gene pyrosequencing, PLoS. One., 8(1), e53350,
542 2013.
543
544 Inskeep, W.P., Rusch, D.B., Jay, Z.J., Herrgard, M.J., Kozubal, M.A., Richardson, T.H., Macur,
545 R.E., Hamamura, N., Jennings, R.d., Fouke, B.W., Reysenbach, A.L., Roberto, F., Young, M.,
546 Schwartz, A., Boyd, E.S., Badger, J.H., Mathur, E.J., Ortmann, A.C., Bateson, M., Geesey, G.,
547 Frazier, M.: Metagenomes from high-temperature chemotrophic systems reveal geochemical
548 controls on microbial community structure and function, PLoS. One., 5(3), e9773, 2010.
549
550 Kambura, A.K., Mwirichia, R.K., Kasili, R.W., Karanja, E.N., Makonde, H.M., Boga, H.I.:
551 Bacteria and Archaea diversity within the hot springs of Lake Magadi and Little Magadi in
552 Kenya, BMC. Microbiol., 16(1), 136, 2016.
553
554 Kearey, P., Wei, H.: Geothermal fields of China, J. Volcanol. Geotherm. Res., 56, 415–428,
555 1993.
556
557 Kong, Y.: Btrim: a fast, lightweight adapter and quality trimming program for next-generation

https://doi.org/10.5194/bg-2019-406
Preprint. Discussion started: 10 December 2019

558 sequencing technologies, Genomics., 98(2), 152-153, 2011.
559
560 Li, J., Peng, X., Zhang, L., Jiang, L., Chen, S.: Linking microbial community structure to S, N
561 and Fe biogeochemical cycling in the hot springs at the Tengchong geothermal fields,
562 Southwest China, Geomicrobiology. J., 32(11), 1-15, 2015.
563
564 Li, J., Liu, X.: Identification and Characterization of a Novel Thermophilic, Organic Solvent
565 Stable Lipase of *Bacillus* from a Hot Spring, Lipids., 52(7), 619-627, 2017.
566
567 Lindsay, M.R., Amenabar, M.J., Fecteau, K.M., Debes, R.V. 2nd., Fernandes Martins, M.C.,
568 Fristad, K.E., Xu, H., Hoehler, T.M., Shock, E.L., Boyd, E.S.: Subsurface processes influence
569 oxidant availability and chemoautotrophic hydrogen metabolism in Yellowstone hot springs,
570 Geobiology., 16(6), 674-692, 2018.
571
572 Louca, S., Parfrey, L.W., Doebeli, M.: Decoupling function and taxonomy in the global ocean
573 microbiome, Science., 353(6305), 1272-1277, 2016.
574
575 Lozupone, C., Knight, R.: UniFrac: a new phylogenetic method for comparing microbial
576 communities, Appl. Environ. Microbiol., 71(12), 8228-8235, 2005.
577
578 Lozupone, C., Hamady, M., Knight, R.: UniFrac--an online tool for comparing microbial
579 community diversity in a phylogenetic context, BMC. Bioinformatics., 7, 371, 2006.
580
581 Lozupone, C.A., Hamady, M., Kelley, S.T., Knight, R.: Quantitative and qualitative beta diversity
582 measures lead to different insights into factors that structure microbial communities, Appl.
583 Environ. Microbiol., 73(5), 1576-1585, 2007.
584
585 Ma, X., Zhao, C., Gao, Y., Liu, B., Wang, T., Yuan, T., Hale, L., Nostrand, J.D.V., Wan, S., Zhou,
586 J., Yang, Y.: Divergent taxonomic and functional responses of microbial communities to field
587 simulation of aeolian soil erosion and deposition, Mol. Ecol., 26(16), 4186-4196, 2017.
588
589 Magoč, T., Salzberg, S.L.: FLASH: fast length adjustment of short reads to improve genome
590 assemblies, Bioinformatics., 27(21), 2957-2963, 2011.
591
592 Marsh, C.L., Larsen, D.H.: Characterization of some thermophilic bacteria from the hot springs
593 of Yellowstone National Park, J. Bacteriol., 65(2), 193-197, 1953.
594
595 McKay, L.J., Dlakić, M., Fields, M.W., Delmont, T.O., Eren, A.M., Jay, Z.J., Klingelsmith, K.B.,
596 Rusch, D.B., Inskeep, W.P.: Co-occurring genomic capacity for anaerobic methane and
597 dissimilatory sulfur metabolisms discovered in the Korarchaeota, Nat. Microbiol., 4(4), 614-622,
598 2019.
599
600 Menzel, P., Gudbergsdóttir, S.R., Rike, A.G., Lin, L., Zhang, Q., Contursi, P., Moracci, M.,
601 Kristjansson, J.K., Bolduc, B., Gavrilov, S., Ravin, N., Mardanov, A., Bonch-Osmolovskaya, E.,

https://doi.org/10.5194/bg-2019-406
Preprint. Discussion started: 10 December 2019

602 Young, M., Krogh, A., Peng, X.: Comparative Metagenomics of Eight Geographically Remote
603 Terrestrial Hot Springs, Microb. Ecol., 70(2), 411-424, 2015.
604
605 Mirete, S., Morgante, V,, González-Pastor, J.E.: Functional metagenomics of extreme
606 environments, Curr. Opin. Biotechnol., 38, 143-149, 2016.
607
608 Nishizawa, M., Sakai, S., Konno, U., Nakahara, N., Takaki, Y., Saito, Y., Imachi, H., Tasumi, E.,
609 Makabe, A., Koba, K., Takai, K.: Nitrogen and Oxygen Isotope Effects of Ammonia Oxidation by
610 Thermophilic *Thaumarchaeota* from a Geothermal Water Stream, Appl. Environ. Microbiol.,
611 82(15), 4492-4504, 2016.
612
613 Park, Y.J., Kim, K.H., Han, D.M., Lee, D.H., Jeon, C.O.: *Sphingobium terrigena* sp. nov.,
614 isolated from gasoline-contaminated soil, Int. J. Syst. Evol. Microbiol., 69(8), 2459-2464, 2019.
615
616 Price, M.N., Dehal, P.S., Arkin, A.P.: FastTree: computing large minimum evolution trees with
617 profiles instead of a distance matrix, Mol. Biol. Evol., 26(7), 1641-1650, 2009.
618
619 Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O.:
620 The SILVA ribosomal RNA gene database project: improved data processing and web-based
621 tools, Nucleic. Acids. Res., 41(Database issue), D590-596, 2013.
622
623 R Development Core Team.: R: A Language and Environment for Statistical Computing. Vienna,
624 Austria : the R Foundation for Statistical Computing, ISBN: 3-900051-07-0, Available online at
625 http://www.R-project.org/, 2012.
626
627 Reysenbach, A.L., Wickham, G.S., Pace, N.R.: Phylogenetic analysis of the hyperthermophilic
628 pink filament community in Octopus Spring, Yellowstone National Park, Appl. Environ.
629 Microbiol., 60(6), 2113-2119, 1994.
630
631 Rozanov, A.S., Bryanskaya, A.V., Malup, T.K., Meshcheryakova, I.A., Lazareva, E.V., Taran,
632 O.P., Ivanisenko, T.V., Ivanisenko, V.A., Zhmodik, S.M., Kolchanov, N.A., Peltek, S.E.:
633 Molecular analysis of the benthos microbial community in Zavarzin thermal spring (Uzon
634 Caldera, Kamchatka, Russia), BMC. Genomics., 15(Suppl 12), S12, 2014.
635
636 Russell, M.J., Nitschke, W.: Methane: Fuel or Exhaust at the Emergence of Life?, Astrobiology.,
637 17(10), 1053-1066, 2017.
638
639 Saxena., R., Dhakan, D.B., Mittal, P., Waiker, P., Chowdhury, A., Ghatak, A., Sharma, V.K.:
640 Metagenomic Analysis of Hot Springs in Central India Reveals Hydrocarbon Degrading
641 Thermophiles and Pathways Essential for Survival in Extreme Environments, Front. Microbiol.,
642 7, 2123, 2017.
643
644 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski,
645 R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van

https://doi.org/10.5194/bg-2019-406
Preprint. Discussion started: 10 December 2019

646 Horn, D.J., Weber, C.F.: Introducing mothur: open-source, platform-independent,
647 community-supported software for describing and comparing microbial communities, Appl.
648 Environ. Microbiol., 75(23), 7537-7541, 2009.
649
650 Schouten, S., van der Meer, M.T., Hopmans, E.C., Rijpstra, W.I., Reysenbach, A.L., Ward, D.M.,
651 Sinninghe Damsté, J.S.: Archaeal and bacterial glycerol dialkyl glycerol tetraether lipids in hot
652 springs of yellowstone national park, Appl. Environ. Microbiol., 73(19), 6181-6191, 2007.
653
654 Shock, E.L., Holland, M., Meyer-Dombard, D.A., Amend, J.P., Osburn, G.R., Fischer, T.P.:
655 Quantifying inorganic sources of geochemical energy in hydrothermal ecosystems, Yellowstone
656 National Park, USA, Geochimica. et. Cosmochimica. Acta., 74(14), 4005–4043, 2010.
657
658 Shi, Z., Yin, H., Van Nostrand, J.D., Voordeckers, J.W., Tu, Q., Deng, Y., Yuan, M., Zhou, A.,
659 Zhang, P., Xiao, N., Ning, D., He, Z., Wu, L., Zhou, J.: Functional Gene Array-Based
660 Ultrasensitive and Quantitative Detection of Microbial Populations in Complex Communities,
661 mSystems., 4(4), e00296-19, 2019.
662
663 Song, Z.Q., Wang, F.P., Zhi, X.Y., Chen, J.Q., Zhou, E.M., Liang, F., Xiao, X., Tang, S.K., Jiang,
664 H.C., Zhang, C.L., Dong, H., Li, W.J.: Bacterial and archaeal diversities in Yunnan and Tibetan
665 hot springs, China, Environ. Microbiol., 15(4), 1160-1175, 2013.
666
667 Spear, J.R., Walker, J.J., McCollom, T.M., Pace, N.R.: Hydrogen and bioenergetics in the
668 Yellowstone geothermal ecosystem, Proc. Natl. Acad. Sci. U S A., 102(7), 2555-2560, 2005.
669
670 Tang, J., Liang, Y., Jiang, D., Li, L., Luo, Y., Shah, M.M.R., Daroch, M.: Temperature-controlled
671 thermophilic bacterial communities in hot springs of western Sichuan, China, BMC. Microbiol.,
672 18(1), 134, 2018.
673
674 Ueno, Y., Yamada, K., Yoshida, N., Maruyama, S., Isozaki, Y.: Evidence from fluid inclusions for
675 microbial methanogenesis in the early Archaean era, Nature., 440(7083), 516-519, 2006.
676
677 Vanwonterghem, I., Evans, P.N., Parks, D.H., Jensen, P.D., Woodcroft, B.J., Hugenholtz, P.,
678 Tyson, G.W.: Methylotrophic methanogenesis discovered in the archaeal phylum
679 Verstraetearchaeota, Nat. Microbiol., 1, 16170, 2016.
680
681 Wang, S., Hou, W., Dong, H., Jiang, H., Huang, L., Wu, G., Zhang, C., Song, Z., Zhang, Y., Ren,
682 H., Zhang, J., Zhang, L.: Control of temperature on microbial community structure in hot
683 springs of the Tibetan Plateau, PLoS. One., 8(5), e62901, 2013.
684
685 Wu, L., Liu, X., Schadt, C.W., Zhou, J.: Microarray-based analysis of subnanogram quantities of
686 microbial community DNAs by using whole-community genome amplification, Appl. Environ.
687 Microbiol., 72(7), 4931-4941, 2006.
688
689 Zhao, M., Xue, K., Wang, F., Liu, S., Bai, S., Sun, B., Zhou, J., Yang, Y.: Microbial mediation of

https://doi.org/10.5194/bg-2019-406
Preprint. Discussion started: 10 December 2019

690 biogeochemical cycles revealed by simulation of global changes with soil transplant and
691 cropping, ISME. J., 8(10), 2045-2055, 2014.
692
693 Zhou, J., He, Z., Yang, Y., Deng, Y., Tringe, S.G., Alvarez-Cohen, L.: High-throughput
694 metagenomic technologies for complex microbial community analysis: open and closed formats,
695 MBio., 6(1), e02288, 2015.
696