Interactive comment on "Distinct microbial composition and functions in an underground high-temperature hot spring at different depths" by Shijie Bai and Xiaotong Peng

Anonymous Referee #1

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This manuscript mainly reported the microbial diversity and functions of three hot springs water samples collected from different depths. The authors found that both bacterial and archaeal community compositions were different along depth, and distinct microbial functions may occur at different depths of hot springs. This study has some merit, because few study focused on surveying the microbial diversity and functions in the subsurface of geothermal systems, which deserves publication. But the authors may need to revise the manuscript thoroughly on its grammar and structure before the ms is acceptable. Some examples were listed below but they are not exhaustive.

Response: Thank the reviewer for his/her time spent providing an in-depth review of our manuscript. We believe this is a valuable work of the hot spring microbiology research. Thank you for your review.

Detail comments below: Page 7 line 135-138: please cite refs for these cutoff.

Response: Thank you for your review. After checked all related GeoChip paper, we did not find "exactly the same" cutoff method. However, these cutoff were made by Joy D. Van Nostrand who is the Scientist of Institute for Environmental Genomics, the University of Oklahoma, and she mentioned in the email like this:

" Shijie,

Here is your GeoChip data for the NJ samples. The numbers are normalized signal intensity and shows all positive probes detected in each sample. To call probes positive, we used a floating SNR so that the hyperthermophile probes accounted for 5% of the positive signals. We then we removed probes as negative if the signal was <1.3 times the background. We did not do any further data transformation. You can use this dataset as-is to do analyses.

.....

Joy D. Van Nostrand, PhD

Scientist

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Thank you so much.

Page 8 line 171-173: awkward grammar issue, please rephrase. Please provide the exact number of reads for normalization.

Response: Thank you for your review. We have made correction according to your comments. Now, reads as follows:
"UPARSE (Edgar, 2013) was used to remove chimeras and cluster sequences into 97% identical operational taxonomy units (OTUs); singletons were kept for further analysis; the bacterium and archaea OTU tables were randomly resampled for the normalization of different sample reads, 48478 sequences for bacterium, and 2317 sequences for archaea.". Thank you.

Page 9 line 187-190: please specify the PCoA analysis.

Response: Thank you for your review. We have made correction according to your comments. Now, reads as follows:
"PyNAST was used to align the selected representative OTU of all samples (Caporaso et al., 2010), the tree file was obtained from FastTree (Price et al., 2009), and the Phylogenetic diversity (PD) was calculated with the Picante package (Kembel et al., 2010). The principal coordinate analysis (PCoA), and detrended correspondence analysis (DCA) were generated by the vegan

package in R.". Thank you.

Page 10 line 211-212: please remove this sentence.

Response: Thank you for this suggestion. We have made correction according to your comments.

Page 10 line 208: Any other parameters were measured in this study?

Response: Thank you for your review. Unfortunately, we did not measure any other parameters except depth, temperature, and pH. We agree that this is the shortcoming of this study. We hope more environmental parameters could be measured by some geologists and scientists in the future, and more discoveries could be expected. Thank you.

Page 11 line 216-218: please remove these sentences.

Response: Thank you for this suggestion. We have made correction according to your comments.

Page 11 line 219: “The Shannon and Inverse Simpson indexes indicated: : :” so weird.

Response: Thank you for your review. Yes, we think the α-diversity indexes of 19 m were weird when we first saw these results. But, the bacterial functions predicted by FAPROTAX were mainly related to chemoheterotrophy at the depth of 19 m, and many microorganisms differ from other two groups were detected, it can cause the high indexes ofα-diversity. Thank you.

Page 11 line 222-214: please remove these sentences.

Response: Thank you for this suggestion. We have made correction according to your comments.

Page 11 line 227: “Gamma-proteobacteria” is not a phylum.

Response: Thank you for your review. We have revised the "Gamma-proteobacteria" to " Proteobacteria (Gamma)", and we checked and corrected all similar mistakes in the manuscript. Thank you.

Page 12 line 236: “twelve samples”? How many samples did you use?

Response: Thank you for your review. Sorry, it is our mistake, it is nine samples, and we corrected it. Thank you so much.

Page 12 line 238-240: why archaeal communities at 0 m and 58m depths were similar?

Response: Thank you for your review. 2317 sequences of each archaeal sample were applied for random resampling, in the samples of 0 m, 2251 to 2262 sequences belong to *Candidatus* Nitrosocaldus, and in the samples of 58 m, 1955 to 2067 sequences belong to *Candidatus* Nitrosocaldus. However, in the samples of 19 m, only 366 to 594 sequences belong to *Candidatus* Nitrosocaldus. That is why the archaeal communities at 0 m and 58 m depths were similar. Thank you.

Page 12 line 238-242: Thaumarchaeota was the most abundant phylum across all samples. You mean uncultured Desulfurococcales archaea are Thaumarchaeota? Please rephrase those sentences. These sentences would mislead the readers.

Response: Thank you for your review. We have made correction according to your comments. Now, reads as follows:
"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 Candidatus *Nitrosocaldus* in hot spring samples at 0 m and 58 m. In contrast, the

most abundant belonged to uncultured archaeon, Candidatus *Nitrosocaldus*, and Candidatus *Nitrocosmicus* in hot spring samples at 19 m (Fig. 4B).". Thank you.

Page 20-21 line 345-369: almost no discussion. This paragraph is too long, please split.

Response: Thank you for your review. We have deleted some sentences and revised this part. Thank you.

Page 22 line 399: too speculative.

Response: Thank you for your review. We have deleted this part, and rewrote discussion part. Thank you.

Page 24 line 429-445: Meaningless. Current discussion is too lengthy and meandering. The authors should re-organize the discussion without too much repeating results. To do so, the authors may follow the following rationale: what finding is notable and why is it notable? Is such a finding consistent or inconsistent with previous related literature and why so?

Response: Thank you for your review. After reading many published articles related to hot springs, we rewrote the results, and discussion part. Please see

below:

Results

Microbial diversity and community taxonomic composition

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. The temperatures and pH of the samples were similar, and the environmental parameters data were collected before sampling and are summarized in Table 1.

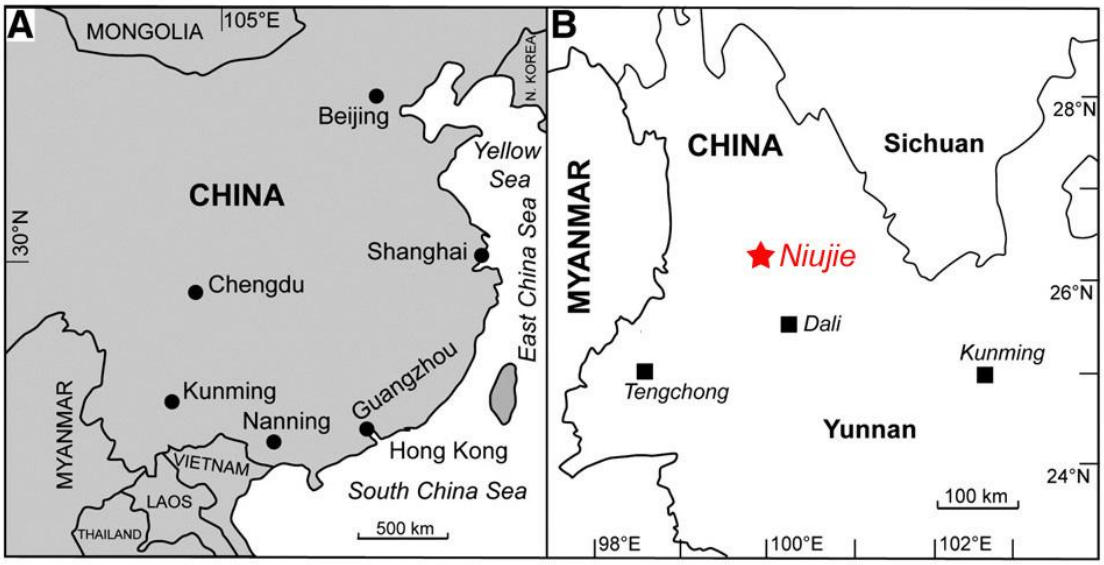

Fig 1. The geographical map showing the hot springs sampling locations in Niujie Town, Eryuan county, Dali city, Yunnan province, China.

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

After quality control, a total of 534875 sequences for bacterium and 111989 sequences for archaea were obtained for nine high-temperature microbial communities sampled from hot spring at three different depths. For the microbial diversity, 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. The bacterial groups at 0 m with the highest relative abundances at the phylum level were members of Aquificae, Proteobacteria (Gamma), and Deinococcus-Thermus. For the 19 m sample, the dominant taxa were Proteobacteria (Alpha), Proteobacteria (Gamma),

and Firmicutes. The bacterial groups Deinococcus-Thermus, Firmicutes, and Proteobacteria (Gamma) dominated in the 58 m samples (Fig. 3A). At the genus level, 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 9 samples, the sequence reads were clustered into 43 OTUs for archaea. 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 *Candidatus* Nitrosocaldus in hot spring samples at 0 m and 58 m. In contrast, the most abundant belonged to uncultured archaeon, *Candidatus* Nitrosocaldus, and *Candidatus* Nitrocosmicus in hot spring samples at 19 m (Fig. 4B).

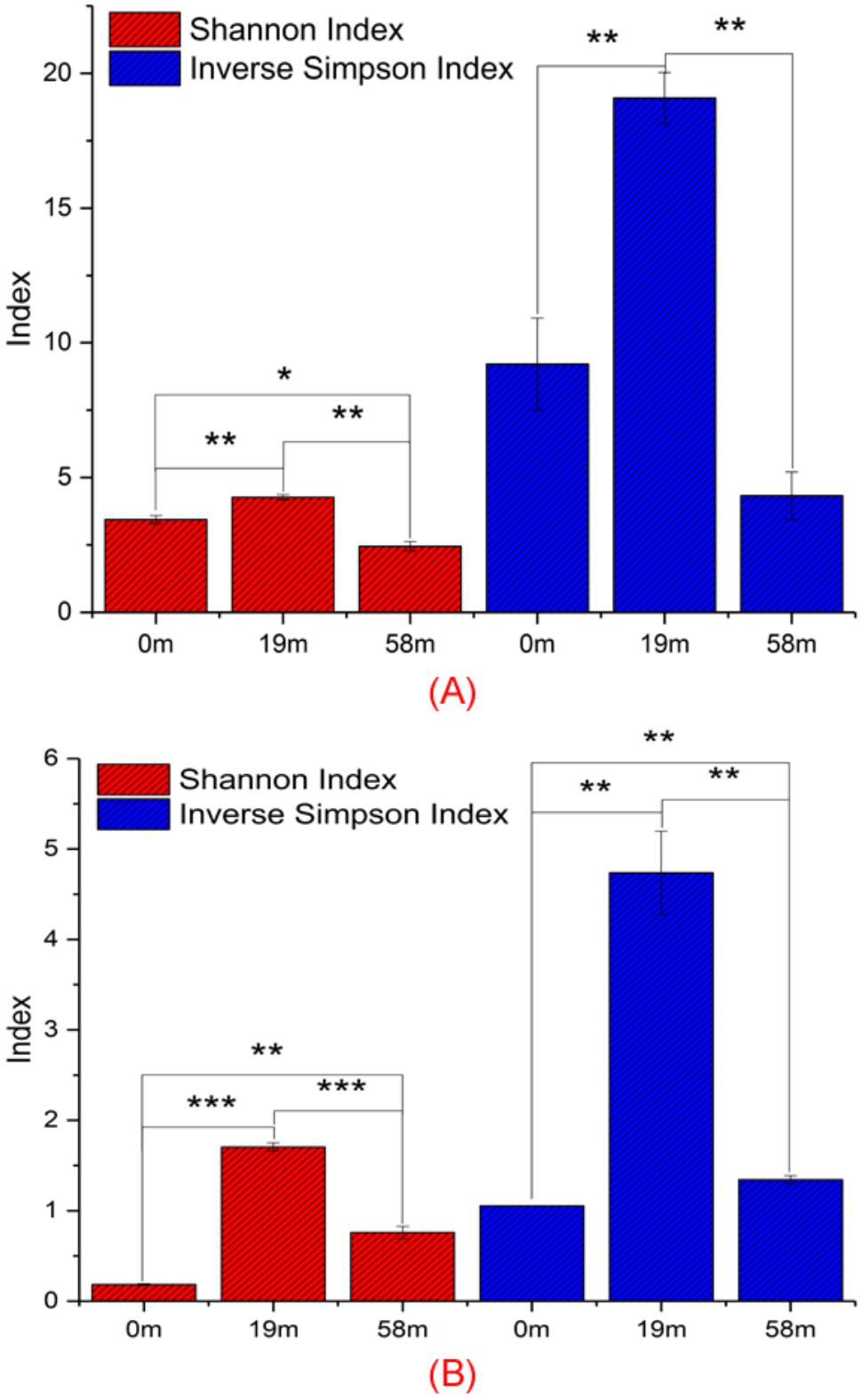

Fig 2. Comparison of the alpha diversity indexes, Shannon index and Inverse Simpson index (A: Bacterial communities; B: Archaeal communities). The value is the mean of the indices within each group. Error bars represent the standard error (SE). *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ based on Student's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

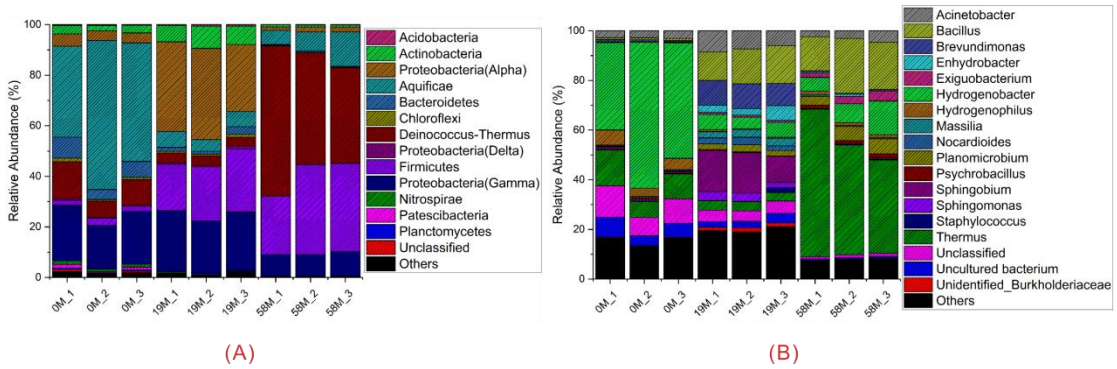

Fig 3. Stacked bar chart showing the relative abundance of the bacterial community composition at the phyla and classes of Proteobacteria level (A), and the genera level (B).

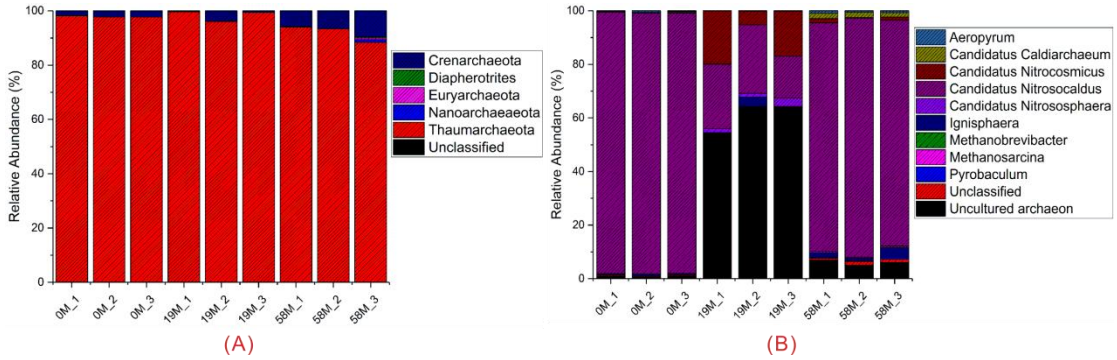

Fig 4. Stacked bar chart showing the relative abundance of the archaeal community composition at the phyla level (A), and the genera level (B).

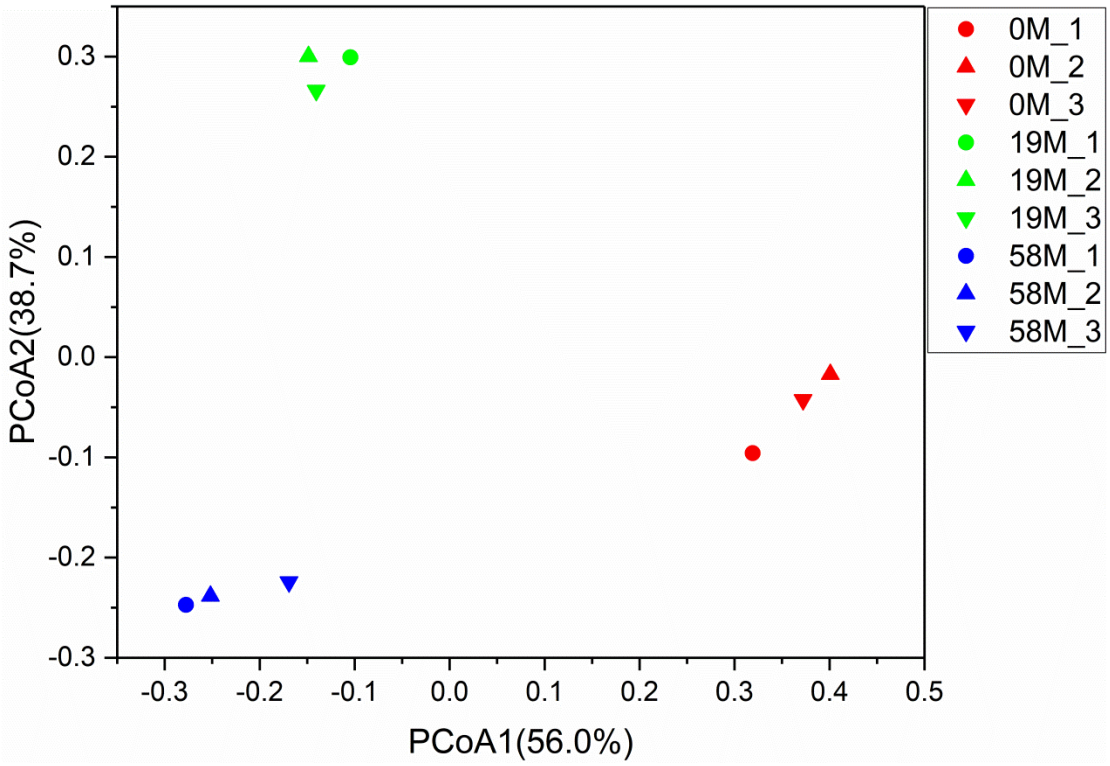

Fig 5. Principal coordinate analysis (PCoA) of bacterial communities from hot springs at different depths. The results are based on weighted the UniFrac distances of the detected OTUs.

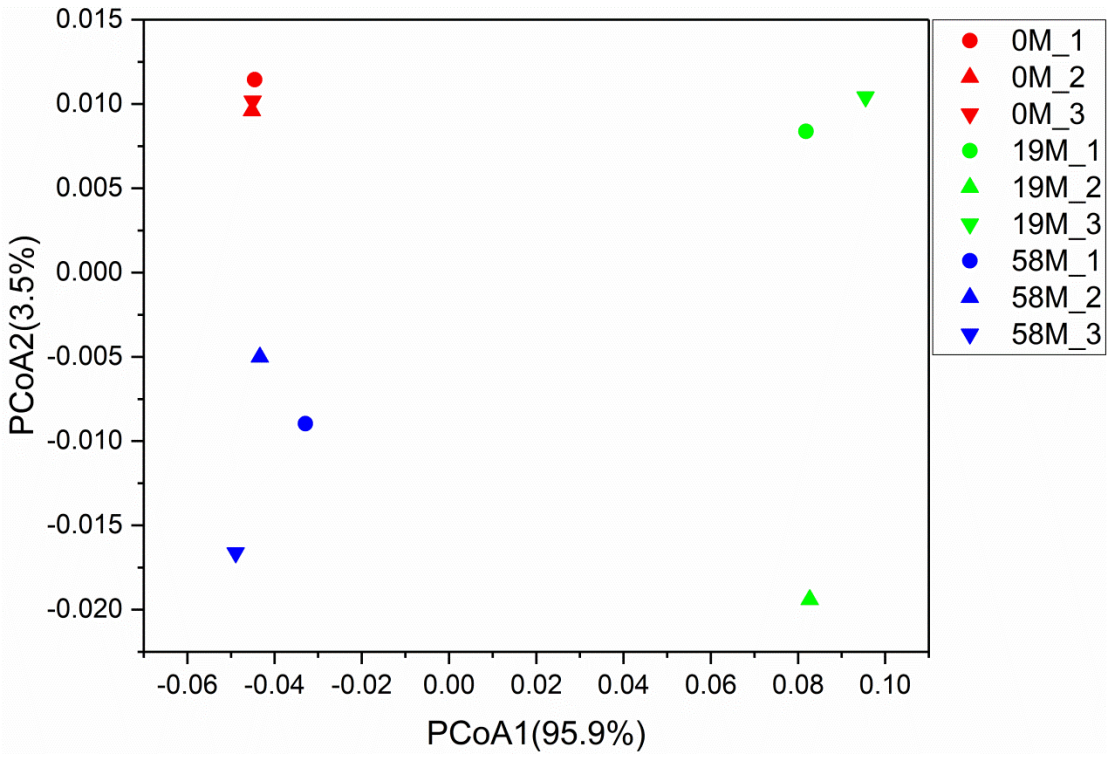

Fig 6. Principal coordinate analysis (PCoA) of archaeal communities from hot springs at different depths. The results based on the weighted UniFrac distances of the

detected OTUs.

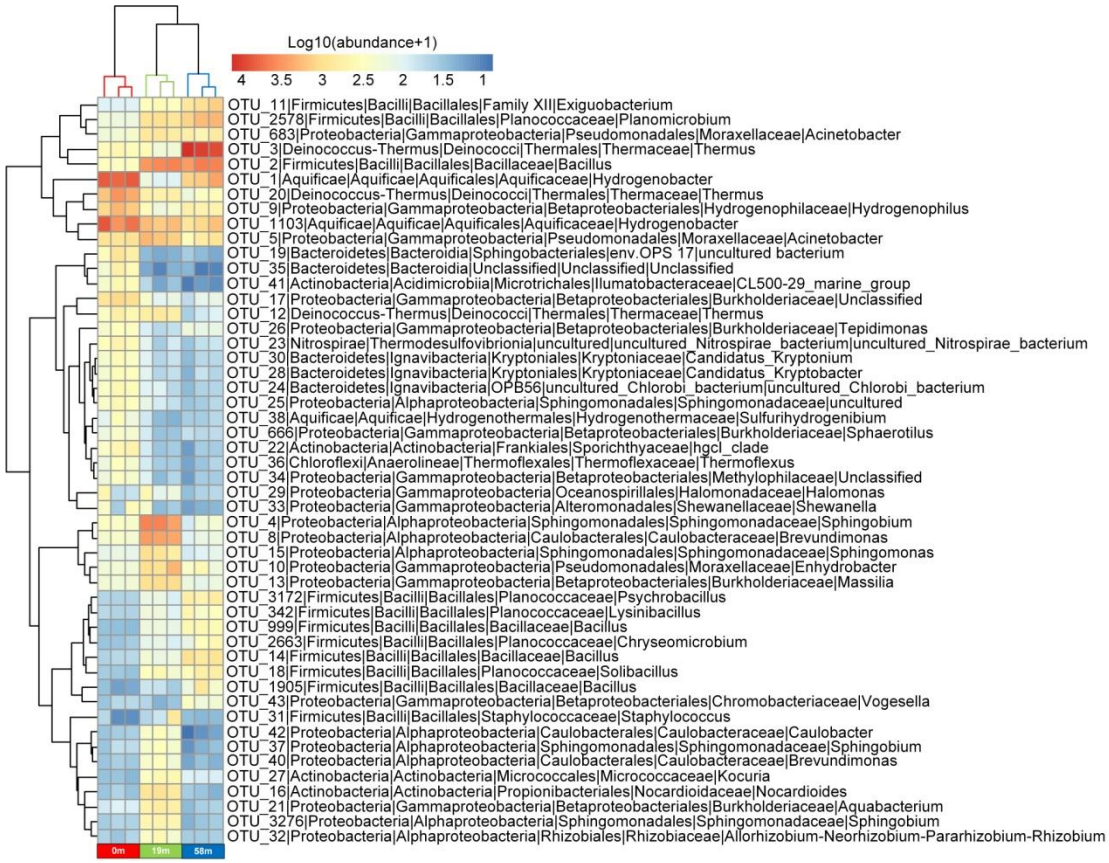

Fig 7. The 50 most abundant bacterial community OTUs from hot springs at different depths. Bacterial abundance was scaled with a log transformation in the heatmap.

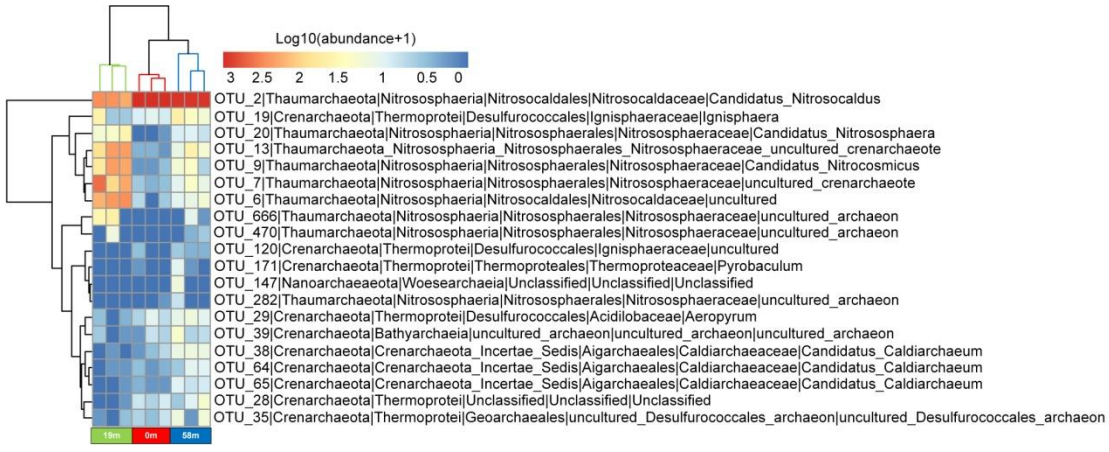

Fig 8. The 20 most abundant archaeal community OTUs from hot springs at different depths. Archaeal abundance was scaled with a log transformation in the heatmap.

Microbial community structure of hot springs at different depths

To examine the microbial community structure of the hot spring at different depths, ß-diversity-based statistical tools were applied, such as principal coordinate analysis (PCoA). The results of PCoA showed that the bacterial and archaeal community structures were distinctly separate from each group at different depths (Fig. 5,6). 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). The OTU3, which annotated to *Thermus* was the most abundant OTU in the bacterial community and mainly contributed from the samples of 58 m. The OTU1 and OTU1103 were both affiliated with *Hydrogenobacter* and dominated in the hot spring samples at 0 m, then the OTU2 and OTU4, which belonged to *Bacillus* and *Sphingobium* were most detected in the samples of 19 m. In the archaeal microbial community, the OTU2, which represent by *Candidatus* Nitrosocaldus, harbored the vast majority of archaeal sequences (69%), and most come from the samples of 0 m and 58 m.

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).

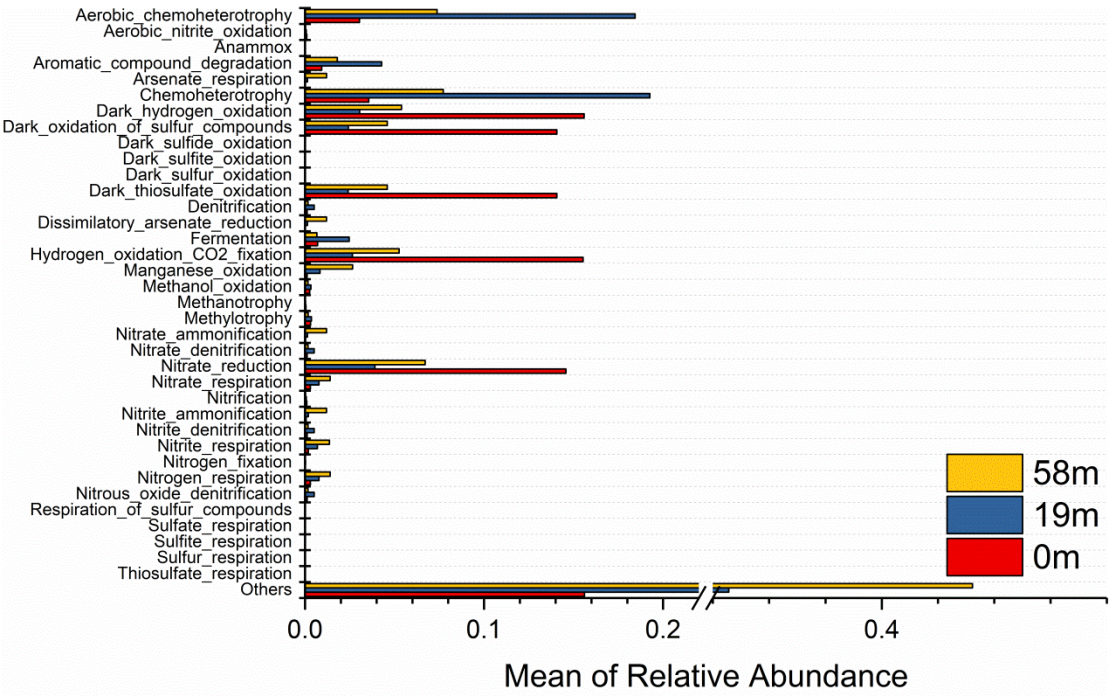

Fig 9. Stacked bar chart showing the mean relative abundance of the predicted metabolic potential of bacterium from hot springs at different depths, as predicted by FAPROTAX.

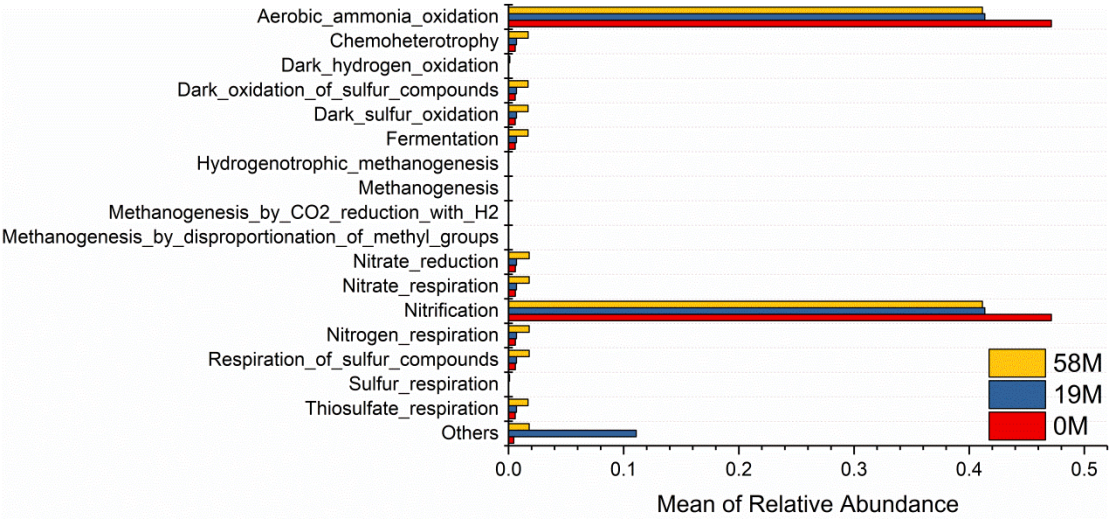

Fig 10. Stacked bar chart showing the mean of the relative abundance of the predicted metabolic potential of archaea from hot springs at different depths, as predicted by FAPROTAX.

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. *nifH*, encoding nitrogenase for nitrogen fixation was detected in all samples, the *nifH* gene abundance of 19 m was slightly lower than other two groups. The samples of 0 m and 58 m showed higher abundances of nitrification related genes, including *hao* (encoding hydroxylamine dehydrogenase) and *amoA* (encoding ammonia monooxygenase). In the process of nitrate reduction, many genes were detected, such as *napA*, *narB*, and *nasA*, encoding nitrate reductase, as well as *nrfA*, and *nirA*, which encoding nitrite reductase. High abundances of genes involved in dissimilatory nitrate reduction was found in the samples of 0 m, followed by 19 m and 58 m, and the results of assimilatory nitrate reduction showed the samples of 19 m harbored more related genes (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 abundances of genes for methane production or methanogenesis were detected at all three hot springs depths. Functional genes of *pmoA* and *mmoX* are both encoding methane monooxygenase, the abundances of these two genes were higher in the samples of 19 m and 58m, rather than the 0 m. On the contrary, the functional genes related to methanogenesis in the samples of 0 m were more abundant than the

other two groups. The heatmap showed the functional genes related to methane cycle were more similar between 19 m and 58 m. (Fig. 12). For the functional genes involved in sulfur metabolism, *soxA*, encoding thiosulfotransferase, *soxV*, and *soxY* encoding sulfur oxidizing protein, *soxB*, encoding sulfohydrolase, and *soxC*, which encoding sulfane dehydrogenase were detected, the abundances of these genes indicated that sulfur and thiosulfate oxidation process were more intense of 0 m, followed by the samples of 19 m, then the 58 m. However, in the samples of 58 m, the abundances of genes related to sulfide oxidation were more intense than the other two groups. For the genes involved in sulfite reduction, the samples of 19 m and 58 showed higher abundances than the samples of 0 m (Fig. 13B), and results of heatmap showed the functional gene structures of the sulfur cycles at different depths were different. (Fig. 13A).

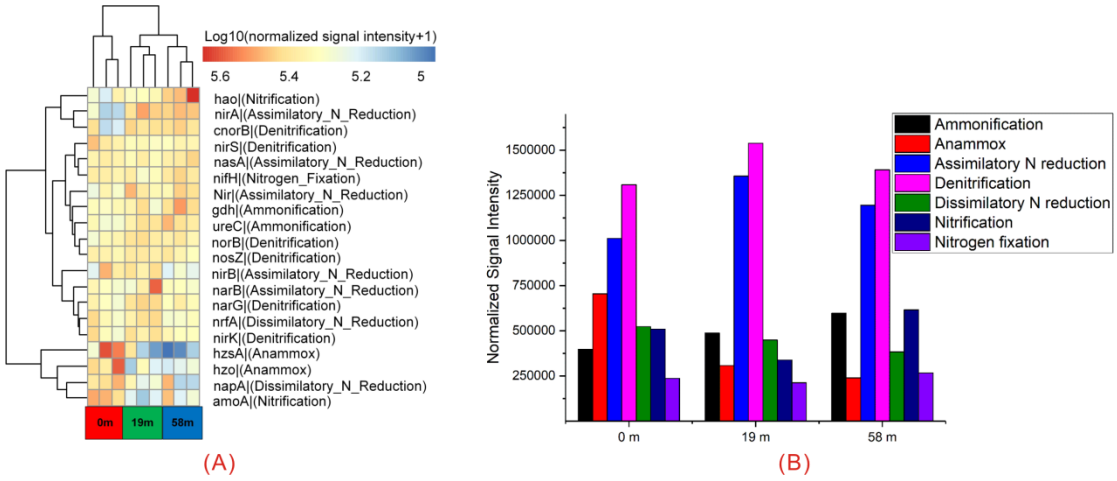

Fig. 11. The normalized signal intensity of the detected key genes involved in the nitrogen cycle (A). The signal intensity for each functional gene category is the average of the total signal intensity from all the replicates, and the heatmap of the functional genes involved in the nitrogen cycle at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

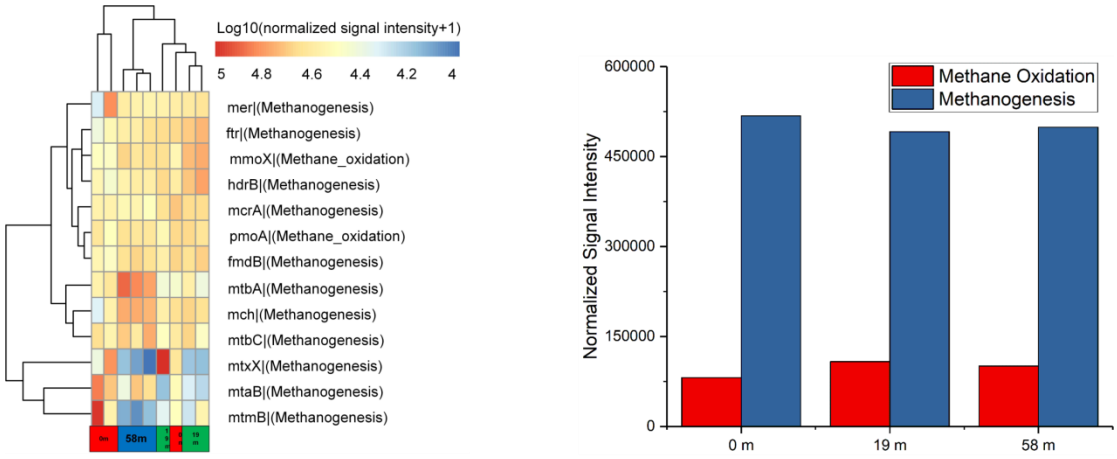

Fig. 12. The normalized signal intensity of the detected key genes involved in the methane cycle (A). The signal intensity for each functional gene category is the average of the total signal intensity from all the replicates, and the heatmap of the functional genes involved in the methane cycle at different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

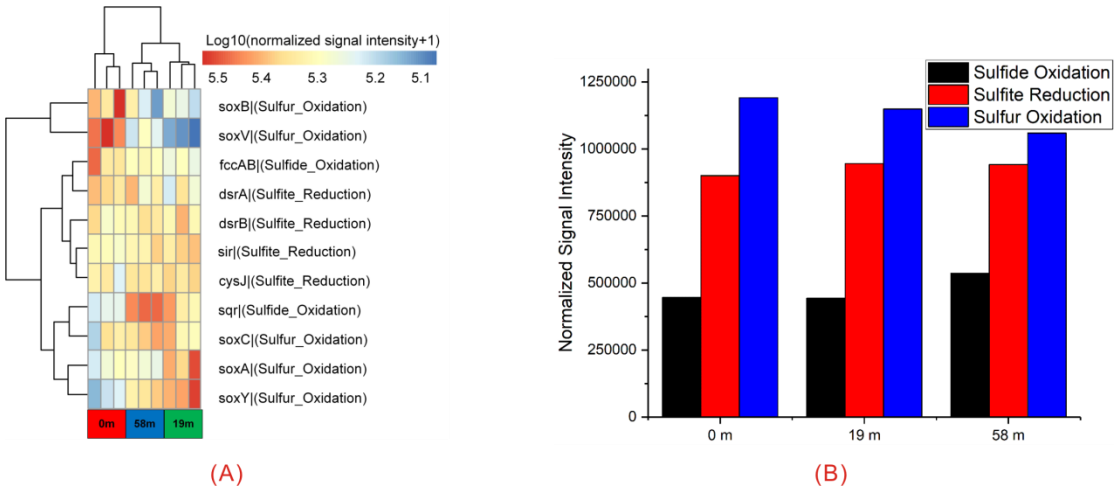

Fig. 13. The normalized signal intensity of the detected key genes involved in the sulfur cycle (A). The signal intensity for each functional gene category is the average of the total signal intensity from all the replicates, and the heatmap of functional genes involved in the sulfur cycle at different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

Discussion

The diversity of archaea and bacteria in hot springs, an extreme environment, has been investigated extensively (Wang et al., 2013; Li et al., 2015; Chen et al., 2016; Power et al., 2018; Zhang et al., 2018). However, 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. Although the temperatures and pH of the samples were similar, the distinct bacterial composition was observed at different depths of hot spring water samples. 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.

The phylum Aquificae is a very common bacterial phylum in neutral and alkaline high-temperature surface hot springs (Hou et al., 2013; Vick et al., 2010; Chan et al., 2015). The *Aquificaceae*, *Hydrogenothermaceae*, and *Desulfurobacteriaceae*, which constitute the single order of Aquificae, *Aquificales* (Hedlund et al., 2015). *Hydrogenobacter* as a thermophilic, hyperthermophilic, chemolithoautotrophic, and aerobic hydrogen oxidizing bacterium affiliated to *Aquificaceae* (Arai et al., 2010; Nishihara et al., 2018) was found in all samples of hot springs at different depths, particularly in the samples of 0 m. 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). *Hydrogenobacter*

not only can utilizes hydrogen but also thiosulfate as the sole source of energy for reducing power, and can fix carbon dioxide as sole carbon source through the reductive tricarboxylic acid (RTCA) cycle (Sato et al., 2012). More than that, Nitrate reductases, such as Dissimilatory nitrate reductase (NAR) and assimilatory nitrate reductase (NAS) also served as key enzymes for nitrate-reducing pathways in *Hydrogenobacter* (Kameya et al., 2017). The functional profiling of the bacterial communities revealed the bacteria in the hot springs at 0 m are mainly involved in hydrogen, sulfur and thiosulfate oxidation, and nitrate reduction, moreover, the results of GeoChip was also supporting this finding.

Sphingobium and *Sphingomonas* are very important genera for their ability to degrade macromolecular organic contaminants, especially polycyclic or heterocyclic aromatic compounds, and can be found in many different environments (Zhao et al., 2017). However, they are very rarely found in hot springs environments. But, this finding may be explained by their diverse environmental adaptations and biodegradative capabilities (Aylward et al., 2013), especially considering that main bacterial functions predicted by FAPROTAX were related to chemoheterotrophy at the depth of 19 m. Besides, *Brevundimonas* and *Bacillus* were also detected in our hot spring samples, these genera were reported that can be isolated from hot spring environments with the ability of their producing extracellular thermostable hydrolytic enzymes (Sahay et al., 2017; Masoudzadeh et al., 2011; Mehetre et al., 2019), and can be exhibited

biodegradation of many macromolecular compounds (Mehetre et al., 2019; Gómez-Acata et al., 2017; Portune et al., 2015). It is therefore likely that such microorganisms exist in hot springs can be one of the potential responses for the chemoheterotroph.

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*. *Thermus* species are thermophilic heterotrophs, with most capable of using a variety of organic and inorganic electron donors for respiration, and can be found in many non-acidic geothermal areas with the temperature > 51 °C (Song et al., 2013; Zhou et al., 2020; Zhang et al., 2018), and even can be detected in hot spring water samples as high as 96 ± 3 °C (Mahato et al., 2019). Our results, *Thermus* dominated the 82 °C hot spring at the 58 m depth, are consistent with previous results for the neutral and alkaline high temperature hot springs. Integration of cultivation-independent and pure cultures method was applied to reveal the diversity and respiratory capability of the *Thermus*, suggesting *Thermus* played a very important role in sulfur, metal, and nitrogen biogeochemical cycles in terrestrial hot springs, such as the oxidation of sulfur and thiosulfate, and the reduction of nitrate (Zhou et al., 2020). The results of GeoChip and predictive functional profiling all support this view.

Previous studies have demonstrated that archaea can ubiquitously coexist in hot springs (Zhang et al., 2018). 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", an ammonia-oxidizing thermophilic archaeon (Abby et al., 2018). Ammonia oxidizing archaea (AOA) can obtain energy solely from the oxidation process of ammonia to nitrite with the first step in nitrification (Tourna et al., 2011). 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. The potential ammonia oxidation capacity of microbial communities from hot spring at different depth were comparable. Even there were a large number of uncultured archaeon and many *Candidatus* Nitrocosmicus in the samples of 19 m, but, those archaeon and *Candidatus* Nitrosocaldus were all affiliated to the same family, order, and class lever, which are *Nitrosocaldaceae*, *Nitrosocaldales*, and *Nitrososphaeria*, respectively.

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 two OTUs affiliated with *Methanobrevibacter* in the sample of 19 m with three sequences and one OTU belonged to *Methanosarcina* with only one sequence. Some methane-oxidizing bacterium, such as *Methylomonas*, *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. The microbial community composition and their metabolic functions in hot springs at different depths should be addressed extensively by further integration of multi-omics and isotope tracer approaches in different geothermal systems.

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