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 #2

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Overall comments:

The manuscript describes the taxonomy and functional potentials of microbial communities at three depths of hot springs. Based on the introduction of this study, it provides new data on microbes from the subsurface biosphere, but more analyses and thinking on these datasets are needed. The manuscript also needs to be rewritten in terms of the language.

Response: Thank you for your comments, we will revise our manuscript address your comments, and using language editing services to improving the English writing of our manuscript, thank you.

Major criticisms:

1. The main conclusion of this study is that the microbial communities were different at different depths. Most figures were used to indicate how similar or different the three samples were. This conclusion is too vague to deliver useful

information from these valuable datasets. More thinking about the results are needed to obtain more specific findings. There was some discussion on specific microbes found in extreme environments from previous studies in the 'discussion' section. However, searching the 16S data in this study for microbial species which were expected in hot springs or analyzing the most abundant species to see whether they were previously recognized as hot spring specialists could make the discussion more solid. Based on functional genes, discuss what metabolic potentials may help the dominant species adapt to the extreme environment. If there was any nutrient data, could nutrients explain the functional potentials of dominant microbes? Solid conclusions are missing in the current manuscript.

Response: Thank you for your comments, we have re-organized the results, and discussion parts. Please see page 15 to page 35, thank you.

2. Many statistical analyses were applied to the data, but explanations and justifications on these analyses are needed. For example, Figs 5 & 6: please justify why both PCoA and DCA are needed and explain what different information could be gained from them in the 'materials and methods' section. If only one is necessary, please update figures and text accordingly. Line 286: this conclusion was drawn from DCA (Fig 6B), but PCoA result (Fig 6A) did not support it. Please explain why the PCoA was not considered. Line 288-290:

different depths harboring distinct microbial communities was indicated by PCoA and DCA analyses (Figs 5 & 6), what could Figs 7 & 8 tell us besides communities were different?

Response: Thank you for your review. We agree that only PCoA is enough, and we will update the figures and text accordingly, thank you. The PCoA results (Fig 6A) still could support the conclusion drawn from DCA, because the percentage of PCoA 1 was 95.9%, and the samples of 0 m and 58 m were very close to each other along the x-axis. We will exclude the figures and text of DCA to avoid misleading the readers, thank you. Yes, we agree that PCoA and Heatmap both could indicate the distinct microbial communities, but, meanwhile, the heatmap also can show some information of OTU, and indicated which OTU dominated in different samples, which did not show in the Stacked bar chart.

3. Microbial function potential was inferred from both 16S data via FAPROTAX and GeoChip 5.0. There was not any comparison between results from these two methods in the manuscript. Why two different methods were used? Did they tell the same story? If not, what is the reason? Is there any information we could get from one but not the other?

Response: Thank you for your review. FAPROTAX, a database that maps prokaryotic clades (e.g., genera, species or subspecies), was used to establish

metabolic or other ecologically relevant functions based on the current literature. FAPROTAX includes software for converting taxonomic microbial community profiles (e.g., in the form of an OTU table) into putative functional profiles based on the taxa identified in a sample. Therefore, the results of these predictive functions are theoretical. Functional gene arrays (FGAs) target genes involved in various functional processes and are valuable for assessing the functional composition and structure of microbial communities. GeoChip, a generic FGA targeting hundreds of functional gene categories important to biogeochemical, ecological, and environmental analyses, is the most widely used. GeoChip is an effective, sensitive, and quantitative tool for examining the functional structure of microbial communities from a variety of environments. Basically, the results of GeoChip are more reliable. However, we did the whole community genome amplification before hybridization, but we did not do the amplification for 16S rRNA gene sequencing. In order to gain more functional information of the microbial community at different depths of hot springs, we applied these two different methods. We have added more information to compare and discuss this issue. Thank you.

Detailed comments:

Abstract: went straight into the methods without any background information. Please moved.

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

Line 200-201 and Line 360-363: the logic seems to be that the environmental conditions at three depths were similar, but microbial communities were different. You cannot say that the environmental conditions were similar based only on two parameters (pH and temperature). How about other important parameters such as oxygen and nutrient concentrations?

Response: Thank you for your review. Yes, you are right. The changes have been made accordingly in the revised manuscript. For line 200-201, " According to the temperatures and pH, there were no significant differences between the samples." has been revised to " The temperatures and pH of the samples were similar, "; For line 360-363, " 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." has been revised to "Although the temperatures and pH of the samples were similar, the distinct bacterial composition was observed at different depths of hot spring water samples. ". Thank you.

Line 239-240: based on Fig 4B, the most abundant purple bar was labeled as

Candidatus Nitrosocaldus, and nothing was labeled as ‘Uncultured Desulfurococcales archaeon’.

Line 241-242: this was not consistent with Fig 4B either. Fig 4B: is ‘uncultured archaeon’ in the legend supposed to be ‘uncultured Desulfurococcales archaeon’ as mentioned in the text? The text kept saying uncultured Desulfurococcales archaeon, but it does not even exist in the legend of Fig 4B. If ‘uncultured archaeon’ is actually ‘uncultured Desulfurococcales archaeon’ which doesn’t belong to Thaumarchaeota, how could it be possible that more than 90% of archaea were from Thaumarchaeota phylum at 19 m (Fig 4A) and more than 50% of archaea in the same sample were uncultured Desulfurococcales archaeon which does not belong to Thaumarchaeota (Fig 4B)? Please check the taxonomic classification carefully.

Response: Thank you for your review. Sorry, It was our fault, The changes have been made accordingly in the revised manuscript. Now, reads as follows:

" 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)."

Figs 9 & 10: (1). Need more explanations. For example, what is ‘mean of relative abundance’? Should the numbers on the x-axis be in percentage such as ‘relative

abundance' in Figs 3 & 4? Do all the numbers of the metabolic potential categories listed in the Figs add up to 1 or 100% for each depth? (2) The metabolic potential categories should be carefully labeled. For example, 'aerobic_chemoheterotrophy' and 'chemoheterotrophy' were both listed in Fig 9. Was the latter 'anaerobic_chemoheterotrophy' or all chemoheterotrophy? Why were 'nitrite oxidation', 'ammonia oxidation' and 'nitrification' all listed while nitrification includes ammonia oxidation and nitrite oxidation? What does 'nitrous_oxide_denitrification' mean? Does it mean the production or consumption of nitrous oxide via denitrification?

Response: Thank you for your review. Yes, more detail explanations have been added. Since each depth has three samples, we calculated the average value of each functional group, so, we used the "mean of relative abundance" to represent those results. Yes, the numbers on the x-axis are in percentage represent the relative abundance of each functional category in different samples. Yes, all the numbers of the metabolic potential categories listed in the Figs add up to 1 or 100% for each depth. After checking the FAPROTAX and published paper " Decoupling function and taxonomy in the global ocean microbiome (Science, 2016)", in the Supplementary Materials, Louca *et al* mentioned: " Strain-specific variations within species were ignored in favor of type strains, which may have led to further inaccuracies in our functional annotations, for example in cases where horizontal gene transfer led to differentiation between strains. This may be particularly

relevant to pathways involved in the breakdown of different carbon compounds, which is partly why we combined aerobic chemoheterotrophs into a single group, as opposed to considering various carbon-catabolic pathways separately (similarly for fermentation). " and " Furthermore, functional groups could be nested: For example, all nitrate denitrifiers were also associated with nitrate respiration as well as nitrate reduction. ". The file of FAPROTAX indicated that the "anaerobic_chemoheterotrophy " groups include the OTUs other than lignin, chitin, xylan, cellulose, methanol, methane, aromatic hydrocarbons, and the condition was aerobic. However, the group of " chemoheterotrophy " indicated the aerobic was variable. Yes, the "nitrous_oxide_denitrification" means the consumption of nitrous oxide via denitrification. Many publications using FAPROTAX showed the same metabolic potential categories in the figures. For example:

1. Kong, Xiao, Jin, Decai, Tai, Xin, Yu, Hao, Duan, Guilan, Yan, Xiulan, Pan, Jiangang, Song, Junhua, Deng, Ye (2019) - Bioremediation of dibutyl phthalate in a simulated agricultural ecosystem by Gordonia sp. strain QH-11 and the microbial ecological effects in soil. Science of The Total Environment, 667:691-700. DOI:10.1016/j.scitotenv.2019.02.385.

2. Hochstein, Rebecca, Zhang, Qian, Sadowsky, Michael J., Forbes, Valery E. (2019) - The deposit feeder Capitella teleta has a unique and relatively complex microbiome likely supporting its ability to degrade pollutants. Science of The Total

Environment, 670:547-554. DOI:10.1016/j.scitotenv.2019.03.255.

3. Bomberg, Malin, Claesson Liljedahl, Lillemor, Lamminmäki, Tiina, Kontula, Anne (2019) - Highly Diverse Aquatic Microbial Communities Separated by Permafrost in Greenland Show Distinct Features According to Environmental Niches. Frontiers in Microbiology, 10:1583. DOI:10.3389/fmicb.2019.01583.

Line 296-298: (1) the expression is weird. (2) how could you tell that all archaea were involved? In Fig 10, none of the bars for ammonia oxidation or nitrification reached 1 or 100%.

Response: Thank you for your review. Yes, you are right, we can not say that all archaea were involved in ammonia oxidation and nitrification. The changes have been made in the revised manuscript. Now, reads as follows:
"The FAPROTAX results based on the archaeal communities showed that the archaea mainly involved in ammonia oxidation and nitrification (Fig. 10)."
Thank you.

Line 313-314: this sounds like that the absolute abundance of nitrogen cycling genes was the lowest. Wasn't it the relative abundance of nitrogen cycling genes among all the genes on GeoChip? Fig 11 & 12: please correct the fond for genes. For example, narb should be narB.

Response: Thank you for your review. Yes, you are right. We rewrote this part, and the gene names have been revised according to your comments. Please see page 24 to page 25, thank you.

Line 314-323: the heatmaps were interesting, but most of the text here was how similar or different the three depths were. I hope to see more detailed insights about the heatmaps.

Response: Thank you for your review. we rewrote the results, and discussion part. Thank you. Please see page 24 to page 26, thank you.

Line 385-393: why Sphingobium and Bacillus were the most abundant genera at 19 m was discussed here. It would be better if the authors could dig further into their data related to the two genera. For example, what was the most abundant species in Bacillus at 19 m? Does this species have enzymes that could remain active at high temperatures?

Response: Thank you for your review. we rewrote this part in the discussion part. Please see page 28 to page 29, thank you.

Line 414-419: I could not follow the logic here: Some studies suggested archaea

were rare, but other studies suggested that archaea and bacteria coexisted. Then I expected to hear about whether archaea in this study is rare or not. The data in this study could only indicate that Thaumarchaeota was the most abundant phylum among archaea but archaea could still be rare (i.e. much fewer than bacteria) in hot springs. Some methods were in the results: The subtitle of the first part of the results 'sampling' sounds like a method section, and the entire section was only one paragraph describing pH and temperature. It could be easily moved to the beginning of another section in the results as one sentence.

Response: Thank you for your review. Yes, we have moved the sampling section of the results to another section in the results. After reading many published articles related to hot springs, we found archaea can ubiquitously coexist in hot springs, and we rewrote this part in the discussion part. Please see page 29 to page 30, thank you.

Line 216-217: 'random resampling was conducted for further analyses' should be removed

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

Line 223: 'with a similarity of 97% for OTU classification', Line 225 'at a 97%

similarity level.', and line 237 'at a 97% similarity level'. The criterium for OTU classification does need to be mentioned but once is enough. Unclear, redundant or awkward expressions (some examples):

Response: Thank you for your review. We have made correction according to your comments, and deleted redundant text in the manuscript . Thank you.

Line 17-18: 'in response to the depths, : : :shifts over the depth profile'. Line 87: 'community functional potential altered by the depth'. I think you wanted to say that the composition of microbial communities depended on the depth.

Response: Thank you for your review. We have made correction according to your comments, and changed the expression as you suggested in the new version of manuscript . Thank you.

Line 112: 'three duplicate samples'. Duplicates mean two replicates. Three duplicates mean six samples. I think here you meant 'triplicates'.

Response: Thank you for your review. You are right, thank you. We have made correction according to your comments.

Line 200-201: 'According to the temperatures and pH, there were no significant

differences between the samples.' The authors may mean that the temperatures and pH of the samples were similar, but the sentence was saying that the samples were considered similar only based on two environmental variables.

Response: Thank you for your review. You are right. The changes have been made accordingly in the revised manuscript. Now, reads as follows:
"The temperatures and pH of the samples were similar, and the environmental parameters data were collected before sampling and are summarized in Table 1".
Thank you.

Line 216:' For the microbial diversity, the composition and structure of each sample could be compared;' What do you mean? This sentence needs to be re-written.

Response: Thank you for your review. Another reviewer also suggested we should remove these sentences, and provide the exact number of reads for normalization in the "Materials and methods" part. We have made correction according to your comments.

Line 225-227: 'The bacterial groups at 0 m with the highest relative abundances at the phylum level were members of Aquificae, Gamma-proteobacteria, and Deinococcus-Thermus.' This sentence needs to be re-written and the

Gamma-proteobacteria is not a phylum.

Response: Thank you for your review. Another reviewer pointed out the same mistake. We have revised " Gamma-proteobacteria" to " Proteobacteria (Gamma)". Thank you.

Line 231: awkward expression: ‘At the genus level, the OTUs were distributed: : :’

Response: Thank you for your review. We have revised "At the genus level, the OTUs were distributed, with the most abundant belonging to *Hydrogenobacter* and *Thermus* in hot spring samples at 0 m," to " At the genus level, the most abundant belonging to *Hydrogenobacter* and *Thermus* in hot spring samples at 0 m,". Thank you.

Line 283-285: the second half of the sentence said the same thing as the first half.

Response: Thank you for your review. The changes have been made accordingly in the revised manuscript. Now, reads as follows:
"such as principal coordinate analysis (PCoA), and the results of PCoA showed that the bacterial community structures were distinctly separate from each group (Fig. 5). However,". Thank you.

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.

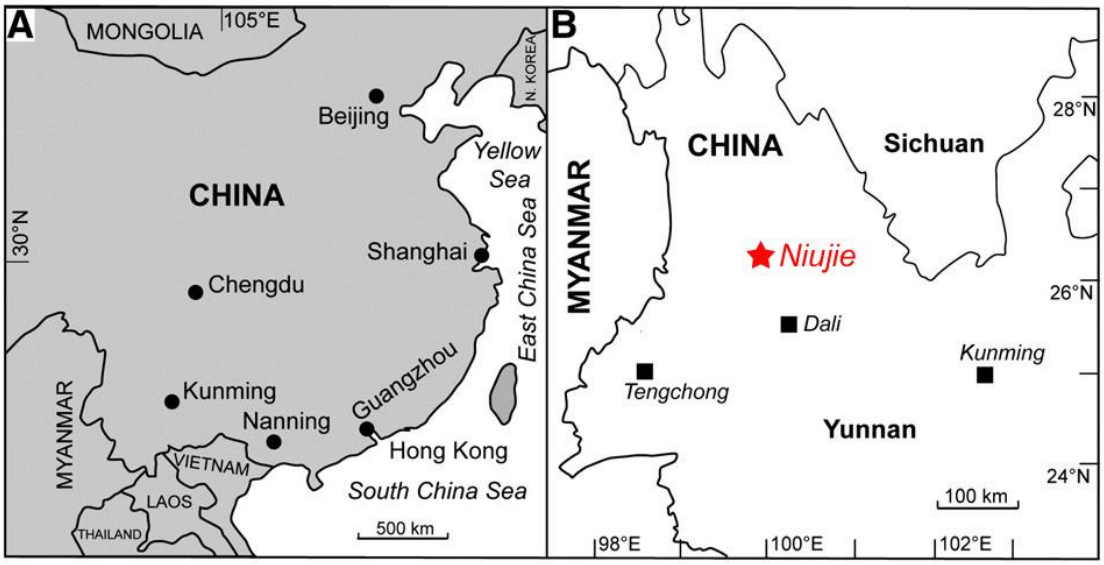

Fig 1. The geographical map showing the hot springs sampling locations in Niujie Town, Eryuan county, Dali city, Yunnan province, China.

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).

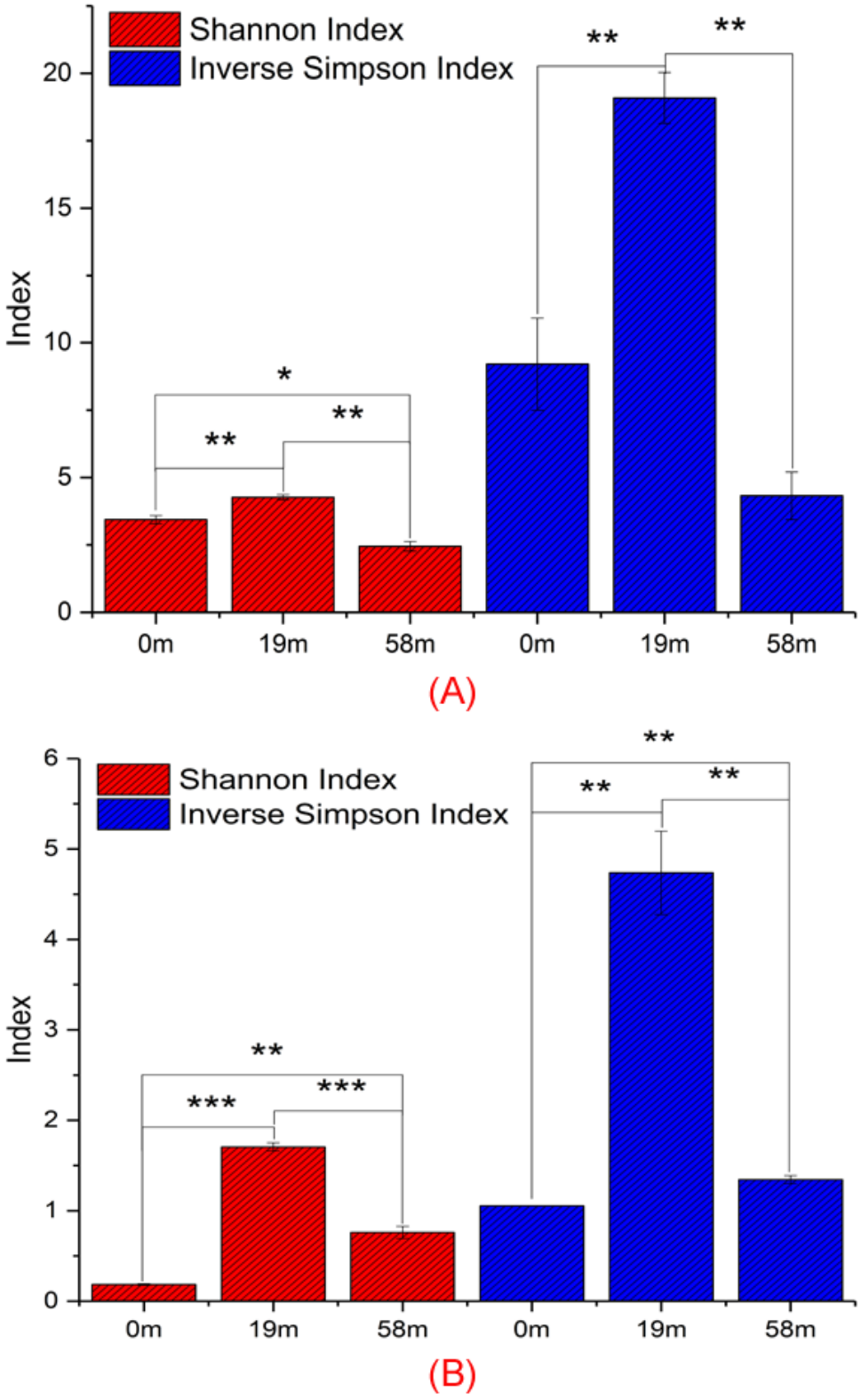

Fig 2. Comparison of the alpha diversity indexes, Shannon index and Inverse Simpson index (A: Bacterial communities; B: Archaeal communities). The value is the mean of the indices within each group. Error bars represent the standard error (SE). *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ based on Student's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

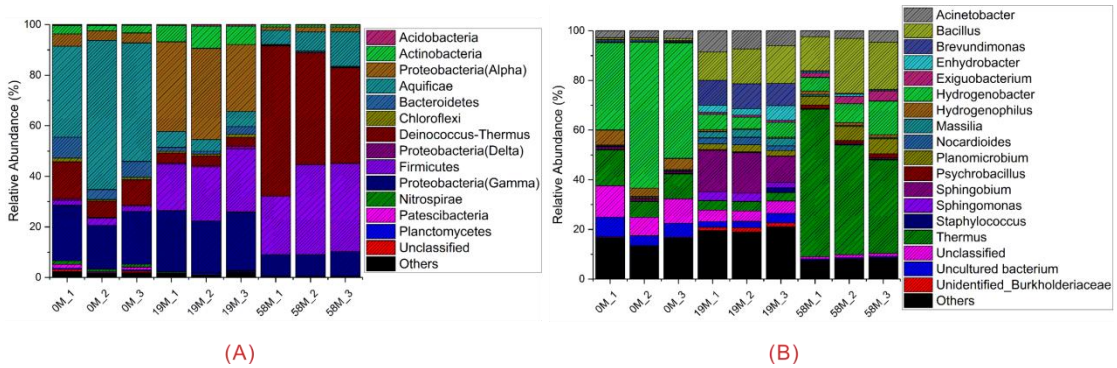

Fig 3. Stacked bar chart showing the relative abundance of the bacterial community composition at the phyla and classes of Proteobacteria level (A), and the genera level (B).

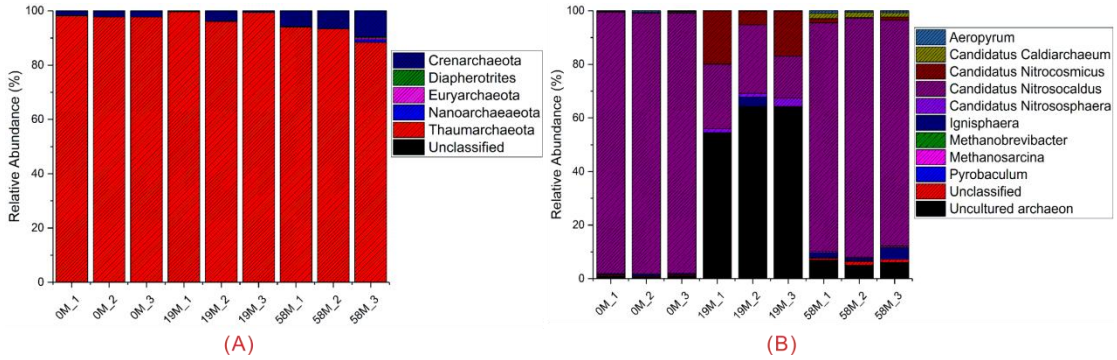

Fig 4. Stacked bar chart showing the relative abundance of the archaeal community composition at the phyla level (A), and the genera level (B).

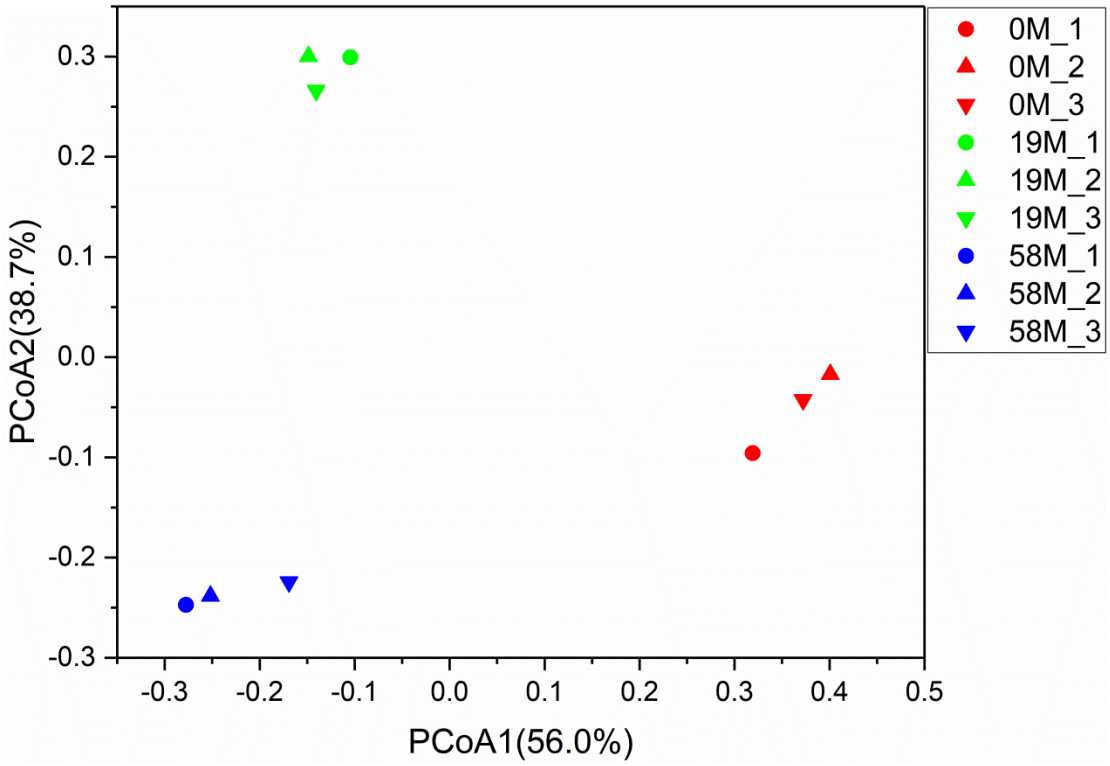

Fig 5. Principal coordinate analysis (PCoA) of bacterial communities from hot springs at different depths. The results are based on weighted the UniFrac distances of the detected OTUs.

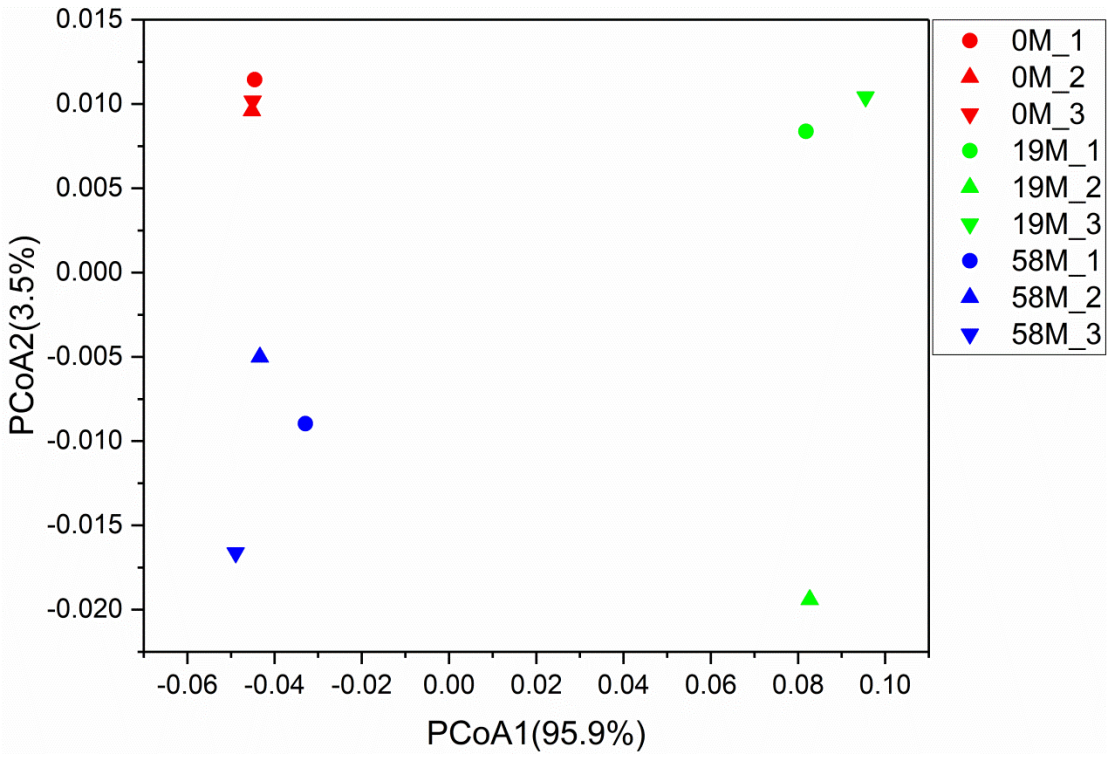

Fig 6. Principal coordinate analysis (PCoA) of archaeal communities from hot springs at different depths. The results based on the weighted UniFrac distances of the

detected OTUs.

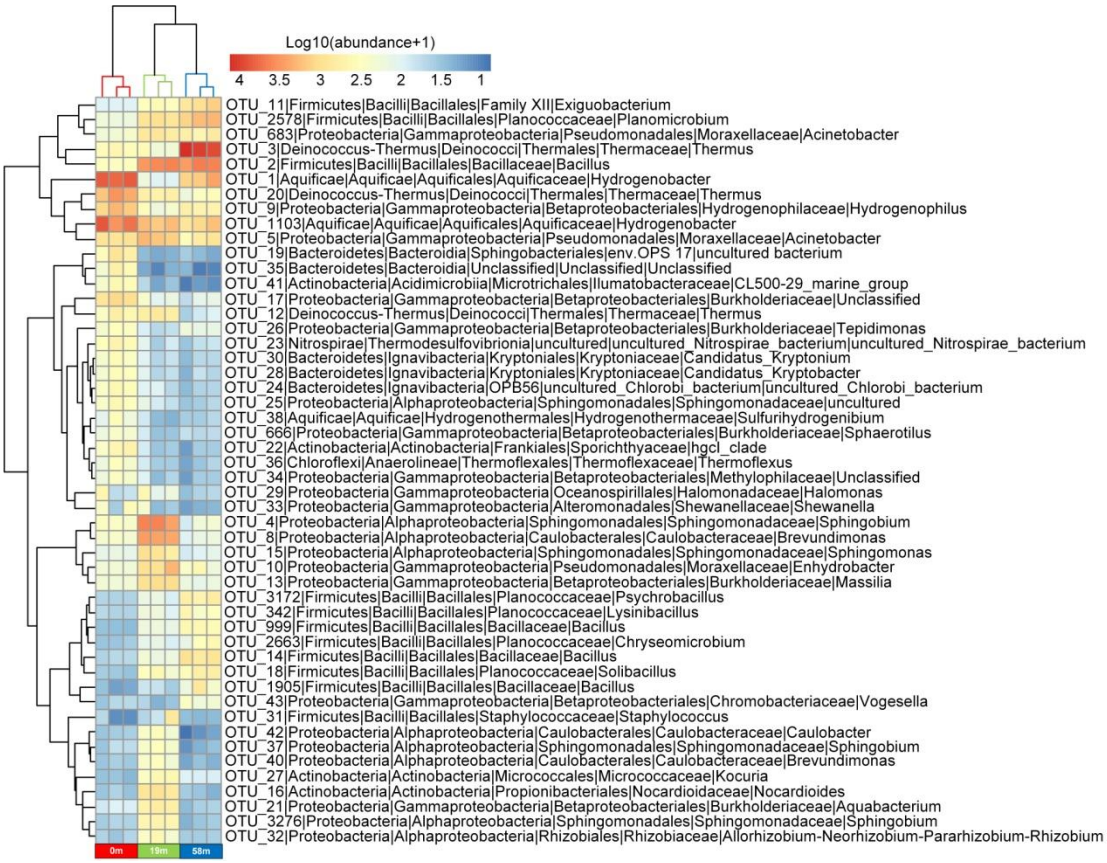

Fig 7. The 50 most abundant bacterial community OTUs from hot springs at different depths. Bacterial abundance was scaled with a log transformation in the heatmap.

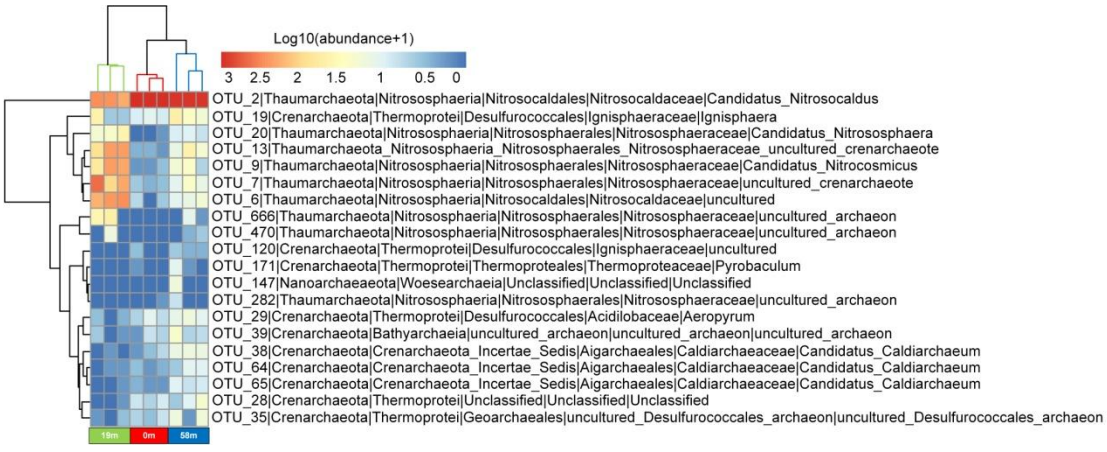

Fig 8. The 20 most abundant archaeal community OTUs from hot springs at different depths. Archaeal abundance was scaled with a log transformation in the heatmap.

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).

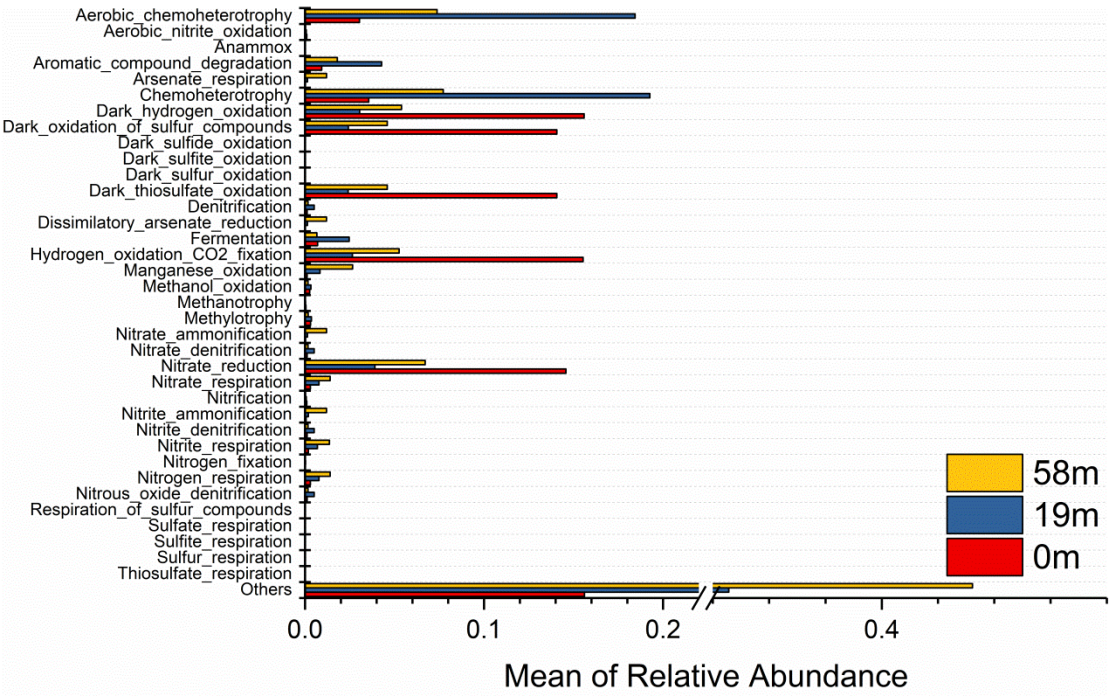

Fig 9. Stacked bar chart showing the mean relative abundance of the predicted metabolic potential of bacterium from hot springs at different depths, as predicted by FAPROTAX.

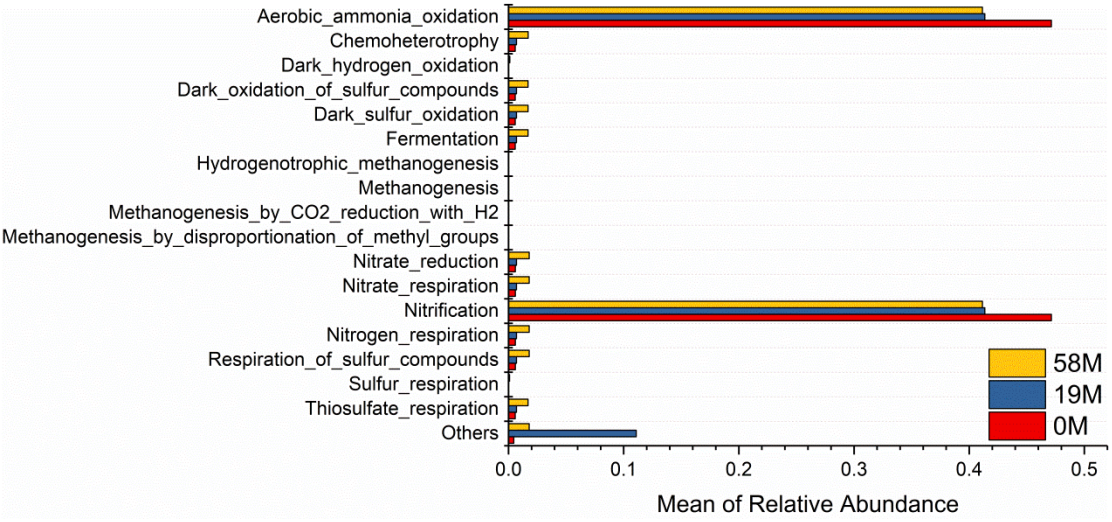

Fig 10. Stacked bar chart showing the mean of the relative abundance of the predicted metabolic potential of archaea from hot springs at different depths, as predicted by FAPROTAX.

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).

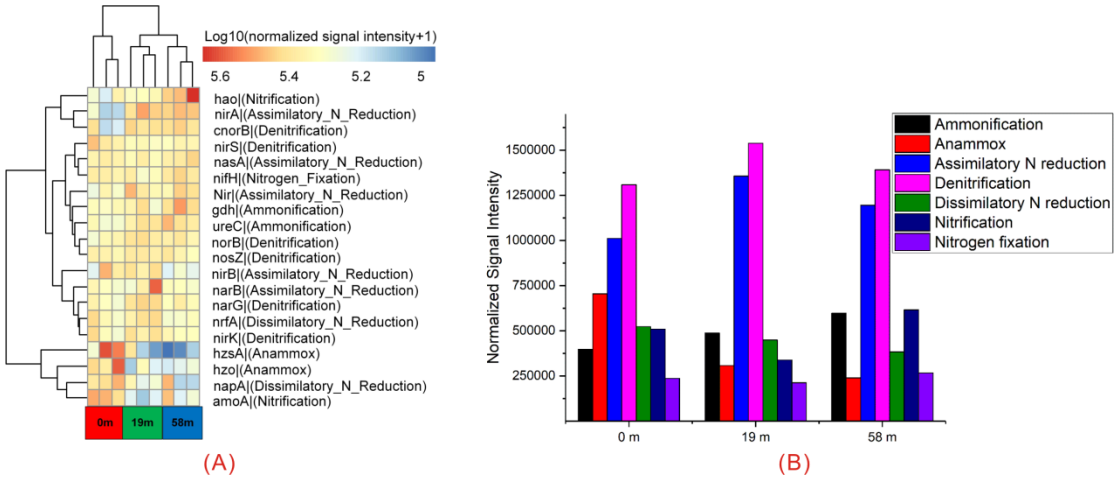

Fig. 11. The normalized signal intensity of the detected key genes involved in the nitrogen cycle (A). The signal intensity for each functional gene category is the average of the total signal intensity from all the replicates, and the heatmap of the functional genes involved in the nitrogen cycle at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

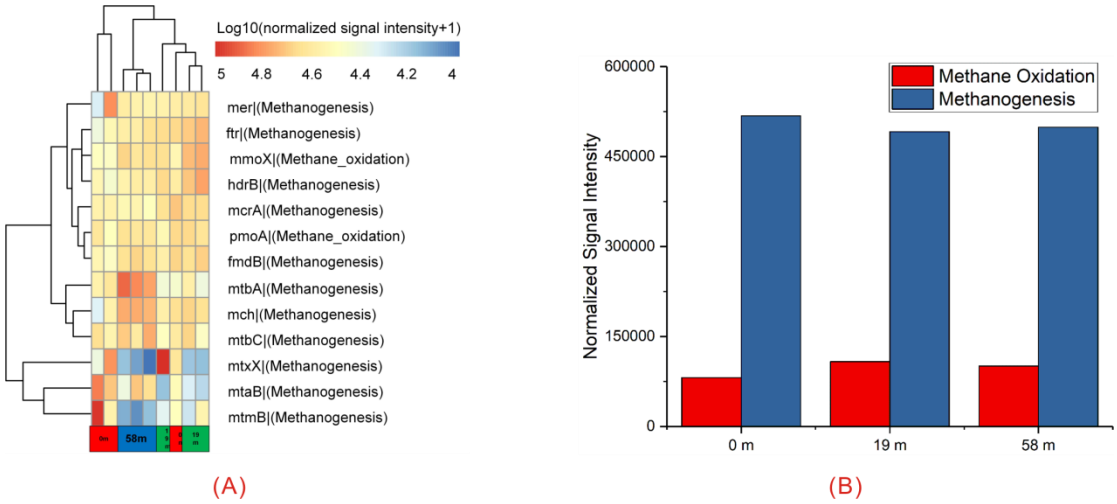

Fig. 12. The normalized signal intensity of the detected key genes involved in the methane cycle (A). The signal intensity for each functional gene category is the average of the total signal intensity from all the replicates, and the heatmap of the functional genes involved in the methane cycle at different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

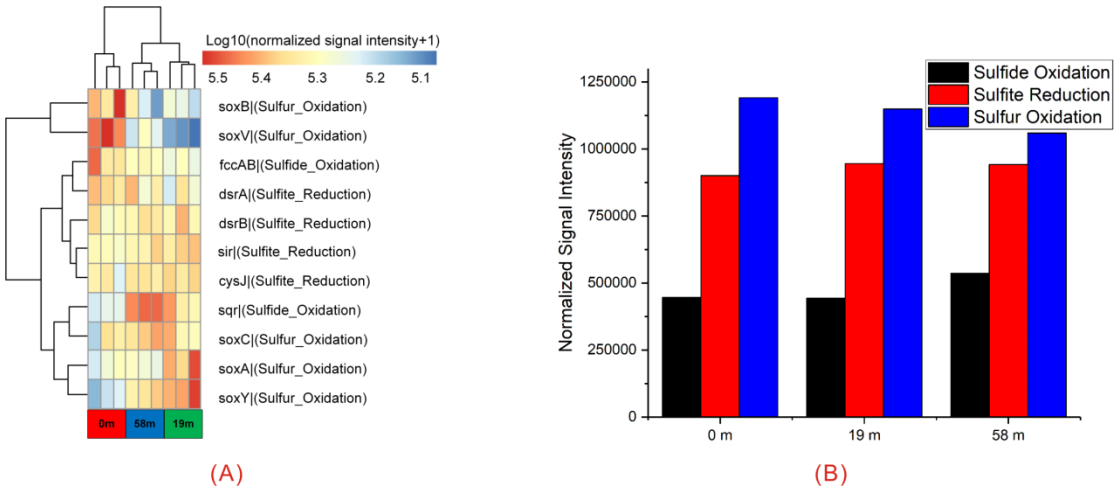

Fig. 13. The normalized signal intensity of the detected key genes involved in the sulfur cycle (A). The signal intensity for each functional gene category is the average of the total signal intensity from all the replicates, and the heatmap of functional genes involved in the sulfur cycle at different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