A Comprehensive Census of Microbial Diversity in Hot Springs of Tengchong, Yunnan Province China Using 16S rRNA Gene Pyrosequencing

Weiguou Hou1,2, Shang Wang1, Hailiang Dong1,2, Hongchen Jiang1, Brandon R. Briggs2, Joseph P. Peacock3, Qiuyuan Huang2, Liuqin Huang1, Geng Wu4, Xiaoyang Zhi5, Wenjun Li5, Jeremy A. Dodsworth3, Brian P. Hedlund3, Chuanlun Zhang6,7, Hilairy E. Hartnett8, Paul Dijkstra9, Bruce A. Hungate9

1 State Key Laboratory of Biogeology and Environmental Geology and Institute of Earth Sciences, China University of Geosciences, Beijing, China, 2 Department of Geology and Environmental Earth Science, Miami University, Oxford, Ohio, United States of America, 3 School of Life Sciences, University of Nevada, Las Vegas, Nevada, United States of America, 4 State Key Laboratory of Biogeology and Environmental Geology, China University of Geosciences, Wuhan, Hubei Province, China, 5 Key Laboratory of Microbial Diversity in Southwest China of Ministry of Education and Laboratory for Conservation and Utilization of Bio-resources of Yunnan Institute of Microbiology, Yunnan University, Kunming, Yunnan Province, China, 6 State Key Laboratory of Marine Geology, School of Ocean of Earth Sciences, Tongji University, Shanghai, China, 7 Department of Marine Sciences, the University of Georgia, Athens, Georgia, United States of America, 8 School of Earth and Space Exploration and Department of Chemistry and Biochemistry, Arizona State University, Tempe, Arizona, United States of America, 9 Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona, United States of America

Abstract

The Rehai and Ruidian geothermal fields, located in Tengchong County, Yunnan Province, China, host a variety of geochemically distinct hot springs. In this study, we report a comprehensive, cultivation-independent census of microbial communities in 37 samples collected from these geothermal fields, encompassing sites ranging in temperature from 55.1 to 93.6 °C, in pH from 2.5 to 9.4, and in mineralogy from silicates in Rehai to carbonates in Ruidian. Richness was low in all samples, with 21–123 species-level OTUs detected. The bacterial phylum *Aquificae* or archaeal phylum *Crenarchaeota* were dominant in Rehai samples, yet the dominant taxa within those phyla depended on temperature, pH, and geochemistry. Rehai springs with low pH (2.5–2.6), high temperature (85.1–89.1 °C), and high sulfur contents favored the crenarchaeal order *Sulfolobales*, whereas those with low pH (2.6–4.8) and cooler temperature (55.1–64.5 °C) favored the *Aquificae* genus *Hydrogenobacter*. Rehai springs with neutral-alkaline pH (7.2–9.4) and high temperature (>80 °C) with high concentrations of silica and salt ions (Na, K, and Cl) favored the *Aquificae* genus *Hydrogenobacter* and crenarchaeal orders *Desulfurococcales* and *Thermoproteales*. *Desulfurococcales* and *Thermoproteales* became predominant in springs with pH much higher than the optimum and even the maximum pH known for these orders. Ruidian water samples harbored a single *Aquificae* genus *Hydrogenobacter*, whereas microbial communities in Ruidian sediment samples were more diverse at the phylum level and distinctly different from those in Rehai and Ruidian water samples, with a higher abundance of uncultivated lineages, close relatives of the ammonia-oxidizing archaeon *Candidatus Nitrosocaldus yellowstonii*, and candidate division O1aA90 and OP1. These differences between Ruidian sediments and Rehai samples were likely caused by temperature, pH, and sediment mineralogy. The results of this study significantly expand the current understanding of the microbiology in Tengchong hot springs and provide a basis for comparison with other geothermal systems around the world.

Introduction

Tengchong County, located in Yunnan Province, Southwestern China, is known for its geothermal features [1–3]. Tectonically, Tengchong is located at the collision boundary between the India and Eurasia plates. Subduction of oceanic crust leads to extensive volcanism. The hot springs of Tengchong are a result of such volcanism with more than 50 volcanoes and 140 geothermal areas throughout the county [4]. In terms of the diversity and scale, the Tengchong springs are comparable to the geothermal systems in Yellowstone National Park (YNP) [5], Japan [6], and Kamchatka, Russia [7].

The Rehai (“Hot Sea”) and Ruidian geothermal fields in Tengchong are two regions of intense hydrothermal activity with numerous springs and pools. Physicochemical conditions span a wide range of temperature (30 to ~97 °C) and pH (<1.8 to >9.3) and these...
conditions provide numerous niches to support phylogenetically and functionally distinct microbial communities. Therefore, the Tengchong geothermal fields likely represent a biodiversity hotspot for thermophiles and provide an opportunity to compare microbial diversity and community structure with other geothermal areas around the world. Past studies in this area have focused on cultivation, physiology, and biotechnological applications of thermophilic microorganisms (reviewed in [8]). Overall, these studies revealed that acidic springs harbor thermoacidophilic bacteria and archaea, particularly *Sulfobolum*, which can grow chemolithotrophically by oxidizing sulfide, sulfur, thiosulfate and ferrous iron. Likewise, alkaline springs harbor many alkaliphilic bacteria, particularly *Firmicutes* and *Thermus*, which grow chemoorganotrophically on a number of organic substrates.

Whereas cultivation-dependent studies are valuable for isolating novel organisms and exploring their properties, the cultivation-independent methods offer a more comprehensive assessment of microbial diversity [9]. In Tengchong, however, only a few molecular-based studies have been reported. Specifically, three orders of Crenarchaeota (Sulfobulbales, Desulfurococcales, and Thermoproteales) were found in Tengchong springs and crenarchaeotal diversity was correlated with temperature [10]. Microbial diversity in two hot spring microbial mats from Tengchong was recently described [11]. Other cultivation-independent studies have focused on ammonia-oxidizing archaea (AOA) [12–14] and these studies suggest that AOA may be a minor but potentially active component of the microbial community in several Tengchong springs.

Despite these previous efforts, a comprehensive census of the microbial communities in Tengchong hot springs is still lacking. The primary objective of this study was to census a large number of high temperature springs in Tengchong and to investigate the relationships between thermophilic microbial communities and physicochemical conditions. To achieve this objective, a coordinated geochemical and molecular survey was conducted for 16 Tengchong hot springs and the relationships among microbial diversity, community structure, and geochemistry were explored. The results of this study expand the current understanding of the diversity and community structure with other geothermal areas worldwide.

**Experimental Procedures**

**Field Measurements and Spring Selection**

No specific permits were required for the described field studies because no animal or human subjects were involved in this research. The sampling locations are not privately owned or protected in any way. The field studies did not involve endangered or protected species.

Field measurements and sample collections were conducted in January 2011. Water temperature, pH, conductivity (portable meters; LaMotte, MD, USA) and the spring size were measured for more than 20 springs. Sixteen springs were chosen for further study, including 14 from Rehai and 2 from Ruidian (Figure 1 and Figure S1). Concentrations of ammonium (NH$_4^+$), total sulfide (ΣS$^{2-}$), ferrous iron (Fe$^{2+}$), nitrate (NO$_3^-$) and nitrite (NO$_2^-$) were measured with spectrophotometry using Hach test kits (Hach Chemical Co., IA, USA) after filtering spring water through 0.22 μm polyethersulfone (PES) membrane filters ( Pall Corp., NY).

**Sample Collection**

Sixteen water samples, 1 microbial streamer, 13 sediment samples, and 6 sinter samples from 16 springs were collected for geochemical and microbiological analyses (Table 1). Depending on the site, 50–360 mL of spring waters were filtered through a syringe filter (0.22 μm pore size, 25 mm diameter, Millipore, USA) to collect biomass. In one spring (Dagunguo), biomass was also collected via tangential flow filtration (TFF) that concentrated 200 L of water to 2 L using a 30 kDa MWCO prep/scale TFF-6 filter (Millipore, USA) followed by centrifugation at 12000 x g (4°C, 20 min.) after four days of storage in the lab at 4°C.

Water samples were collected for the measurements of hydrogen and oxygen isotopic composition and concentrations of cation, anion, trace elements, dissolved organic carbon (DOC), and total nitrogen (TN). Water samples for hydrogen and oxygen isotope measurements were filtered through 0.22 μm polyether sulfone (PES) filters (25 mm diameter; Pall Corp., USA) and collected into 25-mL glass bottles. Water samples were collected to determine concentrations of major cations, anions, and trace elements by filtering 25 mL of water through 0.22 μm PES filters into 60-mL high density polyethylene (HDPE), acid-washed Nalgene® bottles. High-purity nitric acid (2%, V/V) was added to stabilize the trace element samples. Water samples for dissolved organic carbon (DOC) and total nitrogen (TN) measurements were filtered through the same 0.22 μm PES filters. The DOC/ TN samples were stored in acid-washed and rinsed 125 mL fluorinated high-density polyethylene bottles that were pre- acidified with 0.2% (V/V) HCl.

Sediment samples were collected with sterile spatulas and spoons, and homogenized in a pre-sterilized aluminum pan. In springs with little dispersed sediment, fragments of sinter were collected using a sterilized hammer and chisel. Multiple aliquots of sediment or crushed sinter material were placed into 1.5 mL or 50 mL polypropylene tubes. All samples for microbial analysis were immediately frozen in liquid nitrogen or dry ice, stored on dry ice during transportation and at −80°C in the laboratory. Sediment samples for geochemical analyses were stored on ice until analysis.

**Geochemical Analyses**

Anion concentrations were determined via ion chromatography with a Dionex DX-500 chromatograph (AS14A column, with 10 μM Na$_2$CO$_3$/NaHCO$_3$ as an eluent, Dionex, USA). Cation concentrations were determined by direct current plasma emission spectrometry (DCP-OES, Beckman, USA). Trace element concentrations were determined by inductively coupled plasma – mass spectrometry (ICP-MS). DOC and TN concentrations were analyzed by high-temperature combustion [15,16] with NDIR and chemiluminescence detection for C and N, respectively (TOC-V, Shimadzu Corp., Japan). Oxygen and hydrogen isotopic compositions were measured using isotope ratio mass spectrometry after CO$_2$–H$_2$O equilibration for oxygen and reduction to H$_2$ gas for hydrogen, respectively [17].

Frozen sediments were thawed in the lab and dried at 105°C overnight. Total nitrogen (TN) and total organic carbon (TOC) was determined using a NC 2100 Elemental Analyzer interfaced with a Finnigan Delta Plus XL isotope ratio mass spectrometer. TOC was determined by coupled elemental analysis followed by isotope ratio mass spectrometry after fumigating sediment with HCl to remove carbonates. X-ray diffraction was performed to identify the mineralogy using a Scintag X1 powder diffractometer system (Bruker Corporation, USA) according to the procedure described elsewhere [18].

**DNA Extraction**

DNA was extracted from biomass-containing filters or from 0.5 g of sediment using FastDNA SPIN Kit for Soil (MP Biomedical, OH, USA) with a final elution in 70 μL de-ionized water. DNA concentration and quality were assessed based on
Figure 1. A map that shows the nine springs from both the Ruidian and Rehai locations. The open symbols represent the geochemistry and the closed symbols represent the microbial communities. The circles represent the water and the squares represent the sediment geochemistry. The colors of each symbol represent the grouping of either microbial communities (from Figure 2) or water and sediment geochemistry (from Figure S1 and S3 for water and sediment/sinter, respectively) based on hierarchical clustering. The microbial community, sediment geochemistry, and water geochemistry were analyzed separately and color comparisons across different symbols cannot be made; however, a similar color within one symbol (for example solid circle) depicts microbial community or geochemical similarities across the sampling sites. XRD mineralogical analysis of the sediment geochemistry at Dagunguo (Dgg) failed. Huitaijing (Htj) did not have any sediment. There were no microbial data for the Zhenzhuquan (Zzq) sediments because of PCR failure. Abbreviations are Jimeiquan (Jmq), Zhenzhuquan (Zzq), Guminquan (Gmq), Huitaijing (Htj), Jinze (Jz), Diretiyanqu (Drty), Gongxiaoshe (Gxs), Shuirebaozhaqu (Srzb), and Dagunguo (Dgg).

doi:10.1371/journal.pone.0053350.g001
DNA samples were divided into 20 μL aliquots and preserved at −80°C until further processing.

### Amplification of 16S rRNA Genes

The bacterial and archaeal V4–V8 variable regions of the 16S rRNA gene were amplified with the modified forward primer 515F (5'-GTGYCAGCMGCCGCGGTAA-3') in combination with the reverse primer 1391R (5'-GACGCGCCGTWGTRCA-3')

| Samplecode | Pinoyin name – English name | pH  | Temp °C | GPS location | Descriptions |
|------------|----------------------------|-----|---------|--------------|--------------|
| Dgg        | Dagunguo - Great Boiling Pot | 7.20 | 84.5    | N24.95344 E98.43780 | The largest and most prominent spring at Rehai. Roughly cylindrical with diameter 555–590 cm and depth ~150 cm, with two vigorous degassing sources. Sinter composed of amorphous silicate. Sample type includes sinter, water. |
| Drg-1      | Directlylan - Experimental Site (in fault, just under cliff) | 2.58 | 85.1    | N24.95396 E98.43829 | Small acid sulfate pool with small outlet. Irregularly shaped with length ~63 cm, width ~56 cm, and depth ~6.5 cm, with vigorously degassing sources. Turbid. Mixed with fine silicate sands at bottom. Sample type includes sediment and water. |
| Drg-2      | Directlylan - Experimental Site (below Drg-1) | 2.57 | 64.5    | N24.95363 E98.43891 | Small acid sulfate pool with slow outflow. Roughly round-shaped with diameter 40–50 cm, and depth 10.5 cm. Mixed with fine silicate sands at bottom. Sample type includes sediment and water. |
| Drg-3      | Directlylan - Experimental Site (On the right side from Drg-1) | 2.46 | 55.1    | N24.95389 E98.43834 | Small acid sulfate pool with slow outflow. Irregularly shaped with length ~51 cm, width ~26 cm, and depth ~5.5 cm, with some degassing. Grey-white water from suspended clays. Also silicate sands at bottom. Sample type includes sediment and water. |
| GmqS       | Gumingquan – Drum Beating Spring (source) | 9.35 | 93.0    | N24.57060 E98.43615 | Small source pool with a high flow rate (10.4 L/S); with length ~98 cm, width ~79 cm, and depth ~9.5 cm. Clear water. Some silicate sands at bottom. Sample type includes sediment and water. |
| GmqC       | Gumingquan – Drum Beating Spring (right channel) | 9.36 | 89.0    | N24.57060 E98.43615 | Shallow water flow channel (~20 cm width, ~5 cm depth) downstream of GmqS. Clear water and rocky bottom. Sample type includes sediment and water. |
| GmqP       | Gumingquan – Drum Beating Spring (streamer pool) | 9.30 | 82.5    | Close to GmqC | Pool downstream of GmqS, with a depth ~16 cm. Many pink streamers. Clear water. Mixed with fine silicate sands at bottom. Sample type includes sediment, water and streamer. |
| JmqL       | Jiemeiquan - Sisters Spring (Left) | 9.25 | 93.6    | N24.95112 E98.43600 | A shallow pool with spouting spring source and outflow. Length and width 100–110 cm, and depth ~9.5 cm. Lots of red or green mats on the bank of the spring. Clays and sands at the bottom. Sample type includes sediment and water. |
| JmqR       | Jiemeiquan - Sisters Spring (Right) | 9.39 | 83.2    | Close to JmqL | A shallow pool with a small source, downstream of JmqL. Length ~950 cm, width ~100 cm, and depth 4.5 cm. Lots of pink streamers. Sands and clays at bottom. Sample type includes sediment and water. |
| Zq         | Zhenhuquuan - Pearl Spring | 4.79 | 89.1    | N24.95115 E98.43596 | Constructed into a shallow, heart-shaped pool with the longest dimension of 436 cm, and a depth ~6–7 cm. Vigorously degassing. No visible outflow. Silicate sands at bottom. Sample type includes sediment and water. |
| HtlL       | Huitaijing - Pregnancy Spring (Left) | 8.11 | 90.0    | N24.95089 E98.43664 | Constructed into cylindrical well with diameter 65 cm, and depth 63 cm. Visible particles in water. Flow path is sculpted; slow outflow. Sample type includes water. |
| HtlR       | Huitaijing - Pregnancy Spring (Right) | 8.05 | 92.3    | Near HtlL | Similar to HtlL. Sample type includes water. |
| SrzbU      | Shuireb Zhao - Hydrothermal Explosion (upstream) | 8.04 | 79.8    | N24.95002 E98.43728 | Irregularly shaped pool with many bubbling sources. The pool was also fed by rain water. The size was dependent on weather in a given season. Length ~300 cm, width ~240 c. Several hydrothermal explosions occurred in the past. Grey mud at the bottom. Sample type includes sediment and water. |
| SrzbD      | Shuireb Zhao - Hydrothermal Explosion (downstream) | 8.28 | 78.2    | Near SrzbU | Located near the middle of the west pool. Sample type includes sediment and water. |
| GxsS       | Gongxiaoshe – Co-op Hotel (side) | 7.29 | 73.8    | N25.44012 E98.44081 | A large spring in the Ruidian Geothermal area. Constructed into an octagonal pool, with diameter ~297 cm and depth ~130 cm. Sinters growing at the inner wall. Soft cream-colored sediment containing carbonates at bottom. Vigorous degassing sources. No outflow, but the water was pumped out for showering by local residents. Carbonate-dominated sediments from the bottom of the pool were collected. Sample type includes sediment and water. |
| GxsB       | Gongxiaoshe – Co-op Hotel (bottom) | 7.29 | 73.8    | Near GxsS | Sinter samples chipped from the sides of the spring. Sample type includes sinter and water. |
| Jz         | Jinze - Golden Pond Motel | 6.71 | 81.6    | N23.44138 E98.46004 | Constructed into a cubic well with side lengths ~127 and ~134 cm and depth ~103 cm, covered with a shed. Outflow was stored in a cubic tank for showering. Black mud at bottom. Sample type includes sediment and water. |

Table 1. Sample codes, spring name, type and size; sample type; and selected geochemical parameters of spring water.

doi:10.1371/journal.pone.0053350.t001

spectral absorbance at 260 nm wavelength and absorbance ratios of 260/280 nm and 260/230 nm, respectively, using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technology, USA). DNA samples were divided into 20 μL aliquots and preserved at −80°C until further processing.
Pyrosequencing of the V4 region of the 16S rRNA gene was carried out from the 515F-end of the amplicons with a GS FLX sequencer (454 Life Sciences, USA) at the Chinese National Human Genome Center in Shanghai. Quality screening was completed in Mothur by removing low quality reads [23]. All the remaining reads were de-noised [24] and trimmed to a uniform length of 238 bp, followed by clustering analysis at the similarity levels of 80%, 90%, 95% and 97% using UCLUST [25] in the QIIME (quantitative insights into microbial ecology) pipeline [26]. The most abundant sequence from each cluster was chosen as a representative. All representative sequences were aligned with the PyNAST method [27], and chimeric operational taxonomic units (OTUs) were identified with Chimera Slayer [28] and excluded. Taxonomy was assigned to each representative sequence using BLAST with gg_97_otus_4feb2011.fasta (http://greengenes.lbl.gov; GreenGenes training set) as the reference database. All sequences in the Thaumarchaeota phylum were manually moved out of Crenarchaeota [29]. All 454 sequences have been submitted to the Short Read Archive database at NCBI (accession no. SRA056421).

Statistical Analysis of Geochemical and Microbial Data
To identify geochemical parameters that contributed most to microbial variability, seventy-nine water geochemical variables (Table S1 and S2 minus the mineralogical data) were first subjected to principal component analysis (PCA) with the ‘vegan’ package in the R programming environment [30]. Co-correlated geochemical variables were removed, and the remaining 17 variables were rank-ordered based on their correlations to the first three axes. For the sediment geochemistry, all 18 mineralogical variables (Table S1) were used. Seventeen water geochemical variables were subjected to hierarchical clustering of Log10 geochemical values with linkage by the unweighted pair-group method with arithmetic mean (UPGMA) of Euclidean distances to identify groups of sites with similar water geochemistry. Eighteen sediment geochemical variables were subjected to similar clustering analysis but without logarithmic transformation of the values because all values were between 0 and 1.

Chao1 (predicted number of OTUs), Shannon, and equitability indices, based on 16S rRNA gene sequence data, were calculated at the 97% cutoff level after removing four samples with fewer than 978 reads and normalizing all remaining libraries to the one with the smallest number of reads (978 reads). These diversity indices were tested for their correlation with the geochemical data using Mantel test.

Analysis of similarity (ANOSIM) was performed, based on the Bray-Curtis dissimilarity at the 97% similarity level, to test for any significant dissimilarity or similarity in community composition among certain groups of springs defined a priori. The groups were pre-defined based on water and sediment geochemistry. The test was performed in three steps: First, only one factor, e.g., geothermal field (Rehai vs. Ruidian) and sample type (water vs. sediment) was considered individually. Second, geothermal field and sample type were considered together, forming four groups: Rehai water, Rehai sediment, Ruidian water, and Ruidian sediment. Third, because of the large pH range (2.5–9.4) in the Rehai hot springs, the pH factor (low: 2.5–4.8 and high: 6.7–9.4) was added, forming six groups, including Rehai low-pH water, Rehai low-pH sediment, Rehai high-pH water, Rehai high-pH sediment, Ruidian high-pH water, and Ruidian high-pH sediment. There was no low-pH water or sediment in Ruidian. Pairwise comparisons were carried out in ANOSIM and pairwise p- and r values were obtained as a way to assess any significant difference in community composition between any two of the above groups.

Jackknifed UPGMA clustering was performed to compare microbial community similarity among the samples based on the Bray-Curtis dissimilarity at the 97% similarity OTU level. For certain representative groups that were identified by ANOSIM and the UPGMA clustering, SIMPER (similarity percentage) analysis was performed to rank the top ten OTUs that contributed to the observed similarity (or dissimilarity) among two groups. The average abundances of these OTUs in each group were then calculated.

The BIO-ENV procedure [31] was used to identify the best subset of environmental variables to reveal correlations between community data (Bray-Curtis dissimilarity) and scaled environmental data (Euclidean distance) using identified 17 water and 18 sediment geochemical variables. Mantel tests were further performed to confirm correlation between the community structure and selected geochemical variables.

To identify differences between the two primer sets, ANOSIM, non-parametric multivariate ANOVA (Adonis), and multi-response permutation procedure (MRPP) were performed based on the Bray-Curtis dissimilarity distance matrices. Other than geochemical clustering analyses, all statistical analyses were done in QIIME pipeline and/or using the package “vegan” [30] in the R programming environment.
Meteoric water was the main source for all springs, as has been plotted along the local meteoric water line, suggesting that the hydrogen and oxygen isotope compositions (Figure S3) were varying ratios of surface area/volume. The evaporation, consistent with their different temperatures and for Rehai springs suggest that there were different extents of acidic sites in Rehai (W2), Zhenzhuquan and Diretiyanqu-1, -2, and -3, exhibited elevated levels of SO$_4^{2-}$, Mn$^{2+}$, Fe$^{2+}$, Mg$^{2+}$, DOC, and TN (Tables S1 & S2). These data suggest that these acidic springs likely represent vapor condensate-dominated systems that are acidified by oxidation of sulfide to sulfuric acid. The hydrogen and oxygen isotope compositions (Figure S3) were plotted along the local meteoric water line, suggesting that meteoric water was the main source for all springs, as has been previously reported [34]. A large spread in $\delta^1$H and $\delta^{18}$O values for Rehai springs suggest that there were different extents of evaporation, consistent with their different temperatures and varying ratios of surface area/volume. The $\delta^1$H and $\delta^{18}$O values for Ruidian springs were more negative than those for Rehai springs, suggesting Ruidian springs underwent less evaporation, likely due to their moderate temperatures and low ratios of surface area/volume.

Hierarchical clustering divided the spring water chemistry into three well-defined groups based on geographical location (distance) and pH (Figure S2). The neutral-alkaline springs in Rehai (W3) have been previously categorized as Na-Cl-HCO$_3$ or Na-HCO$_3$-Cl or springs that directly discharge from a high temperature subterranean reservoir within granite or granitic clastic sedimentary host rocks [32,33]. In contrast, those in Ruidian (W1) have been categorized as Na-HCO$_3$ springs [32,33] and were characterized by high Mg$^{2+}$ and Ca$^{2+}$ concentrations (Table S1), likely caused by dissolution of carbonates. The four acidic sites in Rehai (W2), Zhenzhuquan and Diretiyanqu-1, -2, and -3, exhibited elevated levels of SO$_4^{2-}$, Mn$^{2+}$, Fe$^{2+}$, Mg$^{2+}$, DOC, and TN (Tables S1 & S2). These data suggest that these acidic springs likely represent vapor condensate-dominated systems that are acidified by oxidation of sulfide to sulfuric acid. The hydrogen and oxygen isotope compositions (Figure S3) were plotted along the local meteoric water line, suggesting that meteoric water was the main source for all springs, as has been previously reported [34]. A large spread in $\delta^1$H and $\delta^{18}$O values for Rehai springs suggest that there were different extents of evaporation, consistent with their different temperatures and varying ratios of surface area/volume. The $\delta^1$H and $\delta^{18}$O values for Ruidian springs were more negative than those for Rehai springs, suggesting Ruidian springs underwent less evaporation, likely due to their moderate temperatures and low ratios of surface area/volume.

Hierarchical clustering of the sediment geochemistry revealed two broad clusters (referred to as S1 and S2) that corresponded to the two geothermal fields (Figure S4). Springs in Ruidian were distinct from those in Rehai in terms of both TOC/TN (51.4% cumulative contribution) and mineralogy (48.6% cumulative contribution). TOC content in the Ruidian spring sediments was generally higher than in the Rehai sediments (Table S1). Silicate minerals dominated the Rehai spring sediments, whereas carbonates (aragonite and calcite) were main minerals in the Ruidian sediments. The solid-phase and aqueous geochemistry of the springs was generally consistent with a granite-hosted system in Rehai and a carbonate-hosted system in Ruidian.

Microbial Diversity

The two primer sets did not result in any significant differences in microbial diversity (Figure S5) and structure (Figures S6 and S7, Table S3). However, more archaean sequences were retrieved by the modified primer set than by the original primer set (Table S4). For this reason, the modified primer pair was used for all the samples. A total of 345,802 reads were obtained for 37 samples with the modified primer set after removal of low-quality and chimeric sequences. A variety of taxa were observed at the 97% OTU level, with 21–123 observed and 50–223 predicted OTUs (based on Chao1) and coverage values ranging from 38.2% to 69.9% (Table 2). In Rehai, richness, Shannon diversity, and equitability were not significantly different between sediments and water communities except for Dagunguo, where these indices were higher for sediment samples (Table 2). Similar to Dagunguo, microbial community diversity and richness were much higher in Ruidian sediments than in waters (Table 2). Richness was slightly higher in neutral-alkaline springs than in acidic springs, but Shannon diversity and equitability showed no significant correlation with temperature and pH (Figure S8), or any other selected geochemical variables, which was also confirmed by Mantel tests with all p > 0.1. Microbial richness, equitability, and Shannon diversity in Ruidian sinters and sediments were markedly higher than in Rehai sediments (Table 2), and this difference was related to TOC content in sediments/sinters (Chao1 and TOC: r = 0.42, p < 0.05; Shannon diversity and TOC: r = 0.30, p < 0.1).

Overall Microbial Community Structure in Relation to Geochemistry

The results of pairwise comparisons using ANOSIM suggested that there was no significant difference in microbial community structure either between Rehai and Ruidian springs or between water and sediment, with the R-statistic value of 0.16 and 0.02, respectively, and p > 0.05 for both comparisons. When the geothermal field and the sample type were considered together, the test results showed that microbial community structure in Ruidian sediment was distinctly different from those of the other three groups: Ruidian water, Rehai water, and Rehai sediment with the R-statistic value of 0.71, 0.28, and 0.41, respectively and p < 0.05 (for all 3 comparisons). Addition of the pH factor in the analysis further divided the Rehai samples to two distinct groups: low (2.5–4.8) and high (6.7–9.4) pH springs (all R-statistic values near 1 and p < 0.01); and Ruidian water samples were similar to Rehai high pH samples in microbial composition. In summary, three distinct groups were identified based on ANOSIM: 1) Ruidian sediment; 2) Rehai low pH, and 3) Rehai high pH samples (both sediment and water) and Ruidian water samples.

A similar grouping pattern was also identified based on Bray-Curtis dissimilarity at the 97% similarity OTU level using a UPGMA cluster tree (Figure 2). Four groups were identified that could be differentiated based on the various combinations of the geothermal field, pH, and temperature (T). The four groups were: Group 1: high T and neutral to alkaline pH (namely Rehai high pH water+sinter and Ruidian water samples); Group 2: moderate T and neutral pH (Ruidian sediments); Group 3: low T and low pH (Rehai); and Group 4: high T and low pH (Rehai). These groups were similar to those identified by the ANOSIM analysis. Mantel tests revealed that community structure was significantly correlated with pH and temperature (r = 0.47 and 0.44, respectively; p = 0.001). The BIO-ENV results confirmed that community structure was significantly correlated with a combination of pH and temperature (r = 0.73).

To reveal associations between microbial composition and geochemistry, geochemical groups were plotted along with the four microbial groups for a direct visual comparison (Figure 1). In general, microbial community structure broadly corresponded to water and sediment geochemistry but with some exceptions. For example, Jiemeiquan, Gumingquan, Huitaijing, Shiirebaozha, and Dagunguo exhibited similar aquatic geochemistry and similar water-borne microbial communities. Likewise, Shiirebaozha, Gumingquan, and Jiemeiquan exhibited similar sediment geochemistry and similar benthic microbial composition, suggesting that sediment geochemistry is important in shaping microbial community structure in sediment. However, in some cases, geochemistry and microbial community structure did not correspond to each other. For example, despite having similar sediment geochemistry to Jiemeiquan, Gumingquan, and Shuirebaozha, the...
acidic hot springs in Rehai (Drty-1, -2, and -3) had a different benthic community.

Microbial Community Composition in Relation to Geochemistry

Average archaeal abundance was much higher in Rehai springs (31±35%) than in Ruidian springs (9±10%). In some high temperature acidic (i.e., Diretiyanqu-1 and Zhenzhuquan) and alkaline (Gumingquan and Huitaijing) springs in Rehai, more than 90% of total sequences in the pyrotag dataset was composed of archaea (Figure 2B, Figure 3A). To reveal differences in specific taxonomic groups between Rehai and Ruidian as well as among the springs of a given region, SIMPER analysis was used to identify the top ten taxonomic groups that contributed to the dissimilarities or similarities among the selected groups of hot springs (Table 3). At the regional scale, there were differences at the family/genus level between Rehai and Ruidian (Table 3, 1st comparison), Hydrogenobacter was present in both Rehai and Ruidian, but its relative abundance was higher in Rehai. Sulfolobus, Desulfurococcales, Hydrogenobaculum, Pyrobaculum, and Thermodesulfobacteriaceae were present in Rehai, but not in Ruidian. Likewise, unclassified bacteria, candidate phylum O1aA90 and OP1, Thermaceae, and Rhodothermaceae were significantly more abundant.

Table 2. Alpha diversity indices at the 97% OTU level of 16S rRNA gene fragments by re-sampling 968 reads in each sample for 1000 replicates with the modified primer set.

| Sample ID | Observed OTUs | Coverage\(^a\) of the observed OTUs (%) | Chao1 | Shannon’s diversity | Equitability |
|-----------|---------------|----------------------------------------|-------|----------------------|-------------|
| Dgg.Water | 21            | 41.90                                  | 50    | 0.77                 | 0.18        |
| Dgg.TFF   | 24            | 40.70                                  | 59    | 0.88                 | 0.19        |
| DggSinter1| 90            | 58.90                                  | 122   | 2.75                 | 0.45        |
| DggSinter2| 93            | 59.44                                  | 156   | 3.48                 | 0.53        |
| Drty-3.Sediment | 46      | 69.89                                  | 66    | 3.04                 | 0.55        |
| Drty-2.Water | 39      | 59.14                                  | 65    | 2.13                 | 0.40        |
| Drty-2.Sediment | 46      | 64.20                                  | 71    | 2.61                 | 0.47        |
| Drty-1.Water | 49      | 56.74                                  | 87    | 1.88                 | 0.33        |
| Drty-1.Sediment | 43      | 53.40                                  | 81    | 1.19                 | 0.22        |
| GmqP.Water | 58            | 40.79                                  | 143   | 1.92                 | 0.33        |
| GmqP.Sediment | 35      | 41.97                                  | 83    | 1.24                 | 0.24        |
| GmqP.Sinter | 30      | 41.94                                  | 73    | 1.18                 | 0.24        |
| GmqC.Water | 102           | 45.80                                  | 224   | 2.98                 | 0.45        |
| GmqC.Sediment | 52      | 48.71                                  | 108   | 2.54                 | 0.45        |
| GmqS.Water | 82            | 56.70                                  | 145   | 2.85                 | 0.45        |
| JmqL.Water | 59            | 41.03                                  | 143   | 2.27                 | 0.39        |
| JmqL.Sediment | 74      | 54.03                                  | 138   | 3.33                 | 0.53        |
| JmqR.Water | 62            | 38.24                                  | 161   | 2.46                 | 0.41        |
| JmqR.Sediment | 42      | 47.26                                  | 88    | 1.69                 | 0.31        |
| Zzq.Water | 46            | 49.98                                  | 93    | 1.88                 | 0.34        |
| HtjR.Water | 63            | 49.28                                  | 128   | 2.79                 | 0.47        |
| SrbzU.Water | 87            | 55.38                                  | 157   | 3.00                 | 0.46        |
| SrbzU.Sediment | 62      | 47.08                                  | 132   | 2.42                 | 0.41        |
| SrbzD.Sediment | 86      | 52.61                                  | 164   | 3.50                 | 0.54        |
| GxsS.Water | 41            | 61.76                                  | 67    | 1.84                 | 0.34        |
| GxsS.Sinter2 | 121     | 46.17                                  | 223   | 4.90                 | 0.71        |
| GxsS.Sinter3 | 121     | 61.30                                  | 198   | 4.80                 | 0.69        |
| GxsB.Water | 37            | 64.58                                  | 57    | 1.73                 | 0.33        |
| GxsB.Sediment1 | 123    | 59.27                                  | 207   | 5.09                 | 0.73        |
| GxsB.Sediment2 | 112     | 57.88                                  | 193   | 4.72                 | 0.69        |
| GxsB.Sediment3 | 110     | 56.36                                  | 195   | 4.78                 | 0.71        |
| Jz.Water   | 40            | 46.15                                  | 86    | 1.32                 | 0.25        |

1 Sinter samples from different locations within the source pool of Dagunguo.
2 Sinter samples from different sites on the edge of the source pool of Gongxiaoshu.
3 Sediment and sinter samples from different sites on the bottom of the source pool of Gongxiaoshu.
4 Coverage is the ratio of the observed OTUs to Chao1.
Sample datasets that contained less than 968 reads were excluded from this table.
doi:10.1371/journal.pone.0053350.t002
in Ruidian. Several of the thermoacidophilic taxa were present only in Rehai because of the presence of acidic springs; therefore, acidic springs were removed from the analysis and the comparison between Rehai and Ruidian was repeated (Table 3, 2nd comparison). This revealed that *Hydrogenobacter*, Desulfurococcaceae, *Pyrobaculum*, and Thermodesulfobacteriaceae were more abundant in Rehai, whereas *Thermus*, yet-uncultivated groups O1aA90, Thermaceae, Rhodothermaceae, and a yet-unnamed novel bacterial group were more abundant in Ruidian.

Within Rehai, the bacterial phylum Aquificae and the archaeal phylum Crenarchaeota (primarily class Thermoprotei) were the predominant groups, comprising 72.2±26% and 92±23% of total bacteria and archaea, respectively (as opposed to 35±41% and 55±46%, respectively within Ruidian). The percentage of Aquificae in water samples (71.1% ± 31.6%) was higher than in sediments or sinters (33.7% ± 28.9%). In addition, the microbial composition within Aquificae and Thermoprotei varied according to environmental conditions. Within the Aquificae, Hydrogenobacter was abundant in springs with circumneutral to alkaline pH (6.7–9.4, Table 3, 3rd comparison and Figure 3B), whereas Hydrogenobaculum was the dominant Aquificae genus in low temperature, acidic, and sulfur-rich springs (T: 55.1–64.5 °C, pH: 2.5–2.6; Table 3, 4th comparison and Figure 3B); however, it was not present in high temperature acidic sites (T: 85.1–89.1 °C and pH: 2.6–4.8).

Similarly, the three orders of Thermoprotei, Sulfolobales, Desulfurococcales and Thermoproteales, showed distinctive distribution patterns. Sulfolobales, predominantly the genus Sulfolobus, was dominant in high temperature, acidic, and sulfur-rich springs (T: 85.1–89.1 °C and pH: 2.5–4.8), comprising >82% of all sequences (Table 3, 3rd and 4th comparisons; Figures 2B & 3C). In contrast, Desulfurococcales and Thermoproteales (mainly *Pyrobaculum*) were the dominant archaea in springs with circumneutral to alkaline pH (pH: 6.7–9.4, Figure 3C) and high concentrations of silica, Na, K and Cl (Table 1). Within acidic sites, temperature exerted a strong control on community composition. With decreased temperature, a Sulfolobus-dominated community gave way to a more diverse community with a relatively high abundance of the bacterial taxa *Hydrogenobaculum*, *Acidisphaera*, and *Desulfurella*, and the archaeal taxon *Metallosphaera* (Table 3, 4th comparison). The relative abundance of Sulfolobus increased linearly with increased temperature in the acidic samples (Pearson correlation = 0.97, p = 0.002).

Some bacterial and archaeal groups were abundant only in specific samples. For example, Thermaceae (*Deinococcus-Thermus*...
phylum) and Rhodothermaceae (Bacteriodetes phylum) were fairly abundant only in samples from Gongxiaoshe in Ruidian. Thermodesulfobacteriaceae were prominent in several Rehai springs with high temperature (78.2–93.6°C) and neutral-alkaline pH (7.2–9.4) (Figure 2 and Table 3). Putative ammonia-oxidizing archaea in the Thaumarchaeota (mainly related to “Candidatus Nitrosocaldus”) were the dominant archaea in Gongxiaoshe spring (74% of total archaea, which was ~10% of total sequences in the dataset), whereas the order Thermoplasmatales in the Euryarchaeota were the predominant archaea in the lowest temperature acidic site, Diretiyanqu 3.

Discussion

Correlation between Microbial Diversity and Geochemistry

Markedly higher microbial richness, equitability, and diversity in Ruidian sinters and sediments than in sediments and waters from Rehai (Table 2) may be due to the circumneutral pH, moderate temperatures, and high TOC contents of the Ruidian springs relative to those from Rehai (Table 1). Different mineralogy between Ruidian and Rehai sediments/sinters (carbonates vs. silicates) may have contributed to the difference in microbial diversity between these two hydrothermal fields as well.
Table 3. Top ten OTUs (at the 97% level) responsible for dissimilarity between certain selected groups of hot spring samples.

### Rehai (A) vs. Ruidian (B)

| Taxon         | Family/Genus            | Contrib. (%) | Avg3.abund.(A) (%) | Avg.abund.(B) (%) |
|---------------|-------------------------|--------------|--------------------|-------------------|
| Aquificae     | Hydrogenobacter         | 30.85        | 43.41              | 34.11             |
| Thermoprotei  | Sulfobacter             | 6.61         | 22.96              | 0.00              |
| Thermoprotei  | Desulfiticrobacteriaceae| 4.08         | 4.68               | 0.00              |
| Aquificae     | Hydrogenobacter         | 3.58         | 2.83               | 0.00              |
| Thermoprotei  | Pyrobaculum             | 3.06         | 3.65               | 0.00              |
| Deinococcus-Thermus | Thermus     | 2.49         | 0.46               | 6.66              |
| Thermodesulfobacteria | Thermodesulfobacteriaceae | 2.27 | 3.05               | 0.00              |
| O1aA90        | Unclassified O1aA90     | 2.18         | 0.02               | 5.78              |
| Deinococcus-Thermus | Thermaceae     | 1.99         | 0.03               | 5.92              |
| Bacteriodetes | Rhodothermaceae         | 1.91         | 0.01               | 7.15              |

### Neutral-Alkaline Rehai springs (A) vs. Ruidian springs (B)

| Taxon         | Family/Genus            | Contrib. (%) | Avg.abund.(A) (%) | Avg.abund.(B) (%) |
|---------------|-------------------------|--------------|-------------------|-------------------|
| Aquificae     | Hydrogenobacter         | 36.77        | 65.22             | 34.11             |
| Thermoprotei  | Desulfiticrobacteriaceae| 5.51         | 20.22             | 0.00              |
| Thermoprotei  | Pyrobaculum             | 4.14         | 19.76             | 0.01              |
| Thermodesulfobacteria | Thermodesulfobacteriaceae | 3.05 | 20.56             | 0.00              |
| O1aA90        | Unclassified O1aA90     | 2.15         | 0.02               | 9.77              |
| Deinococcus-Thermus | Thermaceae     | 1.98         | 0.04               | 6.76              |
| Bacteriodetes | Rhodothermaceae         | 1.91         | 0.01               | 8.76              |
| Unidentified Bacteria | Unidentified Bacteria | 1.71 | 0.02               | 6.12              |

### High T & neutral-alkaline pH (Rehai only, A) vs. High T & low pH (B)

| Taxon         | Family/Genus            | Contrib. (%) | Avg.abund.(A) (%) | Avg.abund.(B) (%) |
|---------------|-------------------------|--------------|-------------------|-------------------|
| Thermoprotei  | Sulfobacter             | 49.18        | 0.04              | 82.21             |
| Aquificae     | Hydrogenobacter         | 21.42        | 64.76             | 0.11              |
| Thermoprotei  | Sulfobacter             | 3.52         | 0.00               | 3.08              |
| Thermoprotei  | Desulfiticrobacteriaceae| 3.23         | 7.17               | 0.00              |
| Thermoprotei  | Pyrobaculum             | 2.48         | 5.59               | 0.00              |
| Thermodesulfobacteria | Thermodesulfobacteriaceae | 1.88 | 4.66               | 0.00              |
| Thermoprotei  | Sulfobacter             | 1.71         | 0.00               | 2.45              |
| Thermoprotei  | Sulfobacter             | 1.43         | 0.00               | 1.79              |
| Thermoprotei  | Sulfobacter             | 1.00         | 0.00               | 1.14              |
| Thermoprotei  | Pyrodictiaceae          | 0.75         | 0.04               | 0.65              |

### Low T & low pH (A) vs. High T & low pH (B)

| Taxon         | Family/Genus            | Contrib. (%) | Avg.abund.(A) (%) | Avg.abund.(B) (%) |
|---------------|-------------------------|--------------|-------------------|-------------------|
| Thermoprotei  | Sulfobacter             | 57.70        | 2.72              | 82.21             |
| Aquificae     | Hydrogenobacter         | 11.61        | 48.74             | 0.00              |
| Alphaproteobacteria | Acidisphaera  | 5.19         | 21.81             | 0.00              |
| Thermoprotei  | Sulfobacter             | 4.26         | 0.00               | 3.08              |
| Thermoprotei  | Sulfobacter             | 2.05         | 0.01               | 2.45              |
| Thermoprotei  | Sulfobacter             | 1.72         | 0.63               | 1.79              |
| Thermoprotei  | Sulfobacter             | 1.46         | 6.39               | 0.02              |
| Thermoprotei  | Sulfobacter             | 1.23         | 0.48               | 1.14              |
However, because of the similarly low diversity in both Ruidian and Rehai waters, it is likely that sediment properties (i.e., high TOC, carbonate mineralogy) may be more important in accounting for the high diversity in Ruidian sediments and sinters. A previous study also revealed a much higher microbial diversity in sediment than in water of Great Boiling Spring in Nevada, which may be related to long water residence time [20]. Ruidian springs were expected to have long resident time and may have accounted for the large difference in diversity between the sediment and water.

Comparison with Previous Studies in Tengchong Hot Springs

Previous studies of thermophiles from Tengchong springs have mostly focused on microbial cultivation studies [8]. Our molecular examination of the springs detected many of the organisms previously isolated, but these taxa tended to be sub-dominant. For example, the bacterial genera *Methanothermus* and *Thermus* and the archaean genera *Acidium* and *Metallosphaera* have been previously isolated from Rehai springs and were detected, but not abundant in our study. Notably, our study revealed the dominance of *Sulfolobus* in high temperature, acidic, and high-sulfur springs in Rehai and a diversity of *Sulfolobales* isolates from Tengchong have been described previously [35]. However, many microorganisms, such as *Fornicutes*, that have been isolated from Tengchong springs were not detected in this study. Although many of the dominant taxa in circumneutral and alkaline springs in Tengchong have close relatives in culture, few, if any, of those organisms have been isolated from Tengchong springs. This discrepancy is particularly clear among the *Aquificae* and the crenarchaeal orders *Desulfurococcales* and *Thermoproteales*.

A comparison between the previous clone library-based studies and this study reveals consistency between the compositions of the community. Orders *Sulfolobales*, *Desulfococcales*, and *Thermoproteales* were all identified in our study, consistent with the results reported for Tengchong springs using a specific primer set for *Crenarchaeota* [36]. A recent clone library-based study showed that both bacterial phylum *Aquificae* and archaean phylum *Crenarchaeota* were detected in a microbial mat sample from Tengchong (near Shuirebaozha) [11], consistent with our results. In contrast, sequences related to putative AOA were not detected in Rehai springs, despite their reported presence using a functional gene approach and the presence of lipid biomarkers (e.g., crenarchaeol) proposed to be diagnostic of AOA [13,14,37]. This discrepancy is likely due to the lower temperatures of the springs previously sampled in Tengchong [13] and is consistent with the low amoA transcript levels detected [14] in some of the spring that were also sampled in this study (i.e., Dagunguo, Diretiyanqu, Zhenzhaquan). It was likely that the abundance of AOA population was low in these springs.

Microbial Community Composition in Tengchong in Comparison to other Geothermal Systems

The composition of microbial communities in Tengchong appeared to be dependent on geothermal region, spring type, residence time, and geochemical conditions. For large source pools with standing water (Dagunguo, Gongxiaoshe, and Jinze), there were substantial differences in microbial community composition between water and sediment. The estimated long water residence times in these springs may allow a genuine planktonic community to develop [20]. For small source pools with high discharge, there was no significant difference between water and sediment, likely because of their dynamic mixing.

Microbial community composition and its correlation with geochemistry in Tengchong can be better understood by a comparison to other geothermal systems in the world. The hot spring systems of YNP and the Great Basin are well-studied examples for this purpose. In terms of mechanisms and genesis, the Tengchong hot springs are similar to those in YNP in that they are both volcanically driven with acidic springs. In contrast, geothermal activity in the Great Basin is attributed to tectonically driven dilution of range-front faults and there are no highly acidic springs (e.g., pH <5.5).

Both Tengchong and YNP host springs with dramatically different geochemical conditions and both display a bi-modal pH distribution due to buffering by either the carbonate system or the sulfuric acid system [38]. As a result, many springs in Rehai and YNP harbor similar microbial communities at the phylum and family/genus levels. Among abundant microbial groups in Tengchong and YNP are the bacteria phyla *Aquificae*, *Deinococcus-Thermus*, *Thermodesulfobacteria*, and the archaean phylum *Crenarchaeota*.

*Aquificae*. Four genera of *Aquificales*, *Thermocrinis*, *Sulfurhydrogenibium*, *Hydrogenobacter* and *Hydrogenobaculum*, are common in terrestrial geothermal environments and have been previously observed in YNP [39–44]. All these genera were present in Tengchong springs; however, their relative abundance was somewhat surprising. *Hydrogenobacter* and *Hydrogenobaculum* were abundant in high temperature (73.8–93.6°C), circumneutral to alkaline pH (6.7–9.4) springs and low temperature (55.1–64.5°C), acidic (pH: 2.3–2.6), sulfur-rich springs, respectively; however, *Thermocrinis* and *Sulfurhydrogenibium* were apparently present at very low relative abundance (<0.02% of all reads). *Thermocrinis* and *Hydrogenobacter* usually occupy similar niches in high temperature, circumneutral to alkaline (~pH 8) terrestrial systems, although *Hydrogenobacter* may be more abundant at lower temperatures (60–
80°C) than Thermocrinis (75–92°C) and is not known to form macroscopically visible streamers [39,43]. Given the high temperatures sampled here (>78.2°C) and the abundance of streamer biomass in alkaline spring sources (pH >8.3) in Rehai, it is surprising that Hydrogenobacter would dominate over Thermocrinis. However, a recent study [44] has shown that Thermocrinis-dominated Aquificales are present in both streamer biofilm community (SBC) and non-SBC locations of YNP, and casted doubt on the hypotheses that Aquificales (especially Thermocrinis) are truly responsible for streamer formation. Our data in Tengchong supported this observation: microbial composition of the streamer sample from Gumingquan was nearly the same as that in the Gumingquan sediment without streamer, consisting of predominantly Aquificae with small amounts of Thermodesulfobacteria and Crenarchaeota (Desulfurococcales) (Fig. 2B). In Tengchong, Hydrogenobacter was the dominant Aquificae in both streamer-forming and non-streamer forming communities. At present, it remains unclear about the mechanisms of streamer formation, although it was possible that some other organisms may be working with Aquificae to either encourage or suppress streamer formation [44].

Sulfahydrogenibium is common and abundant in circumneutral springs in YNP [46–49] and the Great Basin [20]. Its apparent low abundance in Tengchong springs may be related to their low maximum growth temperature of 78°C, which was below the lowest temperature in the circumneutral and alkaline samples reported here except Ruidian springs (Gongxiaosh and Jinze). The Ruidian springs, however, were expected to have a relatively high dissolved O2 content due to the long water residence time, which may be incompatible with the microaerophilic nature of Sulfahydrogenibium (optimal O2 concentration, 6% v/v) [50].

Deinococcus-Termus. Although many isolates from this phylum have been obtained from Rehai [8], our molecular study showed that this group was actually the most abundant in Gongxiaosh and Ruidian, which is cooler and more oxygenated than circumneutral and alkaline springs sampled in Rehai. Thermus has a similar geochemical distribution in YNP [40,44,48,51] and the Great Basin [52,53] as in Tengchong. The distributions of these organisms in Tengchong, YNP, and the Great Basin are consistent with the physiology of cultured representatives within this phylum [54].

Thermodesulfobacteria. Thermodesulfobacteria were found in spring sediments with neutral pH (7.2) and high temperature (84.5°C), consistent with their common presence in sediments of YNP [40,41,44,46] and the Great Basin [52,53] under the conditions of near neutral pH (6.1–7.3) and high temperature (77–90°C). The presence of these organisms in sediments is also consistent with their physiology, which is either anaerobic sulfate or ferric iron reduction.

Crenarchaeota. The crenarchaeal order Thermoprotei was abundant in Rehai and also at many sites in YNP [41,44,47], but only a small component in the Great Basin, where Archaeoglobales, Thaumarchaeota, and yet-uncultivated lineages were more abundant [20,52,53]. This difference is likely due to the fact that there are no highly acidic springs in the Great Basin (pH <5.5) and therefore cannot support the many members of the order Thermoprotei that are generally acidophilic.

The relative proportions of three orders within this class, Sulfolobales, Thermoproteales, and Desulfurococcales, correlated with pH and temperature (Figure 3C), and other geochemical conditions in Tengchong springs. The predominance of Sulfolobales in high temperature, acidic, sulfur-rich sites (Diretiyanqu-1, -2, and -3) in both water and sediment (well mixed with water), is consistent with the oxygen, temperature and pH optima of the genus Sulfolobus (i.e., microaerophilic, 65–85°C and 2–3) and the distribution of Sulfolobus in acidic sulfataras in YNP and elsewhere [35,36]. The dominance of Sulfolobus at these springs with high levels of sulfur (primarily sulfate) and ferrous iron suggests that aerobic respiration of sulfide, Fe2+, organic matter, and H2 are dominant metabolisms in high temperature acid springs in Rehai [53]. In addition to the genus Sulfolobus of the family Sulfolobaceae of the order Sulfolobales, the genera Acidianus and Metallosphaera were also present in low abundance yet neither Stygiolobus nor Sulfuriferae were detected. Stygiolobus-like sequences were present in YNP springs with dissolved oxygen levels at or below detection [43], consistent with the anaerobic nature of this genus [55]. The possible absence of Stygiolobus in Tengchong springs may be due to the toxic nature of these springs.

The predominance of Desulfurococcales in Rehai springs with high temperature (>80°C) and circumneutral to alkaline pH (Figure 3C) is consistent with the known hyperthermophilic of these organisms (temperature optima 84–95°C; [57]), but only partly overlaps with the known pH range of known terrestrial genera of Desulfurococcales (pH range 2.2–8.5; [57]) and their pH distribution in YNP springs based on cultivation-independent studies (pH range 6.1–8.1; [41,43,44]). Thus, it appears that Desulfurococcales in Tengchong have a higher pH range than other terrestrial Desulfurococcales, and even higher than the marine organism Aeropyrum pernix, which is able to grow up to pH 9.0 [57]. Interestingly, the two Rehai springs where abundant Desulfurococcales were observed, i.e., Gumingquan and Jiemeiquan, also had high levels of Si, Cl, K, and Na in the waters (Table S1). At present, it is unclear if these ions affected the pH tolerance of Desulfurococcales. The higher abundance of Desulfurococcales in the sediments of Gumingquan and Jiemeiquan than those in the corresponding waters (Fig. 3C) may suggest that the sediments may have offered some protection against highly alkaline pH.

Similar to Desulfurococcales, Thermoproteales (mainly Pyroboactinum) relatives were the most abundant in Rehai springs with high temperature and neutral-alkaline pH (Table 3, Figure 3C). Again the high temperature of these springs is consistent with the hyperthermophilic nature of the genus Pyroboactinum (74–102°C with optimum 100°C, [58]), but the pH of some Rehai springs is higher than both the pH optimum (6–7) and even the maximum (9) known for this genus [58]. Thus, the Pyroboactinum in Rehai are likely to be more alkaliphilic than any known Thermoproteales including those from YNP [47,59]. Within the order Thermoproteales, the terrestrial genera Thermofilum, Vulcanaisa, Thermoproteus, and Thermocadillum are commonly observed in YNP springs with mildly acidic pH (~5.5–6.1) and moderate to high temperature [41,46,47], which is consistent with the physiology of these genera. The apparent absence of these genera in Tengchong appears to be consistent with the paucity of springs in this pH range.

Physiological Functions

Although it can be difficult to infer physiological functions based on DNA sequences, especially for partial sequences, there are a few taxonomic groups for which tentative functions might be inferred. As expected, phototrophic bacteria were only detected in the Gongxiaosh spring, which is consistent with the observation that the upper temperature limit for photosynthesis is 73–75°C [38,60]. pH was also a limiting factor as no prokaryotic phototrophs were observed in acidic springs even when temperatures were lower than 73°C, as consistent with a previous study [61]. Sulfide or Fe2+ oxidation with O2 as an electron acceptor appears to be an important metabolic pathway in acidic springs of
Rehai (Diretiyanqu and Zhenzhuquan, mostly by Sulfobolus), as it is in YNP [62,63]. For neutral and alkaline hot springs, members of the Aquificales were prominent in springs of Rehai (Tengchong), as they are in many springs in YNP (e.g. [39,40,42–44]), indicating that chemolithotrophy by oxidation of H₂, reduced sulfur compounds (thiosulfate, sulfur, or sulfide), or one-carbon compounds (formate or formaldehyde) are likely important metabolic processes in these springs.

The majority of archaeal sequences in Gongxiaoshe were closely related (>97%) to “Candidatus Nitrosocaldus yellowstonii”, a known AOA [64] within the phylum Thaumarchaeota [65], suggesting a potentially important role for ammonia oxidation in this spring. AOA are also prominent members that have been observed in Great Boiling Spring of the Great Basin [32] and in hydrothermal vent features in Yellowstone Lake [29]. Interestingly, springs from these three different regions share broadly similar pH and temperature conditions, i.e., circumneutral pH (6.2–8.4) and moderate temperature (60–82°C). These conditions are similar to, but extend above, the temperature range of “Candidatus Nitrosocaldus yellowstonii” in enrichment culture [64].

Conclusions

Two geothermal fields in Tengchong, Rehai and Ruidian, were highly diverse in their environmental conditions, encompassing a temperature range from 55.1 to 93.6°C and pH from 2.46 to 9.39 as well as wide ranges of water and sediment geochemistry. The overall microbial diversity was low, and did not show any systematic correlation with geochemical conditions. Ruidian sediments hosted higher microbial diversity than the corresponding waters and Rehai samples, likely due to mild temperature, pH, high organic carbon, and different mineralogy (carbonate-dominated).

The bacterial phylum Aquificae and archaeal phylum Crenarchaeota were dominant in all samples from Rehai and water samples from Ruidian. The specific compositions of Aquificae and Crenarchaeota were highly dependent on pH, temperature, and water/sediment geochemistry. Acidic and sulfur-rich springs were dominated by crenarchaeal order Sulfobacterales and Aquificae genus Hydrogenoacitibacter, whereas neutral to alkaline pH favored the Aquificae genus Hydrogenobacter, and crenarchaeal orders Desulfurococcales and Thermoproteales. A higher pH tolerance was observed for Desulfurococcales and Thermoproteales and this tolerance appeared to be related to water/sediment geochemistry. Whereas many of these organisms have been detected in other terrestrial springs worldwide, some members typically found in other terrestrial hot springs such as Thermococcus were not present or only in minor abundance in Tengchong. This study highlights the importance of temperature, pH and other geochemical conditions and endemism in shaping the microbial community structure in Tengchong hot springs.

Supporting Information

Figure S1 Field photos of Tengchong hot springs. A. Dagunguo (Dgg), the largest spring at Rehai, is a ~5 meter diameter, 1.5 meter deep, cylindrical pool with vigorously degassing sources (84.5°C). This clear, circumneutral chloride spring has hard silica precipitates. Sinter samples were chipped from the sides of the spring in three locations; B. Diretiyanqu (Drty) at Rehai is comprised small acid sulfate pools (pH 2.5, labeled B, C, D) derived from vapor condensate. Springs vary with respect to temperature (53.1–85.1°C) but all are turlid with high dissolved clay content. Pools and fumaroles are temporarily variable; C. Gumingquan is the most alkaline spring in Rehai (pH 9.35). The high discharge source pictured at left (Gmqs, 93°C) has several small downstream pools. GmQC is a large pool (89°C) near the center of the flow path (photo at right). GmQP, the largest pool (82.5°C), is located immediately below a bridge (photo at center) and contained abundant streamer biomass at the time of sampling; D. Jiangmeiquan (also known as Yanjingquan) at Rehai includes a pair of ~1 m diameter, high temperature, alkaline springs (pH 9.3). Left Zimeiquan (JmqL, labeled H) has an extremely active 93.6°C source. Right Zimeiquan (JmqR, labeled J) has a cooler (83.2°C) and less active source; E. Zhenzhuquan (Zzq) at Rehai is a shallow, heart-shaped, acidic spring (pH 4.79) with several vigorously degassing sources (89.1°C). F. Hauitaijing at Rehai includes two 1 m diameter wells, left (Hjtl, 90°C labeled L) and right (Hjtr, 92.3°C, labeled M). Both wells are approximately pH 8.1 and have large boulder bottoms, which precluded sediment sampling; G. Shuirebaozha at Rehai is a shallow pool with a bottom of soft clay and small stones with several degassing sources. SrbzU (79.8°C, pH 8.04 labeled N) is a peninsula between two pools, and SrbzD (78.2°C, pH 8.28, labeled P) is located near the middle of the west pool; H. Gongxiaoshe is a large, circumneutral, carbonate-depositing spring in Ruidian. Samples were collected at temperatures of 73.8°C and included sediments (GxsB) from the bottom of the pool and sinter (GxsS) chipped from the sides of the spring; I. Jinze (Jz) is one of many geothermal wells in Ruidan. Sediment samples were collected from the bottom of the well at pH 6.71 and 81.6°C.

(DOC)

Figure S2 Hierarchical clusters of Euclidean distance for water geochemistry. The geochemical data include TOC, TN, cation/anion concentrations, and trace metal concentrations. The numbers in the site name are the pH and temperature of each site. The scale bar on the top represents Euclidean distance. (TIF)

Figure S3 The δ²H (%o) - δ¹⁸O (%o) relationship for hot springs waters from Ruidian and Rehai. This relationship is compared to the global meteoric water line (GMWL) [66] and local meteoric water line (LMWL). The LMWL is developed based on precipitation data at the Xishuangbanna station, about 400 km south of Tengchong [67]. (DOCX)

Figure S4 Hierarchical clusters of Euclidean distance for sediment/sinter geochemistry. The clustering is based on TOC, TN, and mineral compositions in sediment/sinter. The scale bar on the top represents Euclidean distance. (TIF)

Figure S5 Comparison of diversity indices at the 97% cutoff level between microbial communities retrieved with the two primer pairs. (TIF)

Figure S6 Unweighted UniFrac cluster tree based on microbial communities at the 97% cutoff level. The letter “P” at the end of the sample ID refers to a microbial community retrieved with the unmodified primer pair. (TIF)

Figure S7 Comparison of microbial compositions retrieved with the two primer pairs. (DOC)

Figure S8 Diversity indices of different microbial communities as a function of temperature and pH. The
Table S1 Water and sediment geochemistry for Tengchong springs. (DOCX)

Table S2 Trace metal concentrations of hot spring water samples from Tengchong (values in ppb). (DOCX)

Table S3 Significance tests of the overall microbial community structure between the two primer sets with three different statistical approaches. (DOCX)

References

1. Liao Z, Guo G (1986) Geology of the Tengchong geothermal field and surrounding area, west Yunnan, China. Geomicrobiology 15: 339–345.
2. Tong W, Zhang M (1989) Tengchong geochemistry. Beijing: Science Press (in Chinese). 282 p.
3. Wang F, Peng Z, Chen W, Wang Z, Yang J, et al. (2000) High-precision thermal ionization mass spectrometry dating of young volcanic rocks by using U-series method. Chinese Sci Bull 45: 83–87.
4. Du J, Liu C, Fu B, Nioinonya Y, Zhang Y, et al. (2005) Variations of geochemistry and chemical-isotopic compositions of hot spring fluids in the Rehai geothermal field, southwestern China. J Volcanol Geoth Res 142: 243–261.
5. Fournier RO (1989) Geochemistry and dynamics of the Yellowstone National Park hydrothermal system. Annu Rev Earth Planet Sci 17: 13–53.
6. Yoshida Y (1991) Geochemistry of the Nigorikawa geothermal system, southwestern Hokkaido, Japan. Geochim J 25: 203–222.
7. Kyle JE, Schroader PA, Wiegel J (2007) Microbial silification in sinters from two terrestrial hot springs in the Uzon Caldera, Kamchatka, Russia. Geomicrobiology 24: 627–641.
8. Hedlund BP, Cole JK, Hou W, Zhou E, Li W, et al. (2012) A review of the microbiology of the Rehai geothermal field in Tengchong, Yunnan Province, China. Geosci Front 3: 273–288.
9. Tringe SG, Hugenholtz P (2008) A renaissance for the pioneering 16S rRNA gene. Curr Opin Microbiol 11: 442–446.
10. Song QZ, Chen JQ, Jiang HC, Zhou EM, Tang SK, et al. (2010) Diversity of Crenarchaeota in terrestrial hot springs in Tengchong, China. Extremophiles 14: 287–296.
11. Pangali E, Grant WD, Cowan DA, Jones BE, Ma YH, et al. (2012) Bacterial and archaeal diversity in two hot spring microbial mats from the geothermal region of Tengchong, China. Extremophiles 16: 607–618.
12. Pearson A, P Y, Zhao W, Li WJ, Li Y, et al. (2008) Factors controlling the distribution of archaeral tetraders in terrestrial hot springs. Appl Environ Microbiol 74: 3522–3532.
13. Zhang CL, Ye Q, Huang Z, Li W, Chen J, et al. (2000) Global occurrence of archaeal amoA genes in terrestrial hot springs. Appl Environ Microbiol 74: 6417–6426.
14. Jiang H, Huang Q, Dong H, Wang P, Wang F, et al. (2010) RNA-based investigation of ammonia-oxidizing archaea in hot springs of Yunnan Province, China. Appl Environ Microbiol 76: 4530–4541.
15. Sharp JH, Benner R, Bennett L, Carlsson CA, Dow R, et al. (1993) Re-evaluation of high temperature combustion and chemical oxidation measurements of dissolved organic carbon in seawater. Limnol Oceanogr 38: 1774–1782.
16. Sharp J, Beaucaire A, Burdige D, Cauwe G, Cursels S, et al. (2004) A direct instrument comparison for measurement of total dissolved nitrogen in seawater. Mar Chem 84: 181–193.
17. Bershaw J, Penny SM, Garzione CN (2012) Stable isotopes of modern water across the Himalaya and eastern Tibetan Plateau: Implications for estimates of paleoelevation and paleoclimates. J Geophys Res 117: D020110.
18. Huang Q, Dong CZ, Dong RM, Jiang H, Wang S, et al. (2011) Archaeal and bacterial diversity in hot springs on the Tibetan Plateau, China. Extremophiles 15: 549–563.
19. Lane D (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acids techniques in bacterial systematics. New York: John Wiley and Sons. 115–173.
20. Dodsworth JA, Hungate BA, Hedlund BP (2011) Ammonia oxidation, denitrification and dissimilatory nitrate reduction to ammonium in two US Great Basin hot springs with abundant ammonia-oxidizing archaea. Environ Microbiol 13: 2371–2386.
21. Zhou J, Wu L, Deng Y, Zhi X, Jiang YH, et al. (2011) Reproducibility and quantification of amplicon sequencing-based detection. ISME J 5: 1303–1313.
22. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, et al. (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37: D141–145.
23. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al. (2009) Introducing moduler: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75: 7537–7541.
24. Reeder J, Knight R (2010) Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. Nat methods 7: 668–669.
25. Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26: 2460–2461.
26. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. Nat methods 7: 335–336.
27. Knight R, Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, et al. (2010) PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26: 266–267.
28. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, et al. (2011) Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplions. Genome Res 21: 494–504.
29. Kaj A, Cline P, Zancar RE, Insko K, WoIvlova D, et al. (2011) Archaea in Yellowstone Lake. ISME J. 5: 1784–1795.
30. Oksanen J, Kindt R, Legendre P, O’Hara RB (2007) Vegan: community ecology package version 1.8–6. Available: http://cran.r-project.org/. Accessed December 3 2012.
31. Clarke K, Ainsworth M (1993). A method of linking multivariate community structure to environmental variables. Mar Ecol-Prog Series 92: 205–205.
32. Zhang Z, Liu S, Zhao F (1987) Geochemistry of thermal waters in the Tengchong Volcanic Geothermal Area, West Yunnan Province, China. Geothermics 16: 169–179.
33. Zhang G, Liu CQ, Liu H, Jin Z, Han G, et al. (2008) Geochemistry of the Rehai and Ruidian geothermal waters, Yunnan Province, China. Geothermics 37: 73–83.
34. Shen M, Ni B, Hou F (1982) Hydrogen and oxygen isotopic study of hydrocirculation in the Tengchong geothermal area, Yunnan Province (in Chinese with English abstract). Records Geol Res: 187–191.
35. Xiang X, Dong X, Huang L (2005) Sulfolobus tengchongensis sp. nov., a novel thermococcal archaeon isolated from a hot spring in Tengchong, China. Extremophiles 7: 493–498.
36. Song QZ, Chen JQ, Jiang HC, Zhou EM, Tang SK, et al. (2010) Diversity of Crenarchaeota in terrestrial hot springs in Tengchong, China. Extremophiles 14: 287–296.
37. Dodsworth JA, Hungate BA, Torre J, Jiang H, Hedlund BP (2011) Measuring nitrification, denitrification, and related biomarkers in continental geothermal ecosystems. Methods Enzyomol 486: 171–203.
38. Brock TD (1976) Thermophilic microorganisms and life at high temperatures. New York: Springer-Verlag. 465 p.
39. Reysenbach A-L, Banta A, Civello S, Daly J, Mitchel K, et al. (2005) Aquificales Crenarchaeota in terrestrial hot springs in Tengchong, China. Geosci Front 3: 273–288.
40. Spear JR, Walker JJ, McCollom TM, Pace NR (2005) Hydrogen and carbonic acid levels in the Great Basin hot springs with abundant ammonia-oxidizing archaea. Environ Microbiol 7: 129–139.
Microbial Diversity in Hot Springs of Tengchong

42. Boyd ES, Leavitt WD, Gessey GG (2009) CO2 uptake and fixation by a thermoacidophilic microbial community attached to precipitated sulfur in a geothermal spring. Appl Environ Microbiol 75: 4289–4296.

43. Macur R, Langner H, Kozar B, Imkamp W (2004) Linking geochemical processes with microbial community analysis: successional dynamics in an arsenic-rich, acid-sulfate-chloride geothermal spring. Geobiology 2: 163–177.

44. Meyer-Dombard DR, Swingley WD, Raymond J, Havig J, Shock EL, et al. (2011) Hydrothermal ecotones and streamer biofilm communities in the Lower Geyser Basin, Yellowstone National Park. Environ Microbiol 13: 2216–2231.

45. Eder W, Huber R (2002) New isolates and physiological properties of the Aquificales and description of Thermocrinis albus sp. nov. Extremophiles 6: 309–318.

46. Inkepp WP, McDermont TR (2005) Geomicrobiology of acid-sulfate-chloride springs in Yellowstone National Park. Proceedings of the Thermal Biology Institute Workshop. Yellowstone National Park, WV: Montana State University Publications 143–162.

47. Inkepp WP, Rusch DR, Jay ZJ, Herrgard MJ, Kozubal MA, et al. (2010) Metagenomes from High-Temperature Chemosynthetic Systems Reveal Geochemical Controls on Microbial Community Structure and Function. PLoS ONE 5: e9573. doi:10.1371/journal.pone.0009573.

48. Hall JR, Mitchell KR, Jackson-Weaver O, Kooser AS, Cron BR, et al. (2008) Molecular characterization of the diversity and distribution of a thermal spring microbial community by using rRNA and metabolic genes. Appl Environ Microbiol 74: 4910–4923.

49. Yang T, Lyons S, Aguilar G, Cuhel R, Teske A (2011) Microbial communities and chemosynthesis in yellowstone lake sublactustrine hydrothermal vent waters. Front Microbiol 2: 130.

50. Nakagawa S, Shitah Z, Banta A, Beveridge T, Sako Y, et al. (2005) Sulfurferrogenium yellowstonense sp. nov., an extremely thermophilic, facultatively heterotrophic, sulfur-oxidizing bacterium from Yellowstone National Park, and new and emended descriptions of the genus Sulfurferrogenium, Sulfurferrogenium subterraneum and Sulfurferrogenium azorense. Int J Syst Evol Microbiol 55: 2263–2268.

51. Inkepp WP, Rusch DB, Jay ZJ, Herrgard MJ, Kozubal MA, et al. (2010) Metagenomes from High-Temperature Chemosynthetic Systems Reveal Geochemical Controls on Microbial Community Structure and Function. PLoS ONE 5: e9573.

52. Costa KC, Navarro JB, Shock EL, Zhang CL, Soukup D, et al. (2009) Microbiology and geochemistry of great boiling and mud hot springs in the United States Great Basin. Extremophiles 13: 447–459.

53. Vick TJ, Dodsworth JA, Costa KC, Shock EL, Hedlund BP (2010) Microbiology and geochemistry of Little Hot Creek, a hot spring environment in the Long Valley Caldera. Geobiology 8: 140–154.

54. Da Costa MS, Rainey FA, Nombre MF (2006) The genus Thermus and relatives. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, editors. The Prokaryotes. 3rd ed. New York: Springer. 797–812.

55. Huber H, Sterrer KO (2001) Order III. Sulfidibacteriales. In: Garrity GM, editor. Bergey’s Manual of Systematic Bacteriology. 2nd ed. New York: Springer-Verlag. 169.

56. Whitaker RJ, Grogan DW, Taylor JW (2003) Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science 301: 976–978.

57. Huber H, Sterrer KO (2001) Order II. Desulfurococcales. In: Garrity GM, editor. Bergey’s Manual of Systematic Bacteriology. 2nd ed. New York: Springer-Verlag. 169.

58. Huber H, Sterrer KO (2001) Order I. Thermoproteales. In: Garrity GM, editor. Bergey’s Manual of Systematic Bacteriology. 2nd ed. New York: Springer-Verlag. 170–179.

59. Swingley WD, Meyer-Dombard DR, Shock EL, Alope EB, Falencki HD, et al. (2012) Coordinating environmental genomics and geochemistry reveals metabolic transitions in a hot spring ecosystem. PLoS ONE 7: e50106.

60. Kempner ES (1963) Upper Temperature Limit of Life. Science 142: 1310–1319.

61. Cox A, Shock EL, Havig J (2011) The transition to microbial photosynthesis in hot spring ecosystems. Chem Geol 280: 344–351.

62. Kozubal M, Macur RE, Kofl S, Taylor WP, Ackerman GG, et al. (2008) Isolation and distribution of a novel iron-oxidizing crenarchaeon from acidic geothermal springs in Yellowstome National Park. Appl Environ Microbiol 74: 942–949.

63. Kozubal MA, Macur RE, Jay ZJ, Beam JP, Malatti SA, et al. (2012) Microbial iron cycling in acidic geothermal springs of yellowstone national park: integrating molecular surveys, geochemical processes, and isolation of novel Fe-active microorganisms. Front Microbiol 3: 109.

64. de la Torre JR, Walker CB, Ingalls AE, Konneke M, Stahl DA (2008) Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. Environ Micobiol 10: 810–818.

65. Brock-Adamant C, Bousan B, Gribaldo S, Forterre P (2008) Mesophilic Crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. Nat Rev Microbiol 6: 243–252.

66. Rozanski R, Aragias-Aragias L and Goufi M (1993) Isotopic patterns in modern global precipitation. In: Swart PK, Lohmann KC, MacKenzie J, Savin S, editors. Climate Change in Continental Isotopic Records, Geophys. Monogr. Ser., 78. Washington, DC: American Geophysical Union. 1–36.

67. Liu W, Li P, Li H, Duan W (2006) Estimation of evaporation rate from soil surface using stable isotopic composition of throughfall and stream water in a tropical seasonal rain forest of Xishuangbanna, Southwest China. Acta Ecol Sin 26: 1303–1310.