Microbial Community Structure and Arsenic Biogeochemistry in an Acid Vapor-Formed Spring in Tengchong Geothermal Area, China

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Abstract

Arsenic biogeochemistry has been studied extensively in acid sulfate-chloride hot springs, but not in acid sulfate hot springs with low chloride. In this study, Zhenzhuquan in Tengchong geothermal area, a representative acid sulfate hot spring with low chloride, was chosen to study arsenic geochemistry and microbial community structure using Illumina MiSeq sequencing. Over 0.3 million 16S rRNA sequence reads were obtained from 6-paired parallel water and sediment samples along its outflow channel. Arsenic oxidation occurred in the Zhenxhuquan pool, with distinctly high ratios of arsenate to total dissolved arsenic (0.73–0.86). Coupled with iron and sulfur oxidation along the outflow channel, arsenic accumulated in downstream sediments with concentrations up to 16.44 g/kg and appeared to significantly constrain their microbial community diversity. These oxidations might be correlated with the appearance of some putative functional microbial populations, such as *Aquificae* and *Pseudomonas* (arsenic oxidation), *Sulfolobus* (sulfur and iron oxidation), *Metallosphaera* and *Acidicaldus* (iron oxidation). Temperature, total organic carbon and dissolved oxygen significantly shaped the microbial community structure of upstream and downstream samples. In the upstream outflow channel region, most microbial populations were microaerophilic/anaerobic thermophiles and hyperthermophiles, such as *Sulfolobus*, *Nocardia*, *Fervidicoccus*, *Delftia*, and *Ralstonia*. In the downstream region, aerobic heterotrophic mesophiles and thermophiles were identified, including *Ktedonobacteria*, *Acidicaldus*, *Chthonomonas* and *Sphingobacteria*. A total of 72.41–95.91% unassigned-genus sequences were derived from the downstream high arsenic sediments 16S rRNA clone libraries. This study could enable us to achieve an integrated understanding on arsenic biogeochemistry in acid hot springs.
Introduction

Acid hot springs provide unique environments for the evolution and establishment of microbial communities and their response to various biogeochemical and metabolic processes involving hydrogen (H₂), sulfur (S), iron (Fe) and arsenic (As) [1–6]. Generally, all acid hot springs are classified into two different water types: acid sulfate-Cl water and sulfate water with low Cl [7, 8]. Acid sulfate-Cl hot springs are viewed to form by sulfide oxidation after saturated Na-Cl geothermal water is exposed to the Earth’s surface, and thus have decreased pH values but retain high K, Na, F, Cl, Li, B and As concentrations as geothermal reservoir water [7]. The high As concentration of these environments has attracted attention of several groups studying As redox speciation [1, 9–11]. Results of previous studies illustrated that arsenite (As(III)) was predominant in the source water of acid sulfate-Cl hot springs, and was oxidized to arsenate (As(V)) once being discharged along the outlet by bacterial populations such as *Hydrogenobacter*, *Hydrogenobaculum*, *Sulfurihydrogenibium*, and *Thiomonas* [1, 9, 11, 12]. No As(III) oxidation in the source water of acid sulfate-Cl hot springs was observed because of sulfide inhibition by inactivating expressed As(III) oxidase enzyme (Aio) [4, 13]. Due to high concentrations of sulfide, Fe and As in those acid sulfate-Cl hot springs, elemental S and Fe deposits rich in As successively appeared along the outflow channel [9, 10, 14]. Various microbial populations such as *Sulfolobus*, *Sulfobacillus*, *Metallosphaera*, *Sulfurihydrogenibium*, *Hydrogenobaculum*, *Thiomonas* and *Acidicaldus* had been found to respond to sulfide and Fe oxidations in those acid hot springs [1, 9, 14–16]. Comparatively, acid sulfate hot springs with low Cl have not studied systematically on As biogeochemistry. These geothermal features form by separation of the vapor phase rich in H₂S from the reservoir and subsequent condensation and oxidation in shallow oxygen-rich groundwater or surface water [17].

Tengchong, located in southwestern of China, is a typical volcanic geothermal area and has abundant geothermal resources [18]. Zhenzhuquan in the Rehai geothermal field of Tengchong is a representative acid vapor-formed sulfate hot spring low in Cl, with 128.2 mg/L sulfate, 39.2 mg/L Cl and 71.1 μg/L As, as well as low concentrations of K, Na, Li and B [19]. This geochemistry is distinctly different from the previously studied acid sulfate-Cl hot springs (sulfate: 21.0–144.9 mg/L; Cl: 473.6–1907.0 mg/L; As: 1.8–5.3 mg/L) [1, 9–11]. The As concentrations in the sediments of Zhenzhuquan outflow channel occur at up to 16.44 g/kg, which substantially exceeded the terrestrial abundance of As (1.5–3 mg/kg) and could pose a potential environmental risk [20]. Previous studies indicated that different microbial communities inhabited geothermal environments with distinct geochemistry, including different media such as water and sediment at the same sites of hot springs [21, 22]. Though some microbial studies on As in mats or sediments along those acid sulfate-Cl hot springs outlet had been conducted by clone library and metagenome sequencing [1, 9, 10, 23], arsenic geochemistry and corresponding microbial communities in acid sulfate hot spring with low Cl have yet to be fully understood. Therefore, the objectives of this study were to: (1) investigate the As geochemistry and microbial community structures both in water and sediments along the outflow channel of a typical acid sulfate hot spring with low Cl; (2) evaluate the potential microbially-mediated As oxidation process; and (3) assess the environmental factors shaping the microbial community structures.

Materials and Methods

Site description

No specific permission was 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 were not involved in endangered or protected species.

As mentioned above, Zhenzhuquan (N24.9511°, E98.4361°) located in the Rehai geothermal field of Tengchong geothermal area in Yunnan, southwestern China, was selected for this study (Fig 1A). This spring is a heart-shaped acidic pool with a depth of 6–7 cm and a length of 4.36 m and is fed by numerous very small vigorous degassing vents (Fig 1B) [22]. It has a seasonally fluctuating temperature from 89.1 to 93.3°C and pH from 3.50 to 6.42, which results from the dilution of rainfall [17, 24]. Zhenzhuquan has a low discharge rate of 0.2 L/S and abundant reddish-brown sediments occur downstream from the source (Fig 1C). These sediments contain large amounts of As with concentrations up to 16.44 g/kg, even though As concentration is extremely low (48.16 μg/L) in the pool water [25]. Consequently, a transect of six sampling sites was established along Zhenzhuquan’s outflow channel and each sampling site was assigned a name according to the relative distance from commenced discharge point (0 m), such as 3 m, 6 m and 9 m downstream and -2 m and -1 m in the pool (Fig 1B). Parallel water and sediment samples at each site were collected in August, 2014.

Field measurements and sample collection

Water temperature, pH and dissolved oxygen (DO) were measured in the field at the site of water collection using a hand-held meter. Concentrations of sulfide, ammonium, ferrous iron (Fe(II)) and total iron (FeTot) were also determined in the field with a Hach spectrophotometer (DR850, Hach Corp., USA) according to the manufacturer’s instructions. Water samples for laboratory measurements, e.g., anions, cations and dissolved organic carbon (DOC) were collected into 50 mL acid-washed polypropylene bottles and brown glass bottles respectively by filtration of spring water through 0.22 μm syringe polyethersulfone (PES) membrane filters (Pall Corp., NY, USA). Filtered biomass-containing membranes were placed into 15 mL sterile polypropylene tubes and immediately stored in dry ice. Water samples for cations and DOC were acidified with 1% v/v HNO3. Arsenic species separation was done on site, following the method reported by Le et al. [26]. Briefly, 10 mL of each water sample was passed through a silica-based strong anion-exchange cartridge (Supelco, USA) preconditioned with 50% methanol and deionized water before use. As(V) was retained in the cartridge and As(III) remained in the filtered solution. Subsequently, the cartridge was eluted with 10 mL 1 M HCl to release the bound As(V) to eluate samples. Sediment samples were collected in sterile 50 mL polypropylene tubes in duplicate by using sterile spoons and stored in ice. All samples for microbial community analysis (sediments and biomass-containing membranes) were stored in dry ice in the field and during transport, and then stored at -80°C in the laboratory until further analyses.

Laboratory geochemical analysis

The cation and anion concentrations were measured by inductively coupled plasma-optical emission spectrometry (CAP6300, Thermo, USA) and ion chromatography (ICS1100, Dionex, USA), respectively. AsTot and FeTot in the sediments were extracted by 1:1 aqua regia digestion method in a water bath [27]. Pre-separated As(III) and As(V) from hot spring waters and extracted AsTot from sediments were determined using liquid chromatography-hydride generation -atomic fluorescence spectrometry (LC-HG-AFS, Haiguang AFS-9780, Beijing) according to Jiang et al. [25]. Extracted FeTot from sediments was determined by the 1,10-Phenanthroline-based assay: 10 mL extracted solutions was mixed with 5 mL acetate-sodium acetate buffer (pH = 4.6), 2.5 mL 1% hydroxylamine hydrochloride solution and 5 mL 0.1% 1,10-phenanthroline solution in 50 mL volumetric flask. The mixtures were made up to a volume of 50 mL with deionized water and allowed to stand for 10 min. The absorbance of each
solution at 510 nm was measured with a spectrophotometer (UV1750, Shimadzu, Japan). Dissolved organic carbon (DOC) of water samples and total organic carbon (TOC) of sediment samples were determined using a TOC analyzer (TOC-VCPH, Shimadzu, Japan) and a Macro elemental analyzer (Multi EA 4000, Analytik Jena, Germany), respectively.

**DNA extraction, amplification and sequencing**

DNA was extracted from biomass-containing filters or from 0.5 g sediment samples using the FastDNA SPIN Kit for Soil (MP Biomedical, OH, USA). DNA concentrations were measured by Pico Green using a FLUOstar OPTIMA fluorescence plate reader (BMG LABTECH, Jena, Germany). The V4 region of 16S rRNA gene was amplified using the normal primer pair 515F (5’-GTGCCAGCMGCCGCGGTAA-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’) combined with Illumina adapter sequences, a pad and a linker of two bases, as well as barcodes on the reverse primers [28]. PCR amplification was carried out in a 25 μL reaction containing 2.5 μL 10× PCR buffer II (including dNTPs) (Invitrogen, Grand Island, NY), 0.4 μM of both forward and reverse primers, 10–15 ng DNA and 0.25 U high fidelity AccuPrime™ Taq DNA polymerase (Life Technologies) under the following program: initial denaturation at 94°C for 1 min, followed by 30 cycles of 94°C for 20 s, 53°C for 25 s, and 68°C for 45 s, and then a final extension at 68°C for 10 min. Reactions were performed in triplicate. Amplicons of each sample were combined and confirmed positive PCR products by agarose gel electrophoresis, and then quantified with PicoGreen. Finally, a total of 200 ng PCR product of each sample was pooled together and purified through QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and then was re-quantified with PicoGreen. Sample 16S rRNA clone libraries for sequencing were prepared according to the MiSeqTM Reagent Kit Preparation Guide (Illumina, San Diego, CA).
USA) and the protocol described previously [29]. Briefly, sample denaturation was performed by mixing 10 μL of combined PCR products (2 nM) and 10 μL 0.2 M NaOH and incubated for 8 min at room temperature. Denatured DNA was diluted to 15 pM using HT1 buffer and mixed with a PhiX DNA library (final concentration 14.3%). A total of 600 μL sample mixture, together with customized sequencing primers for forward, reverse, and index reads, were loaded into the corresponding wells on the reagent cartridge of a 500-cycle v2 MiSeq kit. Sequencing was performed for 251, 12, and 251 cycles, respectively for forward, index, and reverse reads on a Illumina MiSeq system (Illumina, San Diego, CA).

Sequence data preprocessing and statistical analysis

Raw sequences with perfect matches to barcodes were split to sample 16S rRNA clone libraries and were trimmed using Btrim with threshold of QC higher than 25 over 5 bp window size and the minimum length of 150 bp [30]. Forward and reverse reads with at least 50 bp overlap and lower than 5% mismatches were joined using Fast Length Adjustment of SHort reads (FLASH) [31]. After trimming of ambiguous bases (i.e. N), joined sequences with lengths between 247 and 258 bp were subjected to chimera removal by Uchime [32]. Operational taxonomic units (OTUs) clustering was through Uclust at 97% similarity level [33], and taxonomic assignment was through Ribosomal Database Project (RDP) classifier [34] with a minimal 50% confidence estimate. The above steps were performed through the Galaxy pipeline of Institute for Environmental Genomics in University of Oklahoma (http://zhoulab5.rccc.ou.edu/). Samples were rarified at 12 955 sequences per sample. Singletons of generated OTU table were removed for downstream analyses.

All statistical analysis in this study was performed based on genus-level OTUs at the 97% similarity level under the Vegan package in R (http://www.r-project.org/), unless otherwise stated. A variety of alpha diversity indices were calculated including Chao1, Shannon and Equitability. Hierarchical cluster tree using unweighted pair group method with arithmetic means (UPGMA), principal coordinates analysis (PCoA) and non-metric dimensional scaling (NMDS) ordination were built to depict the community composition structure based on the Bray-Curtis dissimilarity matrix of detected OTUs. The Envfit function in the package of Vegan was used to overlay the significant environmental variables on the NMDS ordination. Analyses of similarity (ANOSIM), non-parametric multivariate ANOVA (ADONIS), multi-response permutation procedure (MRPP) and Mantel were performed to test for significant differences in microbial community compositions between sample types (i.e., water vs. sediment) and different locations (i.e., pool vs. downstream). The DNA sequences were deposited to the Short Read Archive database at NCBI (Accession number: SRP056673).

Results

Water and sediment geochemistry

Three water samples (-2 m, -1 m and 0 m) located in the pool of Zhenzhuquan showed similar physical and chemical conditions, with a slight fluctuation in temperature (84.3–91.3°C), pH (3.58–4.33), DO (0.23–0.33 mg/L), DOC (0.36–0.52 mg/L) and concentrations of various ions (Table 1 and S1 Fig), which suggested that Zhenzhuquan was derived by vigorous degassing from the same recharged source. The pool with a low DO average of 0.28 mg/L had correspondingly low ratios of Fe(III) and FeTot (Fe(III)/FeTot: ~ 0.15), but contained high ratios of As(V) and AsTot (As(V)/AsTot: ~ 0.81). Once the source water was discharged from the pool, most of physical-chemical parameters dramatically changed (Fig 2). Temperature ranged from 84.3°C to 49.3°C. The pH values showed a slight decline (pH = 3.89 at 0 m and pH = 3.69 at 12 m), which was consistent with acidic Succession Spring in Yellowstone National Park (YNP).
The decrease in pH was primarily caused by evaporation, suggested by slight increase of F and Cl concentrations along the drainage (S1 Fig). Across sampling intervals, DO, sulfate and ratios of Fe(III) to FeTot (Fe(III)/FeTot) significantly increased from 0.29 to 4.74 mg/L, 120.05 to 158.21 mg/L and 0.09 to 0.34, respectively. Dissolved FeTot and AsTot concentrations in water samples gradually declined from 0.64 to 0.45 mg/L and 66.83 to 44.83 μg/L respectively, coupled with significantly elevated concentrations of FeTot (0.05–4.55 g/kg), AsTot (10.53–16.44 g/kg) and TOC (1.37%-2.81%) in the downstream sediments.

### Alpha diversity of microbial communities

A total of 304 743 passing sequences were obtained from six-pair parallel water and sediment samples. After rarefaction at 12 955 sequences per sample, OTU clustering at 97% similarity level and removal of singletons, 155 050 sequences remained. A variety of taxa were present, with 123–834 observed and 172–1173 predicted OTUs (based on Chao1) and coverage values ranging from 47.04% to 83.08% (S1 Table). Along the outlet, microbial community richness, Shannon diversity and equitability from water samples significantly increased, whereas those in downstream sediments.

### Table 1. Geochemistry of water and sediment samples along Zhenzhuquan’s outflow channel in Tengchong geothermal area.

| Distance from discharge (m) | T°C | pH  | DOC | mg/L | Fe(II) | Fe(III) | FeTot  | μg/L | As(III) | As(V) | AsTot  | FeTot  | AsTot  | Solid phase |
|---------------------------|-----|-----|-----|------|--------|---------|--------|------|---------|-------|--------|--------|--------|-------------|
|                           |     |     |     |      |        |         |        |      |         |       |        |        |        |             |
| -2                        | 89.9| 3.58| 0.33| 0.52 | 3.00   | 0.03    | 112.66 | 0.45 | 0.07    | 0.51  | 0.13   | 17.29  | 46.55  | 63.84  | 0.73 | 0.05 | 0.50 | 0.27 |
| -1                        | 91.3| 4.33| 0.23| 0.36 | 3.30   | 0.03    | 114.37 | 0.40 | 0.11    | 0.51  | 0.22   | 9.27   | 52.99  | 62.26  | 0.85 | 0.07 | 0.36 | 0.09 |
| 0                         | 84.3| 3.89| 0.29| 0.52 | 3.10   | 0.05    | 120.05 | 0.58 | 0.06    | 0.64  | 0.09   | 9.20   | 57.63  | 66.83  | 0.86 | 0.09 | 0.28 | 0.57 |
| 3                         | 76.2| 3.64| 0.30| 1.00 | 3.00   | 0.24    | 129.20 | 0.47 | 0.06    | 0.53  | 0.10   | 8.10   | 49.67  | 57.77  | 0.86 | 4.55 | 16.44 | 1.37 |
| 6                         | 56.8| 3.69| 0.60| 0.60 | 3.40   | 0.03    | 150.95 | 0.36 | 0.10    | 0.45  | 0.21   | 7.33   | 38.53  | 45.86  | 0.84 | 1.20 | 10.53 | 2.72 |
| 9                         | 49.3| 3.69| 0.47| 0.47 | 3.40   | 0.38    | 0.01   | 158.21| 0.31    | 0.16  | 0.47   | 0.34   | 7.06   | 33.78  | 40.83 | 0.83 | 1.29 | 11.64 | 2.81 |

bdl: below detection limit (1 μg/L)

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[9]. The decrease in pH was primarily caused by evaporation, suggested by slight increase of F and Cl concentrations along the drainage (S1 Fig). Across sampling intervals, DO, sulfate and ratios of Fe(III) to FeTot (Fe(III)/FeTot) significantly increased from 0.29 to 4.74 mg/L, 120.05 to 158.21 mg/L and 0.09 to 0.34, respectively. Dissolved FeTot and AsTot concentrations in water samples gradually declined from 0.64 to 0.45 mg/L and 66.83 to 44.83 μg/L respectively, coupled with significantly elevated concentrations of FeTot (0.05–4.55 g/kg), AsTot (10.53–16.44 g/kg) and TOC (1.37%-2.81%) in the downstream sediments.

**Fig 2.** Distribution of selected geochemical data in water (A-E) and sediment (F) samples along the outflow channel of Zhenzhuquan. There were two more sampling sites (4.5 m and 7.5 m downstream) of DO. Error bars in D-F represented the standard deviations of duplicates.

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indices of sediment samples had a decline with remarkable minimums at 3 m (Fig 3). Additionally, in the pool of Zhenzhuquan, richness, diversity and equitability in sediment samples were much higher than those in water samples. However, these indices were distinctly lower in sediment samples than those of water samples downstream. These alpha diversity differences between water and sediment samples can also be seen in their correlation with physical-chemical parameters: Shannon diversity and equitability of water samples were significantly correlated with DO, sulfate, Fe(III)/FeTot, Fe(II), dissolved AsTot, temperature and TOC, while these indices of sediment samples were significantly correlated with DOC, solid FeTot and AsTot in the sediments (Table 2).

Microbial community compositions

Microbial community compositions were distinctly different between water and sediment samples (Fig 4). In the pool, water sample 16S rRNA clone libraries were dominated by Crenarchaeota (48.90 to 93.92%) with the rest mainly composed of Actinobacteria (1.42–36.14%) and Proteobacteria (4.12–14.57%), while sediment sample 16S rRNA clone libraries at -2 m and -1 m were predominated by Proteobacteria (74.36–83.31%). Along the outlet from 0 m to 9 m, Crenarchaeota dominated in water samples gradually declined from 93.92% to 34.79%, which was coupled with slight increase of Acidobacteria (0.07–6.06%), Actinobacteria (1.42–2.80%), Armatimonadetes (0.01–5.13%), Bacteroidetes (0.07–8.18%), Firmicutes (0.22–4.36%) and Proteobacteria (4.12–23.10%). However, initial dominant Crenarchaeota in sediment samples was generally substituted by Chloroflexi along the outflow channel, ranging from 0.13% to 37.76%. There were some unclassified phyla in downstream samples 16S rRNA clone libraries, especially the sediment at 9 m (Fig 4). Generally, Acidobacteria, Armatimonadetes, Bacteroidetes and Chloroflexi positively correlated with DO, ammonia, sulfate, Fe(III)/FeTot, dissolved AsTot and AsTot and negatively correlated with temperature, aqueous Fe(II), solid FeTot and AsTot (S2 Table).

At the genus level, water sample 16S rRNA clone libraries in the pool were composed of the Crenarchaeota Sulfolobus (41.38–85.95%), the Actinobacteria Nocardia (1.17–34.90%) and the Proteobacteria Ralstonia (1.66–4.66%), Delftia (0.51–2.76%) and Acinetobacter (0.58–2.57%) (Fig 5, S3 Table). In contrast, sediment sample 16S rRNA clone libraries in the pool (-2 m and -1 m) dominated by Proteobacteria correspondingly were harbored by more Ralstonia (14.96–19.76%), Delftia (20.13–23.29%), Acinetobacter (4.10–6.99%), Undibacterium (7.84–9.29%) and Pseudomonas (2.89–2.92%), and less Sulfolobus (0.30–8.89%). Along the outflow channel, Sulfolobus in water samples gradually decreased from 85.95% to 24.94%, coupled with elevated Gp3 (0.02–4.22%) of Acidobacteria, Chthonomonas (0.5–5.11%) of Armatimonadetes, Alicyclobacillus (0.02–1.93%) of Firmicutes and Acidicaldus (0.10–4.88%) of Proteobacteria. However, in downstream sediment samples, 73.25–96.21% sequences did not belong to any known genus except for minor Gp3 (0.01–5.05%), Chthonomonas (0.03–10.77%) and Acidicaldus (0.01–4.86%). Most of unknown-genus sequences were from class Thermoprotei (86.48–97.18%) of Crenarchaeota at 0 m and 3 m and class Ktedonobacteria (31.73–37.34%) of Chloroflexi at 6 m and 9 m (S2 Fig). Moreover, at the class level, Thermoprotei was predominant in all water samples and sediment samples at 0 m and 3 m, but replaced by Ktedonobacteria in sediment samples at 6 m and 9 m (S3 Fig). Acidicaldus, Gp3, Acidisoma and Chthonomonas positively correlated with DO, ammonia, sulfate, Fe(III)/FeTot, dissolved AsTot and TOC, and negatively correlated with temperature, aqueous Fe(II) and AsTot (S4 Table).

Microbial community structure statistics

Based on Bray-Curtis dissimilarity at the 97% similarity OTU level, an UPGMA cluster tree of the microbial community populations showed that sediment samples were divided into two
groups (pool and downstream), and distinctly separated from the water samples (Fig 6A). A similar result was also revealed by PCoA analysis with explained 68.5% of the observed variation (Fig 6B). Four complimentary non-parametric multivariate statistical tests including ADONIS, ANOSIM, MRPP and Mantel further confirmed the significant differences of microbial communities between not only pool and downstream samples, but also water and sediment samples (S5 Table). Results of Envfit function indicated that six geochemical parameters were significantly correlated (P<0.05) with microbial community structure along the outlet, including temperature, DO, sulfate, aqueous AsTot, solid TOC and AsTot with R² values of 0.63, 0.53,

![Fig 3. Alpha diversity indices distribution of microbial community structures of water and sediment samples along the outflow channel of Zhenzhuquan. The solid circles and open circles represented sediment and water samples respectively.](image)

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Table 2. Correlation between alpha diversity indices at 97% similarity OTU level and environment factors.

| Environment factors | Water samples | Sediment samples |
|---------------------|---------------|------------------|
|                     | Chao1 | Shannon' diversity | Equitability | Chao1 | Shannon' diversity | Equitability |
| Aqueous phase       |       |                   |              |       |                   |              |
| DO                  | +0.492 | +0.978***         | +0.991***    | -0.284 | -0.201            | -0.134        |
| Sulfate             | +0.511 | +0.911*           | +0.935**     | -0.456 | -0.445            | -0.404        |
| Fe(III)/FeTot       | +0.742 | +0.896*           | +0.829*      | +0.181 | +0.127            | +0.105        |
| Fe(II)              | -0.756 | -0.895*           | -0.829*      | -0.144 | -0.045            | +0.023        |
| AsTot               | -0.655 | -0.952**          | -0.938**     | +0.233 | +0.277            | +0.287        |
| T                   | -0.469 | -0.938**          | -0.939**     | +0.486 | +0.460            | +0.411        |
| DOC                 | -0.448 | -0.221            | -0.122       | -0.717 | -0.857*           | -0.896*       |
| Solid phase         |       |                   |              |       |                   |              |
| FeTot               | -0.218 | +0.031            | +0.107       | -0.679 | -0.895*           | -0.964**      |
| AsTot               | +0.107 | +0.480            | +0.544       | -0.694 | -0.856*           | -0.897*       |
| TOC                 | +0.461 | +0.863*           | +0.896*      | -0.508 | -0.504            | -0.496        |

*p<0.05, **p<0.01, ***p<0.001

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0.62, 0.60, 0.65 and 0.57, respectively (Fig 6C). The similar directions of temperature and aqueous AsTot, and the opposite directions of DO, sulfate and TOC indicated correlations among these variables and did not necessarily suggest that all environmental factors were responsible driving forces of community structure. Significant environmental factors shaping microbial communities were selected according to general physiological niche of microbial populations.

**Discussion**

**Potential As, S and Fe oxidation processes**

In the Zhenzhuquan pool, the remarkably high As(V)/AsTot ratio (0.73–0.86) suggested that As(III) oxidation occurred at the discharge source, which were distinctly different from previous studies that showed As(III) was predominate in the source water of acid sulfate-Cl hot springs, such as Dragon Spring (As(V)/AsTot = 0.05) [11], Beowulf Spring (As(V)/AsTot = 0.04) [10] and Succession Spring (As(V)/AsTot = 0.04) [9] in YNP and Champagne Pool in New Zealand (As(V)/AsTot = 0.04) [1]. The As oxidation in the pool might be mediated microbially. Previous studies demonstrated that sulfide was a potent inhibitor of microbially-mediated As(III) oxidation in acid systems by inactivating expressed As(III) oxidase enzyme (Aio) [2, 4, 13]. Consequently, different from acid sulfate-Cl hot springs with sulfide concentrations of 2.02–12.6 mg/L in those previous studies, the distinctly low concentrations of sulfide (0.03–0.05 mg/L) in the Zhenzhuquan pool allowed for microbial As(III) oxidation. Our previous study on aioA genes had demonstrated the presence of several groups of As(III)-oxidizing microorganisms in the pool, including a few unidentified families of *Aquificae* and some postulated archaea [25]. And in this study, based on 16S rRNA sequences, some microbial populations in sample 16S rRNA clone libraries were also highly similar with bacteria *Pseudomonas*. 

![Microbial community structures distribution of water and sediment samples at phylum level.](https://doi.org/10.1371/journal.pone.0146331.g004)
and *Ralstonia* which were found to be capable of As oxidation in geothermal and other environments [35, 36].

Except for degassing in part, the lack of sulfide (0.03–0.05 mg/L) in the pool was primarily due to microbial sulfide oxidation, suggested by high sulfate concentrations (112.66–120.05 mg/L) and the presence of abundant *Sulfolobus* (41.38–85.95% in water sample libraries), a putative thermoacidophilic sulfur-oxidizing archaeon (Table 1 and Fig 5) [22, 37, 38]. Concurrent with sulfide oxidation, Fe oxidation happened in the outflow channel, indicated by the decline of Fe(II) concentrations and increase of Fe(III)/FeTot (Fig 2). Previous studies displayed that Fe(II) oxidation in acid hot springs was mediated by microorganisms, such as *Metallosphaera* str. MK1, *Sulfobacillus* str. MK2, *Sulfolobus* str. MK3, *Sulfolobales* str. MK5 and *Acidicaldus* str. MK6 [9, 14]. In this study, some microbial populations belonging to *Thermoprotei* (such as *Sulfolobus*, and *Metallosphaera*) and *Acidicaldus* dominated in downstream sample 16S rRNA clone libraries and were probably responsible for Fe(II) oxidation in the outflow channel (Fig 2) [14–16].

Due to lack of sulfide, yellow crystalline elemental S deposit presented in those acid sulfate-Cl hot springs did not appear downstream in this study [1, 9, 11]. Coupled Fe and As concentrations decline in water samples and increase in sediments suggested the co-deposition of As and Fe (Fig 2). However, extremely high As concentrations (up to 16.44 g/kg) and As/Fe mole ratios (2.70–6.72) in the sediments was significantly different from previous results from the acid sulfate-Cl hot springs (As/Fe mole ratios: 0.60 to 0.74) [9–11, 39–41]. Previous studies documented the clay minerals in geothermal areas, such as smectite and kaolinite, could host As concentrations up to 4 g/kg [8, 42–44]. Coincidentally, smectite and kaolinite were also
detected in the downstream sediments of Zhenzhuquan by X-ray diffraction, which suggested that As might be also adsorbed on the clay mineral. Further investigation on mineralogy of the As-rich sediments downstream is warranted.

**Significant environmental factors shaping the microbial community structure**

Generally, water and sediment sample 16S rRNA clone libraries along the outflow channel of Zhenzhuquan were dominated by *Thermoprotei* (mostly comprised of the genus *Sulfolobus*) (Fig 4 and S2 Fig), which was significantly different with sediments or waters from acid sulfate-Cl hot springs, mainly colonized by *Hydrogenobacter, Hydrogenobaculum* and
Sulfurihydrogenibium of Aquificae [1, 9, 12]. This difference was probably derived by their distinct temperature and pH. Hydrogenobacter and Sulfurihydrogenibium favor circumneutral pH and Hydrogenobaculum has an optimal growth temperature of 60–70°C [45, 46], and microaerophilic acidophilic Sulfolobus prefer higher temperature of 65–85°C and more acidic pH of 2–3. These are all conditions similar to the Zhenzhuquan pool above at the 3 m site (DO: 0.23–0.33 mg/L; temperature: 76.2–91.3°C; pH: 3.58–4.33) (Table 1) [22]. This result was also consistent with distribution of Sulfolobus in acidic solfataras and hot springs in YNP [47, 48].

As revealed by non-parametric multivariate statistical analysis, there were significant differences in microbial community structures between not only pool and downstream samples, but also water and sediment samples along the outflow channel (S5 Table). Temperature, DO and TOC significantly shaped microbial community structure of upstream and downstream samples (Fig 6). Responding to the relatively low DO (0.23–0.30 mg/L) and high temperature (76.2–91.3°C) in upstream (Table 1), most microbial populations in 16S rRNA clone libraries were microaerophilic/anaerobic, thermophiles and hyperthermophiles, such as the dominant class Thermoprotei (48.90–97.18%, mainly comprised of Sulfolobus in water samples) [49], Nocardia (34.90%) in the water sample at -1 m [50], Fervidicoccus (4.46%) in sediment sample at 0 m [51], Delftia (20.13–23.29%) andRalstonia (14.96–19.76%) in sediment samples at -1 m and -2 m [52] (Fig 5 and S2 Fig). With a decline in temperature (56.8–49.3°C) and increased DO (2.38–4.74 mg/L) as well as TOC (2.72–2.81%), more aerobic heterotrophic mesophiles and thermophiles correspondingly inhabited downstream samples libraries, such as Ktedonobacteria (31.72–37.34% in sediment samples) [53], Acidobacteria Gp3 (0.91–5.05%) [54], Acidicaldus (1.98–10.48%) [55], Chthonomonas (2.12–10.16%) [56] and Sphingobacteria (4.94–10.40%) [57]. A significant positive correlation between sulfate and TOC (r = 0.989, p < 0.001) along the outlet (Fig 2) implied that sulfide oxidation might be coupled to carbon fixation by facultatively chemooautotrophic archaea, such as above Sulfolobus and unclassified genera within the Thermoprotei, leading to TOC accumulation in downstream sediments [23]. Previous studies demonstrated that microbial community diversity increased with temperature decline and TOC or DOC increase in geothermal environments [21, 22]. However, in this study, the remarkably high As concentrations (up to 16.4 g/kg) and abnormal low alpha diversity in the downstream sediments (much lower than those of water at the same site) suggested that solid AsTot in the sediments probably played a significant role in shaping microbial community structure (Fig 3) [58, 59]. Otherwise, it should be noted that even at the cutoff of 0.07, most sequences from the sediments 16S rRNA clone libraries (72.41–95.91%) could still not be assigned to a known genus, which suggested that some novel species possibly inhabit the high As sediments downstream (Fig 5 and S2 Fig).

Conclusions

Arsenic oxidation mainly occurred in acid sulfate Zhenzhuquan pool with low chloride. Coupled with iron and sulfur oxidation along the outflow channel, arsenic was substantially accumulated in downstream sediments and appeared to significantly constrain their microbial community diversity. Temperature, total organic carbon and dissolved oxygen significantly shaped the different microbial communities between upstream and downstream samples of Zhenzhuquan. Some putative functional microbial populations were possibly related to arsenic oxidation (Aquificae and Pseudomonas), sulfur oxidation (Sulfolobus) and iron oxidation (Sulfolobus, Metallosphaera and Acidicaldus). A total of 72.41–95.91% unassigned-genus sequences in downstream high arsenic sediment 16S rRNA clone libraries probably implied the presence of some novel genera.
Supporting Information

S1 Table. Distribution of alpha diversity indices at the 97% similarity OTU level by re-sampling 12,955 reads in each sample.

S2 Table. Correlation between phylum at 97% similarity OTU level and environment factors. Only phylum significantly correlated with environment factors were displayed.

S3 Table. The relative abundances of all genera in different samples. The unit is %.

S4 Table. Correlation between genera at 97% similarity OTU level and environment factors. Only genera significantly correlated with environment factors were displayed.

S5 Table. Significance tests of microbial community structures between different groups with four different statistical approaches.

S1 Fig. Normalized main cations and anions concentrations (divided by the sum) variation along the outflow channel of Zhenzhuquan. The values in parenthesis were averaged concentrations of ions with a unit of mg/L.

S2 Fig. Distribution of microbial community compositions from water sediment samples at the class level. The ratios which exceeded 0.5% were displayed in this figure.

S3 Fig. Change of main microbial community compositions at class level from water samples (A) and sediment samples (B) along the outflow channel of Zhenzhuquan.

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Author Contributions

Conceived and designed the experiments: ZJ PL YXW. Performed the experiments: ZJ PL DJ. Analyzed the data: ZJ PL. Contributed reagents/materials/analysis tools: ZJ PL XD RZ YHW. Wrote the paper: ZJ PL YXW.

References

1. Hug K, Maher WA, Stott MB, Krikowa F, Foster S, Moreau JW. Microbial contributions to coupled arsenic and sulfur cycling in the acid-sulfide hot spring Champagne Pool, New Zealand. Front Microbiol. 2014 Nov; 5:569. doi: 10.3389/fmicb.2014.00569 PMID: 25414696

2. Clingenpeel SR, D’Imperio S, Oduro H, Druschel GK, McDermott TR. Cloning and in situ expression studies of the Hydrogenobaculum arsenite oxidase genes. Appl Environ Microbiol. 2009 May; 75(10):3362–3365. doi: 10.1128/AEM.00336-09 PMID: 19304831

3. D’Imperio S, Lehr CR, Oduro H, Druschel G, Kuhl M, McDermott TR. Relative importance of H₂ and H₂S as energy sources for primary production in geothermal springs. Appl Environ Microbiol. 2008 Sep; 74(18):5802–5808. doi: 10.1128/AEM.00852-08 PMID: 18641166
4. D’Imperio S, Lehr CR, Breary M, McDermott TR. Autecology of an arsenite chemolithotroph: sulfide constraints on function and distribution in a geothermal spring. Appl Environ Microbiol. 2007 Nov; 73(21):7067–7074. PMID: 17827309

5. Ogawa Y, Ishiyama D, Shikazono N, Iwane K, Kajiwara M, Tsuchiya N. The role of hydrous ferric oxide precipitation in the fractionation of arsenic, gallium, and indium during the neutralization of acidic hot spring water by river water in the Tama River watershed, Japan. Geochim Cosmochim Acta. 2012 Jun; 86:367–383.

6. McCleskey RB, Nordstrom DK, Maest AS. Preservation of water samples for arsenic(III/V) determinations: an evaluation of the literature and new analytical results. Appl Geochem. 2004 Mar; 19(7):995–1009.

7. López DL, Bundschuh J, Birkle P, Armienta MA, Cumal B, Sracek O, et al. Arsenic in volcanic geothermal fluids of Latin America. Sci Total Environ. 2012 Jul; 429:57–75. doi: 10.1016/j.scitotenv.2011.08.043 PMID: 22825066

8. Birkle P, Bundschuh J, Sracek O. Mechanisms of arsenic enrichment in geothermal and petroleum reservoirs fluids in Mexico. Water Res. 2010 Nov; 44(19):5605–5617. doi: 10.1016/j.watres.2010.05.046 PMID: 20691459

9. Macur R, Langner H, Kocar B, Inskeep W. Linking geochemical processes with microbial community analysis: successional dynamics in an arsenic-rich, acid-sulphate-chloride geothermal spring. Geobiology. 2004 Jul; 2(3):163–177.

10. Inskeep WP, Macur RE, Harrison G, Bostick BC, Fendorf S. Biominalization of As(V)-hydrous ferric oxyhydroxide in microbial mats of an acid-sulfate-chloride geothermal spring, Yellowstone National Park. Geochim Cosmochim Acta. 2004 Aug; 68(15):3141–3155.

11. Langner HW, Jackson CR, McDermott TR, Inskeep WP. Rapid oxidation of arsenite in a hot spring ecosystem, Yellowstone National Park. Environ Sci Technol. 2001 Jul; 35(16):3302–3309. PMID: 11529568

12. Jackson CR, Langner HW, Donahoe-Christiansen J, Inskeep WP, McDermott TR. Molecular analysis of microbial community structure in an arsenite-oxidizing acid thermal spring. Environ Microbiol. 2001 Aug; 3(6):532–542. PMID: 11578314

13. Donahoe-Christiansen J, D’Imperio S, Jackson CR, Inskeep WP, McDermott TR. Arsenite-oxidizing Hydrogenobaculum strain isolated from an acid-sulfate-chloride geothermal spring in Yellowstone National Park. Appl Environ Microbiol. 2004 Mar; 70(3):1865–1868. PMID: 15006819

14. Kozubal MA, Macur RE, Jay ZJ, Beam JP, Malfatti SA, Tringe SG, et al. 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. 2012 Mar; 3:109. doi: 10.3389/fmicb.2012.00109 PMID: 22470372

15. Jay ZJ, Rusch DB, Tringe SG, Bailey C, Jennings RM, Inskeep WP. Predominant Acidilobus-like populations from geothermal environments in Yellowstone National Park exhibit similar metabolic potential in different hypoxic microbial communities. Environ Microbiol. 2014 Jan; 80(1):294–305. doi: 10.1128/AEM.02860-13 PMID: 24162572

16. Kozubal M, Macur RE, Korf S, Taylor WP, Ackerman GG, Nagy A, et al. Isolation and distribution of a novel iron-oxidizing crenarchaeon from acidic geothermal springs in Yellowstone National Park. Appl Environ Microbiol. 2006 Feb; 74(4):942–949. PMID: 18083951

17. Guo QH, Liu ML, Li JX, Zhang XB, Wang YX. Acid hot springs discharged from the Rehai hydrothermal system of the Tengchong volcanic area (China): formed via magmatic fluid absorption or geothermal steam heating? Bull Volcanol. 2014 Oct; 76(10):868.

18. Guo Q. Hydrogeochemistry of high-temperature geothermal systems in China: A review. Appl Geochem. 2012 Oct; 27(10):1887–1898.

19. Guo Q, Wang Y. Geochemistry of hot springs in the Tengchong hydrothermal areas, Southwestern China. J Volcanol Geoth Res. 2012 Feb; 215:61–73.

20. Mandal BK, Suzuki KT. Arsenic round the world: a review. Talanta. 2002 Aug; 58(1):201–235. PMID: 18968746

21. Wang S, Dong H, Hou W, Jiang H, Huang Q, Briggs BR, et al. Greater temporal changes of sediment microbial community than its waterborne counterpart in Tengchong hot springs, Yunnan Province, China. Sci Rep. 2014 Dec; 4:7479. doi: 10.1038/srep07479 PMID: 25524763

22. Hou W, Wang S, Dong H, Jiang H, Briggs BR, Peacock JP, et al. A comprehensive census of microbial diversity in hot springs of tengchong, Yunnan Province China using 16S rRNA gene pyrosequencing. PLoS One. 2013 Jan; 8(1):e53350. doi: 10.1371/journal.pone.0053350 PMID: 23326417

23. Inskeep WP, Jay ZJ, Tringe SG, Herrgard MJ, Rusch DB, Committee YNPMP, et al. The YNP metagenome project: environmental parameters responsible for microbial distribution in the Yellowstone
geothermal ecosystem. Front Microbiol. 2013 May; 4:67. doi: 10.3389/fmicb.2013.00067 PMID: 23653623

24. Hedlund BP, Cole JK, Williams AJ, Hou W, Zhou E, Li W, et al. A review of the microbiology of the Rehai geothermal field in Tengchong, Yunnan Province, China. Geosci Front. 2012 May; 3(3):273–288.

25. Jiang Z, Li P, Jiang D, Wu G, Dong H, Wang Y, et al. Diversity and abundance of the arsenite oxidase gene aioA in geothermal areas of Tengchong, Yunnan, China. Extremophiles. 2014 Jan; 18(1):161–170. doi: 10.1007/s00792-013-0608-7 PMID: 24292445

26. Le XC, Yalcin S, Ma MS. Speciation of submicrogram per liter levels of arsenic in water: On-site species separation integrated with sample collection. Environ Sci Technol. 2000 Jun; 34(11):2342–2347.

27. Jiang Z, Li P, Jiang D, Wu G, Dong H, Wang Y, et al. Diversity and abundance of the arsenite oxidase gene aioA in geothermal areas of Tengchong, Yunnan, China. Extremophiles. 2014 Jan; 18(1):161–170. doi: 10.1007/s00792-013-0608-7 PMID: 24292445

28. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global patterns of 16S rRNA diversity at depth of millions of sequences per sample. Proc Natl Acad Sci U S A. 2011 Mar; 108:4516–4522. doi: 10.1073/pnas.1000080107 PMID: 20534432

29. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 2012 Aug; 6(8):1621–1624. doi: 10.1038/ismej.2012.8 PMID: 22402401

30. Kong Y. Btrim: A fast, lightweight adapter and quality trimming program for next-generation sequencing technologies. Genomics. 2011 Aug; 98(2):152–153. doi: 10.1016/j.ygeno.2011.05.009 PMID: 21651976

31. Magoc T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 2011 Nov; 27(21):2957–2963. doi: 10.1093/bioinformatics/btr507 PMID: 21903629

32. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010 Oct; 26(19):2460–2461. doi: 10.1093/bioinformatics/btq461 PMID: 20709691

33. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007 Aug; 73(16):5261–5267. PMID: 17586664

34. Cavalca L, Corsini A, Zaccheo P, Andreoni V, Muyzer G. Microbial transformations of arsenic: perspectives for biological removal of arsenic from water. Future Microbiol. 2013 Jun; 8(6):753–768. doi: 10.2217/fmb.13.38 PMID: 23586329

35. Macur RE, Jay ZJ, Taylor WP, Kozubal MA, Kocar BD, Inskeep WP. Microbial community structure and sulfur biogeochemistry in mildly-acidic sulfidic geothermal springs in Yellowstone National Park. Geobiology. 2013 Jan; 11(1):86–99. doi: 10.1111/gbi.12015 PMID: 23231658

36. Xiang X, Dong X, Huang L. Sulfolobus tengchongensis sp. nov., a novel thermoacidophilic archaeon isolated from a hot spring in Tengchong, China. Extremophiles. 2003 Dec; 7(6):493–498. PMID: 12955604

37. Drahota P, Filippi M. Secondary arsenic minerals in the environment: a review. Environ Int. 2009 Nov; 35(8):1243–1255. doi: 10.1016/j.envint.2009.07.004 PMID: 19665230

38. Song J, Jia SY, Yu B, Wu SH, Han X. Formation of iron (hydr)oxides during the abiotic oxidation of Fe (II) in the presence of arsenate. J Hazard Mater. 2015 Aug; 294:70–79. doi: 10.1016/j.jhazmat.2015.03.048 PMID: 25855615

39. Ford RG. Rates of hydrous ferric oxide crystallization and the influence on coprecipitated arsenate. Environ Sci Technol. 2002 Jun; 36(11):2459–2463. PMID: 12075804

40. Pascua C, Charnock J, Polya D, Sato T, Yokoyama S, Minato M. Arsenic-bearing smectite from the geothermal environment. Mineral Mag. 2005 Oct; 69(5):897–906.

41. Pascua C, Sato T, Golla G. Mineralogical and geochemical constraints on arsenic mobility in a Philippine geothermal field. Acta Geol Sin-Engl. 2006; 80(2):230–235.

42. Ilgen AG, Rychagov SN, Trainor TP. Arsenic speciation and transport associated with the release of spent geothermal fluids in Mutnovsky field (Kamchatka, Russia). Chem Geol. 2011 Sep 25; 288(3–4):115–132.
45. Hedlund BP, Reysenbach AL, Huang L, Ong JC, Liu Z, Dodsworth JA, et al. Isolation of diverse members of the Aquificales from geothermal springs in Tengchong, China. Front Microbiol. 2015 Feb; 6:157. doi: 10.3389/fmicb.2015.00157 PMID: 25774153

46. Briggs BR, Brodie EL, Tom LM, Dong HL, Jiang HC, Huang QY, et al. Seasonal patterns in microbial communities inhabiting the hot springs of Tengchong, Yunnan Province, China. Environ Microbiol. 2014 Jun; 16(6):1579–1591. doi:10.1111/1462-2920.12311 PMID: 24148100

47. Inskeep WP, Jay ZJ, Herrgard MJ, Kozubal MA, Rusch DB, Tringe SG, et al. Phylogenetic and functional analysis of metagenome sequence from high-temperature archaeal habitats demonstrate linkages between metabolic potential and geochemistry. Front Microbiol. 2013 May; 4:95. doi:10.3389/fmicb.2013.00095 PMID: 23720654

48. Whitaker RJ, Grogan DW, Taylor JW. Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science. 2003 Aug; 301(5635):976–978. PMID:12881573

49. Garrity GM, Holt JG, Reysenbach A-L, Huber H, Stetter KO, Zillig W, et al. Phylum Al. Crenarchaeota phy. nov. Bergey’s Manual of Systematic Bacteriology: Springer; 2001. pp. 169–210.

50. Song Z, Zhi X, Li W, Jiang H, Zhang C, Dong H. Actinobacterial diversity in hot springs in Tengchong (China), Kamchatka (Russia), and Nevada (USA). Geomicrobiol J. 2009 May; 26(4):256–263.

51. Perevalova AA, Bidzhieva SK, Kublanov IV, Hinrichs K-U, Liu XL, Mardanov AV, et al. Fervidicoccus fontis gen. nov., sp. nov., an anaerobic, thermophilic crenarchaeote from terrestrial hot springs, and proposal of Fervidicoccaceae fam. nov. and Fervidicoccales ord. nov. Int J Syst Evol Microbiol. 2010 Sep; 60(9):2082–2088.

52. Aminin ALN, Warganegara FM, Aditiawati P. Culture-Independent and culture-dependent approaches on microbial community analysis at Gedongsongo (GS-2) hot spring. Int J Integr Biol. 2008 May; 2(3):145–152.

53. King CE, King GM. Description of Thermogemmatispora carboxidivorans sp. nov., a carbon-monoxide-oxidizing member of the class Ktedonobacteria isolated from a geothermally heated biofilm, and analysis of carbon monoxide oxidation by members of the class Ktedonobacteria. Int J Syst Evol Microbiol. 2014 Apr; 64(Pt 4):1244–1251. doi:10.1099/ijs.0.059675-0 PMID: 24425739

54. Barns SM, Cain EC, Sommerville L, Kuske CR. Acidobacteria phylum sequences in uranium-contaminated subsurface sediments greatly expand the known diversity within the phylum. Appl Environ Microbiol. 2007 May; 73(9):3113–3116. PMID: 17337544

55. Johnson DB, Stallwood B, Kimura S, Hallberg KB. Isolation and characterization of Acidicaldus orginovorus, gen. nov., sp. nov.: a novel sulfur-oxidizing, ferric iron-reducing thermo-acidophilic heterotrophic Proteobacterium. Arch Microbiol. 2006 Apr; 185(3):212–221. PMID: 16432746

56. Lee KC-Y, Dunfield PF, Morgan XC, Crowe MA, Houghton KM, Vyssotski M, et al. Chthonomonas calidirosea gen. nov., sp. nov., an aerobic, pigmented, thermophilic micro-organism of a novel bacterial class, Chthonomonadetes classis nov., of the newly described phylum Armatimonadetes originally designated candidate division OP10. Int J Syst Evol Microbiol. 2011 Oct; 61(10):2482–2490.

57. Song Z-Q, Wang F-P, Zhi X-Y, Chen J-Q, Zhou E-M, Liang F, et al. Bacterial and archaeal diversities in Yunnan and Tibetan hot springs, China. Environ Microbiol. 2013 Apr; 15(4):1160–1175. doi: 10.1111/1462-2920.12025 PMID: 23126508

58. Wang Y, Li P, Li B, Webster G, Weightman AJ, Jiang Z, et al. Bacterial diversity and community structure in high arsenic aquifers in Hetao Plain of Inner Mongolia, China. Geomicrobiol J. 2014 Feb; 31(4):338–349.

59. Li P, Wang Y, Jiang Z, Jiang H, Li B, Dong H, et al. Microbial diversity in high arsenic groundwater in Hetao Basin of Inner Mongolia, China. Geomicrobiol J. 2013 Aug; 30(10):897–909.