Seasonal patterns in microbial communities inhabiting the hot springs of Tengchong, Yunnan Province[†], China

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Summary

Studies focusing on seasonal dynamics of microbial communities in terrestrial and marine environments are common; however, little is known about seasonal dynamics in high-temperature environments. Thus, our objective was to document the seasonal dynamics of both the physicochemical conditions and the microbial communities inhabiting hot springs in Tengchong County, Yunnan Province, China. The PhyloChip microarray detected 4882 operational taxonomic units (OTUs) within 79 bacterial phylum-level groups and 113 OTUs within 20 archaeal phylum-level groups, which are additional 54 bacterial phyla and 11 archaeal phyla to those that were previously described using pyrosequencing. Monsoon samples (June 2011) showed increased concentrations of potassium, total organic carbon, ammonium, calcium, sodium and total nitrogen, and decreased ferrous iron relative to the dry season (January 2011). At the same time, the highly ordered microbial communities present in January gave way to poorly ordered communities in June, characterized by higher richness of *Bacteria*, including microbes related to mesophiles. These seasonal changes in geochemistry and community structure are likely due to high rainfall influx during the monsoon season and indicate that seasonal dynamics occurs in high-temperature environments experiencing significant changes in seasonal recharge. Thus, geothermal environments are not isolated from the surrounding environment and seasonality affects microbial ecology.

Introduction

The effect of seasonality on the microbial community structure in high-temperature geothermal environments is less obvious than for many other environments possibly because the physicochemical conditions of the source water were assumed to be stable with no significant seasonal changes. However, few studies have examined the effect of seasonality on hot spring environments (Ferris and Ward, 1997; Norris *et al.*, 2002; Lacap *et al.*, 2007; Mackenzie *et al.*, 2013). For example, in temperate ecosystems such as Yellowstone National Park (YNP), seasonal variations were not identified in phototrophic microbial mats and spring geochemistry from circumneutral hot springs (Ferris and Ward, 1997), even when light intensity was manipulated (Norris *et al.*, 2002). However, increased light during the summer increased the abundance and activity of eukaryotic algae in acidic hot springs (Lehr *et al.*, 2007). Similarly, in a tropical Philippines hot spring ecosystem, increased abundance of *Chloroflexi* and *Synechococcus*, and increased phosphate concentration were observed during the summer

monsoonal rains (Lacap *et al.*, 2007). Minor microbial and geochemical changes were also observed seasonally in phototrophic mat communities in hot springs from Patagonia (Mackenzie *et al.*, 2013). While these results conflict to some degree, they do provide evidence that phototrophic mats respond to the seasonal environment in some hot spring ecosystems.

The earlier studies provide a framework for seasonal dynamics of phototrophic mats; however, the seasonality of microbes that exist in the water and sediment of hot spring ecosystems have not been studied. In addition, the earlier studies have focused on a few springs with temperature ranges of 40–75°C and mostly circumneutral pH (Lehr *et al.*, 2007 is an exception at a pH of 2–4). Thus, questions remain as to whether sediment and water (planktonic) communities display seasonal dynamics and if geochemically distinct hot springs respond differently to seasonal changes.

Tengchong County, Yunnan Province is a subtropical climate and is known for its geothermal features (Liao and Guo, 1986; Liao *et al.*, 1986). The county contains over 50 volcanoes and 140 geothermal features (Du *et al.*, 2005), several of which have been the focus of microbiology studies. Culture-dependent methods have resulted in isolation of novel thermophiles (Xiang *et al.*, 2003; Wang *et al.*, 2012), and culture-independent methods have identified moderately diverse microbial communities (Huang *et al.*, 2010; Jiang *et al.*, 2010; Song *et al.*, 2010; 2013; Hedlund *et al.*, 2012; Pagaling *et al.*, 2012; Hou *et al.*, 2013). Hou and colleagues (2013) performed an extensive 16S rRNA gene pyrotag survey of sediment and water microbial communities from 11 hot springs in the Rehai geothermal field and the two largest geothermal sources in Dientan (formerly known as Ruidian). These reports show that temperature, pH and sediment/water geochemistry are the major conditions that control the moderately diverse array of bacterial and archaeal communities in these springs.

Tengchong hot springs are an ideal place to improve our understanding of how physicochemical conditions and microbial communities respond to seasons. The rainfall in Tengchong increases from on average 30 mm per month for winter to 150 mm per month during the summer monsoon season. We hypothesized that the chemistry of hot springs in Tengchong experiences seasonal variation because of increased rainfall in the summer months and that the microbial community responds to those changes. To test this hypothesis, a study of geochemistry was combined with a cultivation-independent study of sediment and water communities using a 16S rRNA gene microarray (the PhyloChip) on samples obtained from a large number of Tengchong hot springs (temperature 53–93°C; pH 2.5–9.5) collected during the subtropical dry season (January 2011) and the summer monsoon season (June 2011).

Results

Site description

Samples were collected in January and June of 2011 (Table 1) from the Dientan and Rehai geothermal areas (Fig. 1). Dientan is a carbonate-hosted system and characterized as Na-HCO$_3$ springs (Zhang *et al.*, 1987; 2008b). Both springs sampled in Dientan, Gongxiaoshe and Jinze were large pools with standing water surrounded by retaining walls. These and other springs in Dientan are pumped for showering and cooking by local residents. A roof covered Jinze. Rehai is a granite-hosted system characterized by neutral-alkaline, Na-Cl-HCO$_3$ or Na-HCO$_3$-Cl springs (Zhang *et al.*, 1987; 2008b). Five circumneutral springs were sampled in Rehai. Jiemeiquan and Gumingquan were small high discharge springs that contained pink streamers. Direchi spring was shallow with multiple sources. Huitaijing was comprised of two small open wells, and Shuirebaozha had a variety of geothermal sources. The acidic springs in Rehai represented vapour condensate-dominated systems that were acidified by oxidation of sulphide to sulphuric acid (Hou *et al.*, 2013). Samples were collected from several acidic springs that were shallow with relatively low discharge. Diretiyanqu is a small acidic site located on a fault just under a cliff. Tourists are encouraged to 'experiment' with this site by placing eggs and other food in the springs, thus the name 'experimental site'. Zhenzhuquan had a wall constructed around it and exhibits strong outgassing from multiple sources. Meteoric water is the main source for all springs (Shen *et al.*, 2011; Hou *et al.*, 2013).

Geochemistry

Geochemical data associated with samples from January have been described previously (Hou *et al.*, 2013), while the geochemical conditions in June are reported in this study (Table 1 and S1). For January, the pH ranged from 2.5 to 9.4, and temperature ranged between 64.5 and 93.6°C. In June, the range in pH was similar, while temperature varied between 53.0 and 91.7°C. The acidic springs typically had higher concentrations of ferrous iron, total iron and sulphate, typical of springs sourced by condensed hydrothermal vapor (Nordstrom *et al.*, 2009), while the circumneutral to alkaline springs typically had higher fluorine, bromine, sodium and chloride concentrations.

Differences in geochemistry were observed between January and June samples. June water samples contained higher concentrations of potassium, calcium, ammonia, sodium and dissolved organic carbon (DOC), while June sediment samples contained higher concentrations of total nitrogen and total organic carbon (TOC) (Fig. 2). In addition, water δ^{18}O values were higher in June

Table 1. Sample names, date sampled, sample type, select physicochemical parameters of spring water and spring descriptions.

Site	Spring	Month sampled	Sample type	pH	Temp °C	Descriptions
Drty-1	Diretiyanqu	January, June	Water, Sediment	2.6, 3.2	85.1, 87.8	length ~ 63 cm, width ~ 56 cm, depth ~ 6.5 cm. Fine silicate sand sediment
Drty-2	Diretiyanqu	January, June	Water, Sediment	2.6, 2.8	64.5, 66.0	diameter ~ 50 cm, depth 10.5 cm. Fine silicate sand sediment
Drty-3	Diretiyanqu	January, June	Water, sediment	2.5, 2.7	55.1, 53.0	length ~ 51 cm, width ~ 26 cm, depth ~ 5.5 cm. Fine silicate sand sediment
Drty-4	Diretiyanqu	June	Sediment	2.6	73.0	diameter ~ 60 cm, Fine silicate sand sediment
GmqS	Gumingquan	January	Water, sediment	9.4	93.0	length ~ 98 cm, width ~ 79 cm, depth ~ 9.5 cm, Silicate sand sediment
GmqP	Gumingquan	June	Water, sediment	9.4	83.5	depth ~ 16 cm. Fine silicate sand sediment
GxsB	Gongxiaoshe	January, June	Water, sediment	7.3, 7.7	73.8, 75.0	Constructed diameter ~ 297 cm, depth 130 cm. Soft cream-coloured sediment containing carbonate
GxsS	Gongxiaoshe	January	Water, sediment	7.3	73.8	Similar to GxsB
HtjL	Huitaijing	January	Water	8.1	90.0	Constructed diameter ~ 65 cm, depth ~63 cm
HtjR	Huitaijing	January	Water	8.1	92.3	Similar to HtjL
JmqL	Jiemeiquan	January, June	Water, sediment	9.3, 9.0	93.6, 84.7	Length and width ~ 100 cm, depth ~ 9.5 cm. Clay and sand sediment
JmqR	Jiemeiquan	January, June	Water, sediment	9.4, 9.0	83.2, 84.7	length ~950 cm, width ~ 100 cm, depth ~ 4.5 cm. Clay and sand sediment
Jz	Jinze	January, June	Water, sediment	6.7, 7.0	81.6, 80.7	Constructed length ~ 127 cm, width ~ 134 cm, depth ~ 103 cm. Black mud sediment
Drc-1	Direchi	June	Sediment	8.3	74.3	Length ~ 2 m, width ~ 6 m. Muddy sediment
Drc-2	Direchi	June	Sediment	8.4	69.4	Similar to Drc-1
Drc-3	Direchi	June	Water, sediment	8.6	61.2	Similar to Drc-1
Drc-4	Direchi	June	Water, sediment	8.3	83.0	Dimensions similar to Drc-1, sandy sediment
SrbzD	Shuirebaozha	June	Water, sediment	8.3	72.1	Length ~ 300 cm, width ~ 240 cm. Grey mud sediment
ZzqR	Zhenzhuquan	January	Water, sediment	4.8	89.1	Constructed heart-shaped pool. Longest dimension 436 cm, depth ~ 7 cm. Sillicate sand sediment
ZzqL	Zhenzhuquan	June	Water, sediment	4.4	91.7	Similar to ZzqR

Additional geochemical data are presented in Table S1.

(Fig. 2). In contrast, concentrations of ferrous iron decreased in June for most springs. Hierarchical clustering of sites based on the change in the geochemistry indicated that acidic sites (Diretiyanqu) responded to seasonal changes differently than circumneutral and alkaline springs (Jinze, Gongxiaoshe, Jiemeiquan). For example, from January to June chloride and phosphate concentrations increased in the acidic springs but decreased or did not change in the circumneutral and alkaline springs.

Microbial diversity

The PhyloChip detected a total of 4882 bacterial operational taxonomic units (OTUs) from 79 phyla and 113 archaeal OTUs from 20 phyla in the 45 samples collected in January and June. The number of OTUs ranged from 34 to 1102 per sample. Many of the detected phyla (42 bacterial and 10 archaeal phyla) were candidate phyla with no cultured representatives. The 22 samples collected in January contained 1966 bacterial OTUs from 61 phyla and 76 archaeal OTUs from 19 phyla, while the 23 sites collected in June contained 4260 bacterial OTUs from 79 phyla and 94 archaeal OTUs from 19 phyla.

A subset of 10 samples that were collected in January was resampled in June and used to identify the temporal changes in the community structure and composition. The 10 January samples contained 1397 bacterial OTUs from 57 phyla and 70 archaeal OTUs across 18 phyla. The 10 June samples had significantly higher bacterial richness than January, with 2224 bacterial OTUs within 53 phyla ($P < 0.05$); however, archaeal richness was slightly lower in June, with 55 OTUs within 14 phyla.

Seasonal variations

Non-metric multidimensional scaling (NMDS) was used to visualize the community structure and correlated physicochemical conditions. The microbial community in January was highly structured with significant correlations to temperature, ferrous iron and magnesium concentrations ($r^2 = 0.38$, 0.34 and 0.28 respectively; $P < 0.05$; Fig. 3A). Three groups of samples were identified in the January NMDS (Fig. 3A). Group 1 contained mainly low pH and low temperature water and sediment samples from Diretiyanqu, and grouped along the ferrous iron vector because these acidic springs also had the highest
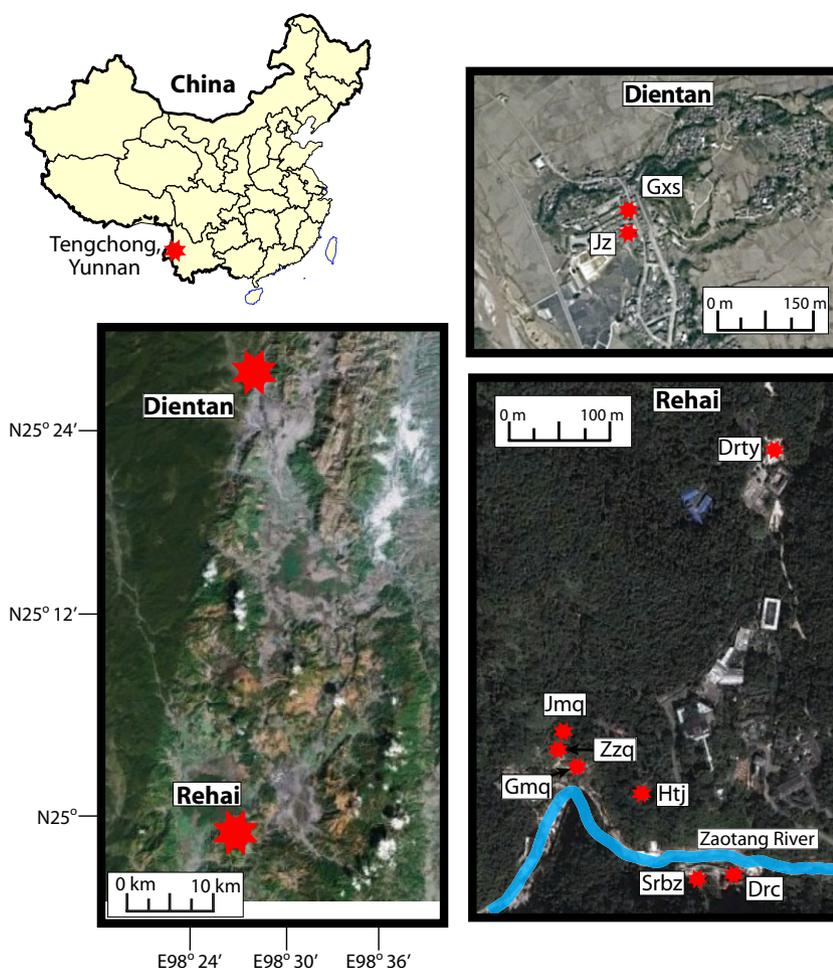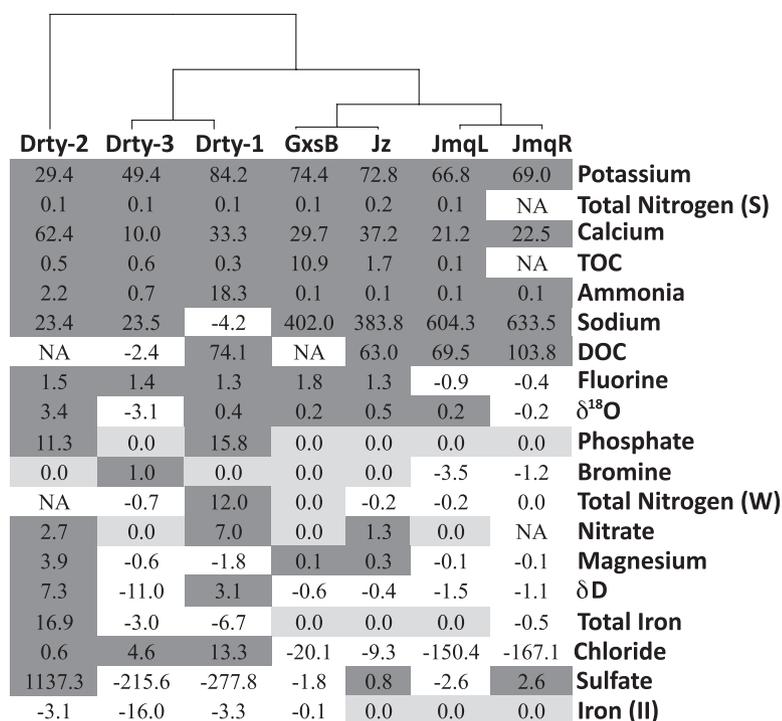
Fig. 1. A map of geothermal springs sampled in Tengchong, Yunnan, China, modified from Hou and colleagues (2013). Dientan was formerly known as Ruidian. Gxs, Gongxiaoshe; Jz, Jinze; Drty, Diretiyanqu; Jmq, Jiemeiquan; Zzq, Zhenzhuquan; Gmq, Gumingquan; Htj, Huitaijing; Srbz, Shuirebaozha; Drc, Direchi.

ferrous iron concentration (Table S1). Group 2 contained sediment samples mostly from Dientan; however, Jiemeiquan-R from Rehai was present in this group as well. Group 2 typically had higher magnesium concentrations. Group 3 was the largest and most diverse group. Most samples in Group 3 had neutral or alkaline pH; however, acidic Diretiyanqu-1 and Zhenzhuquan-R were present in Group 3 (Table 1). Among the acidic springs sampled, both Diretiyanqu-1 and Zhenzhuquan-R had the highest temperatures (85 and 89°C respectively), and their position along the temperature vector indicates a stronger influence of temperature than pH on microbial community structure in these acidic springs in January.

In June, the microbial community was not highly structured, and no clear grouping of samples was observed. Also unlike January, seven geochemical parameters were significantly correlated ($P < 0.05$) to community structure in June: ferrous iron, pH, bromide, sodium, chloride, sulphate and fluorine ($r^2 = 0.58, 0.56, 0.52, 0.41, 0.37, 0.34$ and 0.34 respectively) (Fig. 3B). The similar direction of vectors for pH, sodium, chloride, fluorine and bromine,

and the opposite direction of ferrous iron and sulphate indicate co-correlations among these variables and do not necessarily suggest that all are environmentally relevant driving forces of community structure. Temperature was not significantly correlated to community structure but instead pH was significant. Diretiyanqu-1 and Zhenzhuquan-L grouped more closely with other low pH springs instead with the high temperature springs, suggesting a shift in the control of the community structure for these sites.

The microbial community changed significantly from January to June in most springs; however, three sediment sites (Diretiyanqu-3, Jiemeiquan-L and Jiemeiquan-R) did not significantly change ($P > 0.05$). To depict the change of OTUs (family level) from January to June for the 10 paired samples, a canonical correspondence analysis (CCA) ordination was performed (Fig. 4). Axis one represents an artificial gradient in time where OTUs that were more abundant in January are to the left and OTUs that were more abundant in June are to the right. Extreme values along axis one depict OTUs that were only found during one season, while OTUs placed in the middle were

	Drty-2	Drty-3	Drty-1	GxsB	Jz	JmqL	JmqR	
	29.4	49.4	84.2	74.4	72.8	66.8	69.0	**Potassium**
	0.1	0.1	0.1	0.1	0.2	0.1	NA	**Total Nitrogen (S)**
	62.4	10.0	33.3	29.7	37.2	21.2	22.5	**Calcium**
	0.5	0.6	0.3	10.9	1.7	0.1	NA	**TOC**
	2.2	0.7	18.3	0.1	0.1	0.1	0.1	**Ammonia**
	23.4	23.5	-4.2	402.0	383.8	604.3	633.5	**Sodium**
	NA	-2.4	74.1	NA	63.0	69.5	103.8	**DOC**
	1.5	1.4	1.3	1.8	1.3	-0.9	-0.4	**Fluorine**
	3.4	-3.1	0.4	0.2	0.5	0.2	-0.2	**δ^{18}O**
	11.3	0.0	15.8	0.0	0.0	0.0	0.0	**Phosphate**
	0.0	1.0	0.0	0.0	0.0	-3.5	-1.2	**Bromine**
	NA	-0.7	12.0	0.0	-0.2	-0.2	0.0	**Total Nitrogen (W)**
	2.7	0.0	7.0	0.0	1.3	0.0	NA	**Nitrate**
	3.9	-0.6	-1.8	0.1	0.3	-0.1	-0.1	**Magnesium**
	7.3	-11.0	3.1	-0.6	-0.4	-1.5	-1.1	**δD**
	16.9	-3.0	-6.7	0.0	0.0	0.0	-0.5	**Total Iron**
	0.6	4.6	13.3	-20.1	-9.3	-150.4	-167.1	**Chloride**
	1137.3	-215.6	-277.8	-1.8	0.8	-2.6	2.6	**Sulfate**
	-3.1	-16.0	-3.3	-0.1	0.0	0.0	0.0	**Iron (II)**

Fig. 2. Unweighted pair group method with arithmetical mean hierarchal clustering of sites based on the change in geochemistry from January to June using the Euclidean distance measure. The values depict the difference between June and January. Values highlighted in black increased in June and values not highlighted decreased in June. S, sediment; W, water.

found in both January and June. Axis two was not constrained, and similar to an NMDS shows the overall variability within the artificial temporal gradient. Archaeal OTUs were predominantly placed to the left, demonstrating that most archaeal OTUs were more abundant in January. Three archaeal OTUs representing three phyla and 42 bacterial OTUs representing 12 phyla were detected only in January (Table S2). All archaeal OTUs detected in June were also detected in January; however, 198 bacterial OTUs from 18 phyla were detected only in June (Table S3). Many of these latter groups of bacterial OTUs are often associated with mammal and faecal environments (80 OTUs). For example, many of these OTUs are common inhabitants on human skin (Grice *et al.*, 2008) or in mammal faeces (Ley *et al.*, 2008) (i.e. *Sphingomonas*, *Roseococcaceae*, *Clostridia*, *Oxalobacteraceae*).

In January, thermoacidopilic organisms such as *Hydrogenothermus*, *Acidianus*, *Sulfobacilli* and *Aciduliprofundaceae* were primarily detected in acidic sites (Diretiyanqu) (Table 2). In June, Diretiyanqu-3 still contained thermoacidophilic microorganisms; however, Diretiyanqu-1 and Diretiyanqu-2 contained non-thermophilic organisms (i.e. *Sphingomonas*, and *Oxalobacteraceae*). Similarly, the Jiemeiquan sites contained thermophilic organisms in January but shifted to non-thermophilic, organisms such as *Sphingomonas* and *Clostridia* in June (Table 2). The Dientan sites, Gongxiaoshe and Jinze, contained candidate phyla EM19, GN02, OP9, OP8 and SAGMEG-1 in both January and June.

Distribution of Aquificae and Crenarchaeota

A prior study that used pyrosequencing identified either *Aquificae* or *Crenarchaeota* as the dominant groups of organisms in Rehai hot springs (Hou *et al.*, 2013). NMDS ordination was performed to depict similarities in taxa distributions within these phyla and the correlated geochemical parameters for both sampling seasons (Fig. 5). Three genera of *Aquificae* were detected: *Hydrogenobacter*, *Hydrogenobaculum* and *Hydrogenothermus*. Consistent with an earlier report using pyrosequencing, *Thermocrinis* was not detected by PhyloChip (Hou *et al.*, 2013). *Hydrogenobaculum* formed a distinct group and separated from *Hydrogenobacter* and *Hydrogenothermus* along axis 1. Parameters that were correlated to axis 1 were pH ($r^2 = 0.58$) and analytes that are typically abundant in acidic springs (i.e. ferrous iron, total iron and sulphate; $r^2 = 0.51$, 0.71 and 0.80 respectively; Fig. 5A). *Hydrogenobacter* and *Hydrogenothermus* distribution was positively correlated with fluorine, sodium and chloride concentrations ($r^2 = 0.30$, 0.28 and 0.26 respectively). One OTU of *Hydrogenobaculum* (accession #EF156469) was found in the alkaline springs Gumingquan, Shuirebaozha and Direchi, which typically had higher nitrate, ammonium and phosphate concentrations. Temperature was not a significant parameter as all three genera were detected across the full range of temperatures sampled (53–93°C).

Within the *Crenarchaeota*, the orders *Thermoproteales*, *Desulfurococcales*, candidate group CH2a62 and

A

January

B
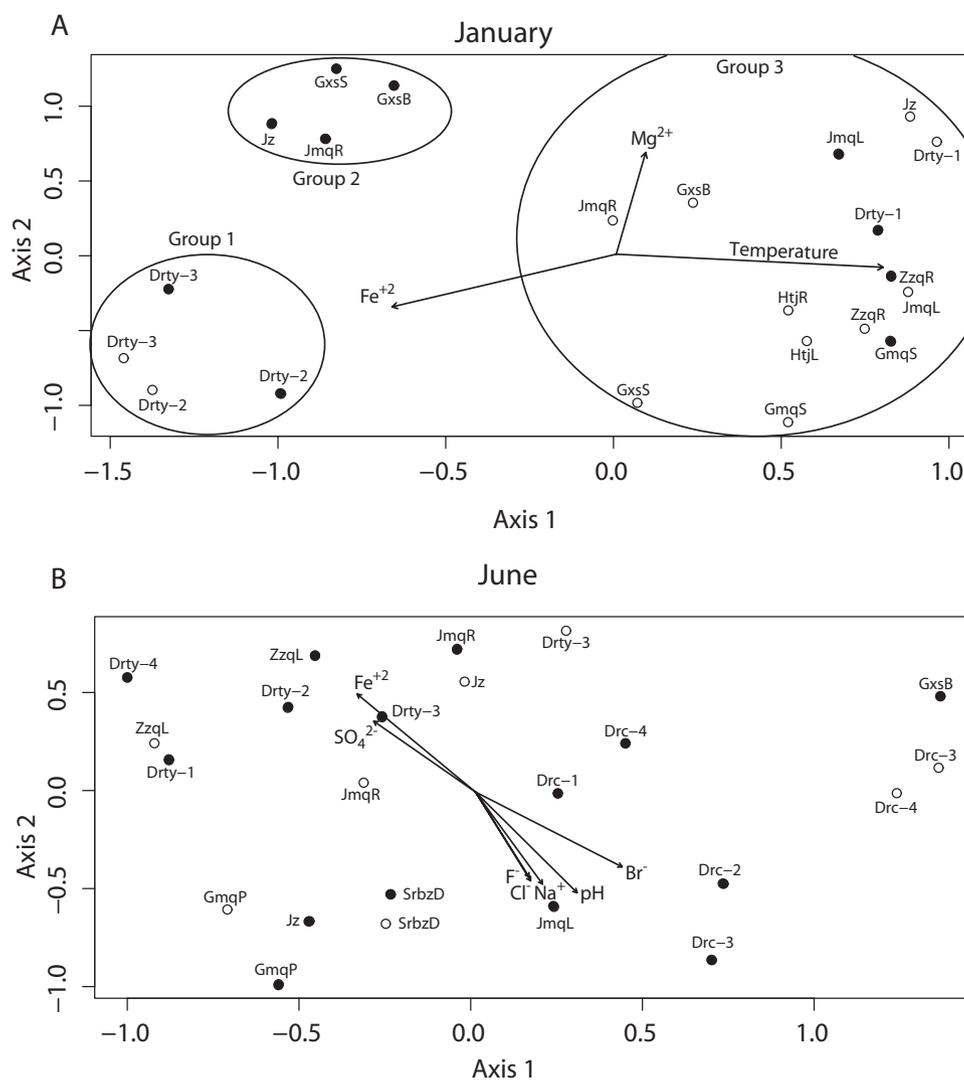
June

Fig. 3. An NMDS ordination for samples collected during January or June. The solid circles and open circles represent the central tendency of all OTUs detected in each sample from the sediment and water samples respectively. Symbols that are closer to each other depict samples that contain similar taxa detected by the PhyloChip. A biplot is overlaid on the ordination to identify environmental parameters that were correlated with the microbial community structure. The length of the line corresponds to the degree of the correlation. Only variables that had a significant correlation ($P < 0.05$) are depicted. The stress value was 0.16 and 0.14 for January and June respectively. The sample codes follow abbreviations defined in Table 1 and depicted on Fig. 1.

unclassified *Crenarchaeota* were detected along the pH, sodium, fluorine, chloride, bromide and potassium vectors ($r^2 = 0.63$, 0.54, 0.53, 0.43, 0.36 and 0.29 respectively) (Fig. 5B). In contrast, *Sulfolobales* were found at lower pH along the sulphate, total iron, ferrous iron and phosphate vectors ($r^2 = 0.53$, 0.20, 0.44 and 0.18 respectively). The distribution of *Desulfurococcales* and *Thermoproteales* in high pH springs was also consistent previously published pyrosequencing results (Hou *et al.*, 2013). Two OTUs within candidate group SK213 were detected at widely different pH (Fig. 5B). One taxon was only found in the acidic Diretiyanqu spring, while the other taxon was found

in several alkaline springs (Gumingquan, Jiemeiquan, Direchi).

Discussion

Seasonal geochemical variations

The source water for these springs was meteoric not deeply sourced hydrothermal fluids (Hou *et al.*, 2013). Therefore, seasonal differences in geochemistry are a consequence of precipitation and evaporation. The monthly precipitation increased from 75 mm in January to 150 mm in June (Fig. S1). Several geochemical param-
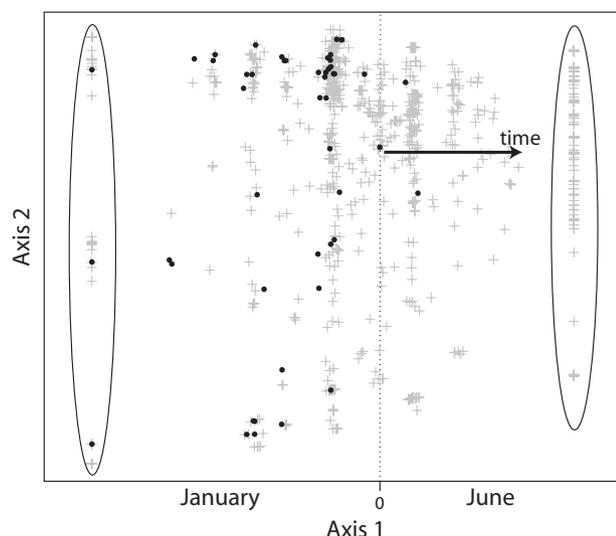
Fig. 4. CCA of differences in community composition between the January and June samples. Grey crosses and black circles represent bacterial and archaeal taxa respectively. OTUs with higher abundance in January or June are to the left or right of zero respectively. The circled taxa were only detected during their respective times of sampling. The second axis was not constrained and represents the overall variability in the community structure.

eters varied between the two sampling times, suggesting surface run-off from surrounding surfaces or shallow recharge of the springs in June. For example, June samples had increased levels of ammonium, DOC and TOC. June samples also had increased cations such as sodium, potassium and calcium likely because of interactions of surface run-off with minerals such as sulphates, kaolinite, illite, calcite/aragonite, opal, feldspars and other silicates that were present around the Tengchong springs (Zhu and Tong, 1987; Hou *et al.*, 2013). An alkaline solution is more effective in dissolving silicates and can account for increases of sodium, potassium and calcium in alkaline springs (Fig. 2). Furthermore, seasonal changes in chloride appeared to be decoupled from sodium. The neutral-alkaline springs in Rehai have been categorized as Na-Cl-HCO$_3$ or Na-HCO$_3$-Cl (Zhang *et al.*, 1987; 2008b), indicating that the decoupling of sodium and chloride in June may be due to surface run-off input, and sodium and chloride are derived from different sources. One input of sodium could be from weathering of volcanic rocks, especially from plagioclase in this region. Another indication of the effect of runoff on the

Table 2. Percentages of the contribution to the overall Bray–Curtis dissimilarity between January and June samples are shown for the top three families for each site.

Site	January Phylum; Family	%	June Phylum; Family	%
Drty1_S	*Firmicutes; Thermoactinomycetaceae*	2.9	*Proteobacteria; Oxalobacteraceae*	2.9
	Proteobacteria; Unclassified	2.8	*Proteobacteria; Sphingomonaceae*	2.9
	SAGMEG-1; Unclassified	2.7	*Firmicutes*; Unclassified	2.8
Drty2_S	*Euryarchaeota; Thermoplasmataceae*	2.6	BRC1; Unclassified	2.4
	Proteobacteria; Roseococcaceae	2.5	*Proteobacteria; Sphingomonaceae*	2.4
	Proteobacteria; Unclassified	2.5	*Proteobacteria; Sphingomonaceae*	2.3
Drty3_S	*Proteobacteria; Comamonadaceae*	1.3	*Aquificae; Hydrogenothermaceae*	1.3
	Proteobacteria; Unclassified	1.3	BRC1; Unclassified	1.3
	Proteobacteria; RCP2–54	1.2	*Proteobacteria; Roseococcaceae*	1.2
Drty3_W	SAGMEG-1; Unclassified	1.9	*Crenarchaeota; Sulfolobaceae*	1.9
	Proteobacteria; Comamonadaceae	1.8	*Proteobacteria*; Unclassified	1.9
	Euryachaeota; Aciduliprofundaceae	1.8	*Sulfobacilli*; Unclassified	1.9
JmqL_S	*Crenarchaeota; Aeropyraceae*	1.1	*Chloroflexi; Chloroflexaceae*	1.1
	Proteobacteria; Comamonadaceae	1.1	*Proteobacteria; Hyphomicrobiaceae*	1.1
	Cyanobacteria; Pleurocapsa	1.1	*Thermotogae; Thermotogaceae*	1.1
JmqR_S	*Crenarchaeota; Thermoproteaceae*	1.9	*Aquificae*; Unclassified	1.8
	Bacteroidetes; Spirosomaceae	1.8	*Actinobacteria; Pseudonocardiaceae*	1.8
	Crenarchaeota; Desulfurococcaceae	1.8	*Proteobacteria; Sphingomonacea*	1.7
JmqR_W	SAGMEG-1; Unclassified	2.0	*Thermodesulfobacteria*; Unclassified	2.1
	Lentisphaerae; Unclassified	1.8	GN02; Unclassified	2.0
	OP11; Unclassified	1.8	*Firmicutes; Clostridiaceae*	1.9
GxsB_S	*Proteobacteria; Comamonadaceae*	1.8	*Thermodesulfobacteria*; Unclassified	1.7
	Crenarchaeota; CH2a62_SP	1.8	*Acidobacteria*; Unclassified	1.6
	Actinobacteria; Pseudonocardiaceae	1.7	OP8; Unclassified	1.6
Jz_W	*Aquificae*; Unclassified	3.1	EM19; Unclassified	2.9
	Proteobacteria; Comamonadaceae	2.9	*Chloroflexi*; Unclassified	2.9
	Caldicellulosiruptor; Unclassified	2.9	GN02; Unclassified	2.8
Jz_S	OP9; Unclassified	1.3	SAGMEG-1; Unclassified	1.4
	Chloroflexi; Unclassified	1.3	*Cyanobacteria; Synechococcaceae*	1.3
	Deferribacteres; Unclassified	1.3	*Verrucomicrobia*; Unclassified	1.3

Those that were more abundant in January (left column) and June (right column) are shown separately. Phyla and families highlighted in grey are within the archaea. The letter code following the site abbreviations indicates the sampling location, whether sediment (S) or water (W).
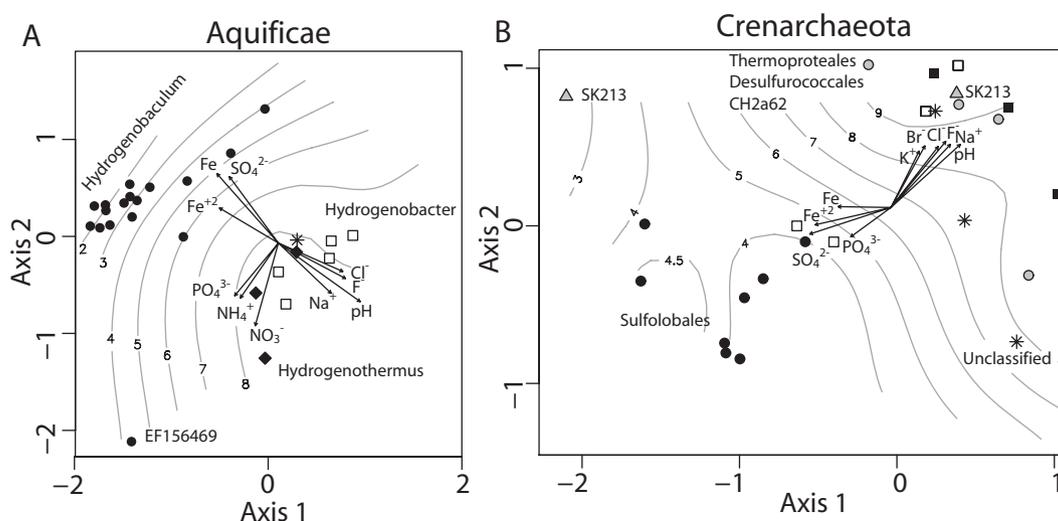
Fig. 5. NMDS plots of distributions of OTUs and correlated geochemical parameters classified as either (A) *Aquificae* or (B) *Crenarchaeota*. (A) The genera *Hydrogenobacter* (open squares), *Hydrogenobaculum* (closed circles), *Hydrogenothermus* (closed diamonds) and unclassified *Aquificae* (asterisk) are shown. (B) The orders *Sulfolobales* (closed circles), *Thermoproteales* (grey circles), *Desulfurococcales* (open squares), candidate divisions within *Crenarchaeota* SK213 (grey triangle), CH2a62 (closed squares) and unclassified *Crenarchaeota* (asterisk) are shown. Only significant ($P < 0.05$) physicochemical parameters are depicted on the biplot. Contour lines depict the pH that each taxon was predominantly detected. The sample codes follow abbreviations defined in Table 1 and depicted in Fig. 1. Fe, total iron.

geochemistry is the decrease of iron in June. Iron concentrations are higher in hot springs than the surrounding soils, especially in the acidic springs where the low pH solubilizes iron minerals. Increased meteoric water would dilute the iron in the hot springs.

Phosphorus was expected to increase in June for all springs because of the increased input of organic matter. However, our data show that the seasonal effect on phosphorus was dependent on the pH of the spring. Phosphorus increased in acidic springs but remained the same in circumneutral and alkaline springs. While phosphorus could have increased in alkaline springs as well, we were not able to detect it likely because of the preferential sorption of phosphate onto sediment particles (Jahnke, 1992). At acidic pH, phosphorus occurs as H_3PO_4, which does not sorb onto sediment particles. At neutral and alkaline pH, phosphorus occurs as $H_2PO_4^-$ and HPO_4^{2-}, and in the presence of various ions, pairs can form that can sorb onto sediment particles. In addition, precipitation of phosphate minerals occurs more readily in neutral to alkaline pH (Jahnke, 1992). All these factors may have accounted for the similar phosphorus concentration in January and June in alkaline springs.

Comparison of microbial diversity to past studies at Tengchong

Previously identified cultured representatives of *Firmicutes*, *Deinococcus-Thermus* and archaeal members of *Thermoprotei* (reviewed in Hedlund *et al.*, 2012) were

detected using the PhyloChip. Previous clone library-based studies detected *Crenarchaeota* (Song *et al.*, 2010) and putative ammonia-oxidizing archaea (Zhang *et al.*, 2008a; Jiang *et al.*, 2010), both of which were also detected by the PhyloChip. However, relative to these culture-dependent and clone library approaches, the PhyloChip detected more rare microbes, which are important to reveal seasonal variations of microbial communities in Tengchong hot springs.

DNA aliquots from 20 of the January samples were also used for pyrosequence analysis (Hou *et al.*, 2013), which allowed for a direct comparison of the detected taxonomic groups between the two techniques. The number of OTUs detected by the PhyloChip (34–548 per sample) was similar to the number of predicted OTUs based on pyrosequencing (50–223 per sample). At the phylum level, the PhyloChip detected an additional 54 bacterial and 11 archaeal phyla that were not detected by pyrosequencing (Table S4). The *Dictyoglomi* phylum was the only exception, i.e. it was not detected by the PhyloChip but was detected using pyrosequencing. Thus, the PhyloChip detected a greater number of microbes and a greater diversity at the phylum level than previous reports.

Distribution of Aquificae and Crenarchaeota in comparison with pyrosequencing data set

NMDS ordinations of OTUs of the *Aquificae* and *Crenarchaeota* depicted a similar response to the geochemistry as previously reported for both water and sedi-

ment samples (Hou *et al.*, 2013). Both culture-dependent and -independent methods have identified the distribution of *Aquificae* and have shown that the genus *Hydrogenobaculum* is present in acidic environments, while *Hydrogenobacter* is found in circumneutral to alkaline environments (Reysenbach *et al.*, 2005; 2009; Hou *et al.*, 2013). *Hydrogenothermus* was also found in circumneutral to alkaline springs that contained high sodium chloride concentrations (Fig. 5A), consistent with the finding that *Hydrogenothermus* growth increases with the addition of sodium chloride (Stohr *et al.*, 2001).

Hou and colleagues (2013) did not detect *Thermocrinis* representatives in Tengchong hot springs. *Thermocrinis* occupies a similar niche as *Hydrogenobacter* but is typically more abundant at higher temperatures and is believed to produce macroscopic streamers (Eder and Huber, 2002; Reysenbach *et al.*, 2005). Given the high temperatures and the abundance of streamer biomass in Tengchong hot springs, it was expected that *Thermocrinis* would be abundant; however, it was not detected by pyrosequencing (Hou *et al.*, 2013). The PhyloChip data supported the conclusion from pyrosequencing that *Thermocrinis* was absent in Tengchong springs, and its absence was not caused by misassignment of short pyrosequencing reads. Instead, *Thermus* species were detected. *Thermus* species were also found in high-temperature streamers in YNP (Meyer-Dombard *et al.*, 2011).

Similar to *Aquificae*, OTUs within *Crenarchaeota* were structured mainly by pH. This observation is also consistent with the pyrosequencing results from Tengchong (Hou *et al.*, 2013) and other hot spring environments (Meyer-Dombard *et al.*, 2005; Huang *et al.*, 2011; Kozubal *et al.*, 2012; Pagaling *et al.*, 2012). The tolerance of *Desulfurococcales* and *Thermoproteales* in Rehai to higher pH ranges than previously known, as suggested by pyrosequencing (Hou *et al.*, 2013), was confirmed by PhyloChip data in this study (Fig. 5). Cultivation-based approaches can provide further evidence for their alkaline-tolerant or alkaliphilic physiologies.

Seasonal variation in microbial communities

Seasonal variation in microbial communities has been documented in marine, freshwater and soil environments (Auguet *et al.*, 2011; Rasche *et al.*, 2011; Tšertova *et al.*, 2011; Gilbert *et al.*, 2012). In addition, seasonal variability of phototrophic mats communities in hot spring ecosystems has been reported (Ferris and Ward, 1997; Norris *et al.*, 2002; Lacap *et al.*, 2007; Mackenzie *et al.*, 2013). However, until now, the seasonal variation in high-temperature hot springs water and sediment microbial communities has not been reported. We observed that microbial communities in most Tengchong hot springs significantly changed from January to June; the change in the community structure in June was mainly due to the increase of organisms typically considered non-thermophilic (Table 2 and S3).

The possible non-thermophilic OTUs detected in June are unlikely to be contaminants caused by our methodology because the same personnel performed DNA extraction and polymerase chain reaction (PCR) reactions for both January and June samples, and samples from both January and June were processed at the same time. The likelihood that only samples from June were contaminated during the laboratory analysis is therefore minimal. Instead, similar to observations from Bison Spring in YNP (Swingley *et al.*, 2012), these OTUs are likely non-endemic and transported to the hot spring environment during rain events that typically occur in June.

It is possible that increased surface water input to the springs in June could have delivered non-indigenous microbes to the hot spring environment that originated in the surrounding environments (soils, forests and rocks). This speculation is supported by our geochemical data, where analytes typical of soils (i.e. ammonia, potassium, total nitrogen, calcium, TOC, DOC and sodium) increased in June, but those typical of geothermal water (i.e. chloride, iron, sulphate) decreased because of dilution. Moreover, the amount of input of non-indigenous microbes to hot springs appears to be dependent on the topographical setting of the spring. For example, Diretiyanqu-1 and Diretiyanqu-2 contained more of these non-endemic OTUs in June than the other sites (Table 2). For example, the pools at Diretiyanqu are located within an erosional feature where we have witnessed surface water flowing into the springs during heavy rain events. Furthermore, in contrast with other springs, tourists are encouraged to approach and experiment. The number of visitors in 2011 was nearly half a million (J. Fang, pers. comm.; vice manager of Yunnan Tengchong Volcano and Spa Tourist Attraction Development Corporation). In addition, $\delta^{18}O$ values slightly increased in June for Diretiyanqu-1 and Diretiyanqu-2 suggesting input of surface water (Fig. 2). However, not all sites at Diretiyanqu responded the same way. Diretiyanqu-3 community structure did not significantly change with season, $\delta^{18}O$ slightly decreased, and similarity percentage (SIMPER) analysis found that more acidophilic and thermophilic organisms contributed to the dissimilarity for this site compared with the other acidic sites (i.e. *Hydrogenothermus*, *Acidianus* and *Sulfobacilli*), suggesting less effects of surface water input in Diretiyanqu-3 than the other acidic sites. Future source tracking efforts could verify whether the detected non-thermophilic organisms are truly non-endemic by sampling and identifying similar organisms in the surrounding soils and along flow paths from the soils to the springs.

Conclusions

The changes in geochemistry and community structure (increased abundance of non-thermophilic microbes) in June compared with January is a consequence of input of rainwater or shallow underground recharge. These results demonstrate that seasonal changes can occur in the sediment and water of high-temperature geothermal environments. Similar to marine and terrestrial environments, geothermal environments are not isolated from the surrounding environment and seasonality affect the microbial ecology.

Experimental procedures

Sampling and geochemistry

The Rehai and Dientan geothermal springs are located in the Tengchong volcanic area in southwestern China (Fig. 1) (Zhang *et al.*, 2008b; Hedlund *et al.*, 2012; Hou *et al.*, 2013). Two sampling trips to this area were made, one in January (22 samples) and the other in June (23 samples) 2011 to collect a total of 45 samples from Gongxiaoshe, Diretiyanqu, Gumingquan, Shuirebaozha, Jinze, Jiemeiquan, Direchi, Huaitaijing and Zhenzhuquan springs (Table 1). The same locations for 10 of these springs were sampled in January and June. Water and sediment samples were collected as previously described (Hou *et al.*, 2013). Briefly, biomass in the water was concentrated by filtering 360 ml of spring water through a 0.22 μm pore size syringe filter (Millipore, Billerica, MA, USA). Sediment samples were collected with sterile spoons and transferred sterile 50 ml polypropylene tubes. Both water and sediment samples were immediately placed in liquid nitrogen and stored at −80°C until DNA extraction. Water pH and temperature were measured in the field (portable metres; LaMotte, Chestertown, MD, USA) at the site of water collection. Concentration of ammonia, nitrate, nitrite and ferrous iron were also measured in the field by spectrophotometry using Hach kits (Hach Chemical Co., Ames, IA, USA) after filtering through a 0.2 μm polyethersulfone membrane filter (Pall Corp., Port Washington, NY, USA). Concentration of total soluble iron, calcium, potassium, sodium, chlorine, bromine, fluorine, phosphate, nitrate, sulphate and magnesium were measured using ion chromatography as previously described (Hou *et al.*, 2013). DOC and total nitrogen in filtered water samples were analysed by high-temperature combustion with non-dispersive infrared and chemiluminescence as previously described (Hou *et al.*, 2013). TOC and total nitrogen present in the sediment was determined using a NC 2100 Elemental Analyzer interfaced with a Finnigan Delta Plus XL isotope ratio mass spectrometer as previously described (Hou *et al.*, 2013). Water samples were analysed for oxygen and hydrogen isotope composition using LGR's (Los Gatos Research) Off-Axis Integrated Cavity Output isotope analyser at the Colorado Plateau Stable Isotope Laboratory after filtration through a 0.2 μm Acrodisc® GHP membrane filter.

DNA extraction and PhyloChip

DNA extraction from water and sediment samples was performed as previously described using either filters (water samples) or 0.5 g of sediment with the FastDNA SPIN Kit (MP Biomedical, Solon, OH, USA) according to manufacturer's protocol (Hou *et al.*, 2013). The 16S rRNA genes were amplified from extracted DNA using either the general bacterial primers 27F and 1492R, or general archaeal primers 4Fa and 1492R in a Veriti thermocycler with the same PCR conditions as previously described (Briggs *et al.*, 2011). Each 25 μl PCR mixture contained 0.625 units of ExTaq polymerase (Takara, Tokyo, Japan), 1x PCR buffer, 800 μM of each dNTP and 300 nM of each primer. The amplified products were purified using a Wizard SV PCR clean up kit (Promega, Madison, WI, USA). Amplified DNA quantities were determined by measuring absorbance at 260 nm using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

The G3 PhyloChip contains 1 100 000 probes targeting 16S rRNA genes representing 59 959 OTUs, 10 993 subfamilies, 1219 orders and 147 phyla demarcated within bacteria and archaea. The G3 chip has been previously validated and shown to have a high reproducibility between replicates (coefficient of variation = 0.097) (Hazen *et al.*, 2010; Mendes *et al.*, 2011). PhyloChip version G3 sample preparation and data analysis were performed as previously described (Hazen *et al.*, 2010). Briefly, for each sample, eight PCR reactions were pooled. The full-length 16S rRNA genes were fragmented and biotin-labelled. Hybridization to the array was performed with 500 ng of bacterial 16S rRNA gene amplicons, and 100 ng of archaeal 16S rRNA gene amplicons and 100 ng of internal spike DNA (control to determine background fluorescence).

Statistical analyses

All statistical analyses were performed in R (R Development Core Team, 2012). Sites were clustered based on the change in geochemistry from January to June using unweighted pair group method with arithmetic mean and Euclidean distance measure.

Statistical analyses of the microbial community were done by first removing singletons (OTUs found in only one sample) from the PhyloChip data, and a Bray-Curtis dissimilarity matrix was constructed (Bray and Curtis, 1957). The community structure for January and June samples was depicted separately using NMDS (Kruskal, 1964) based on the Bray–Curtis dissimilarity matrix of detected OTUs (Briggs *et al.*, 2012). The NMDS plot was created using the metaMDS function in the 'vegan' package in R with 500 random starts to minimize the likelihood of finding local optima (Oksanen *et al.*, 2011). Physiochemical parameters were overlaid on the NMDS ordination as a biplot where the length and angle of the arrow represented the magnitude and direction of the correlation to the community structure. Only parameters with a significant P value (< 0.05) were displayed on the ordination. In addition, an NMDS ordination overlaid with a biplot was performed as discussed earlier to identify geochemical variables correlated to *Aquificae* and *Crenarchaeota* distributions. Contour lines depicting pH were overlaid on the NMDS plots using the ordisurf function in the 'vegan' package.

To graphically display OTU assemblages along environmental gradients, CCA of the Bray–Curtis dissimilarity measurement was performed using the 'vegan' package in R

(Oksanen *et al.*, 2011). CCA is a constrained ordination that associates two or more data sets in the ordination itself. In this study, a CCA ordination was constrained to depict that the seasonal variation in OTU composition was performed on the 20 paired samples collected in January and in June.

Pairwise *t*-test was used to determine if spring community composition was significantly different between January and June using the R function 't.test'. SIMPER analysis was performed to identify differences between January and June samples for each site with the Bray–Curtis dissimilarity using the PAST statistical software (Hammer *et al.*, 2001). SIMPER is a method to determine which OTUs are primarily responsible for a difference between two groups (Clarke, 1993).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Monthly precipitation and solar radiance for the Tengchong hot spring region in 2011.

Table S1. Geochemical parameters measured for January (Hou *et al.*, 2013) and June. All units are ppm except for measurements performed on sediment samples and isotopes, which are mg g^{-1} (dry wt.) and per millilitre respectively.

Table S2. Family-level taxa predominantly found in January for both water and sediment identified by the CCA analysis. Some taxa could not be classified to the family level, in which case the most specific taxonomic assignment that could be assigned is shown.

Table S3. Family-level taxa predominantly found in June for both water and sediment identified by the CCA analysis. Some taxa could not be classified to the family level, in which case the most specific taxonomic assignment that could be assigned is shown.

Table S4. Bacterial and archaeal phyla and candidate phyla detected on the PhyloChip for both January and June samples. The number of OTUs (species level) detected in each phylum is also listed. Phyla in bold have not been detected before in the Tengchong hot springs.