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An analysis of geothermal and carbonic springs in the western United States sustained by deep fluid inputs

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ABSTRACT

Hydrothermal springs harbor unique microbial communities that have provided insight into the early evolution of life, expanded known microbial diversity, and documented a deep Earth biosphere. Mesothermal (cool but above ambient temperature) continental springs, however, have largely been ignored although they may also harbor unique populations of micro-organisms influenced by deep subsurface fluid mixing with near surface fluids. We investigated the microbial communities of 28 mesothermal springs in diverse geologic provinces of the western United States that demonstrate differential mixing of deeply and shallowly circulated water. Culture-independent analysis of the communities yielded 1966 bacterial and 283 archaeal 16S rRNA gene sequences. The springs harbored diverse taxa and shared few operational taxonomic units (OTUs) across sites. The Proteobacteria phylum accounted for most of the dataset (81.2% of all 16S rRNA genes), with 31 other phyla/candidate divisions comprising the remainder. A small percentage (~6%) of bacterial 16S rRNA genes could not be classified at the phylum level, but were mostly distributed in those springs with greatest inputs of deeply sourced fluids. Archaeal diversity was limited to only four springs and was primarily composed of well-characterized Thaumarchaeota. Geochemistry across the dataset was varied, but statistical analyses suggested that greater input of deeply sourced fluids was correlated with community structure. Those with lesser input contained genera typical of surficial waters, while some of the springs with greater input may contain putatively chemolithotrophic communities. The results reported here expand our understanding of microbial diversity of continental geothermal systems and suggest that these communities are influenced by the geochemical and hydrologic characteristics arising from deeply sourced (mantle-derived) fluid mixing. The springs and communities we report here provide evidence for opportunities to understand new dimensions of continental geobiological processes where warm, highly reduced fluids are mixing with more oxidized surficial waters.

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INTRODUCTION

Microbial studies in continental and oceanic hydrothermal systems have significantly expanded known microbial taxonomic and physiological diversity (Huber *et al.*, 1992; Barns *et al.*, 1994; Hugenholtz *et al.*, 1998; De La Torre *et al.*, 2008), knowledge of the deep subterranean biosphere (Huber *et al.*, 2003; Takai *et al.*, 2004), and have contributed significantly to the fields of microbial ecology and evolution (Ward *et al.*, 1998, 2008; Whitaker *et al.*, 2003). Chemolithotrophic microbial communities dominate hydrothermal ecosystems, which generally exhibit high temperatures, anoxic and reducing conditions, and an abundance of inorganic compounds used in chemolithotrophy (H_2, S, and Fe among others). High-temperature hydrothermal systems have received the most research attention in tectonically

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active areas such as Yellowstone National Park, Kamchatka, and Iceland (Barns *et al.*, 1994; Hugenholtz *et al.*, 1998; Ward *et al.*, 1998; Kelley *et al.*, 2001, 2005; Rudolph *et al.*, 2001; Hall *et al.*, 2008; Inskeep *et al.*, 2010). Similarly, high-temperature marine hydrothermal vent systems have been the subject of considerable research since their discovery in the 1970s (for example: Longnecker & Reysenbach, 2001; Huber *et al.*, 2003; Nakagawa *et al.*, 2005). More recently, the discovery of warm, serpentinite-hosted vents and diffuse-flow hydrothermal sediment sites on the seafloor has revealed a new dimension of microbial diversity in hydrothermal systems. These systems are dominated by Archaea and Bacteria that use reduced substrates produced from the water–rock interactions occurring at these sites (Kelley *et al.*, 2001, 2005; Teske *et al.*, 2002). The geobiological interactions, taxonomic composition, and geochemistry at these low-temperature vents are distinct from their high-temperature vent analogs and provide evidence of widespread geobiological ecosystems that were previously unknown.

Mesothermal continental springs have received less attention as microbial environments distinct from other freshwater environments. Although there is no widely accepted definition for what temperatures demarcate thermal springs from cold springs, here we define mesothermal as those springs above ambient temperature, but below the minimum threshold (45 °C) for optimal thermophile growth (Reysenbach & Shock, 2002). The few culture-independent studies of cold and mesothermal springs suggest that they differ markedly in community composition, which is likely due to local geochemical characteristics. Sulfur-rich mesothermal springs have been investigated in detail and generally are dominated by or harbor sulfur-metabolizing bacterial lineages like *Beggiatoa*, *Thiothrix*, *Thiomicrospira,* among other genera, sulfide-dependent phototrophs, and Epsilonproteobacteria (Elshahed *et al.*, 2003; Engel *et al.*, 2004; Rudolph *et al.*, 2004; Barton & Luiszer, 2005; Perreault *et al.*, 2007; Chaudhary *et al.*, 2009). Novel, division-level lineages of micro-organisms have also been detected in mesothermal sulfurous springs (Rudolph *et al.*, 2001; Elshahed *et al.*, 2003). Communities in the few non-sulfurous mesothermal springs that have been analyzed with culture-independent techniques are more similar to those in freshwater and marine habitats with a predominance of the Gamma, Beta, and Alpha- subdivisions of Proteobacteria, Bacteroidetes, and Nitrospira (Farnleitner *et al.*, 2005; Ball & Crawford, 2006; Weidler *et al.*, 2007), although Epsilonproteobacteria may be present as minor populations (Ball & Crawford, 2006). Little is known overall about the diversity of microbial inhabitants of these largely understudied environments; nonetheless, they may be unique microbial systems with implications for understanding subsurface microbial communities. A recent metagenomic analysis of a cold terrestrial spring in the Tablelands region of Newfoundland, Canada, supports this hypothesis with the presence of dominant microbial populations in the Burkholderiales order of Betaproteobacteria that are putatively capable of chemolithoautotrophic H_2 oxidation (Brazelton *et al.*, 2012). It is hypothesized that H_2 is produced in abundance from deep subsurface serpentinization from abducted Ophiolites in the Tablelands region and provides a potential terrestrial analog to warm-vent marine sites, such as at the Lost City hydrothermal field.

The aim of this study was to investigate the diversity and microbial community ecology of 28 carbonic (generally characterized by higher than atmospheric pCO_2) springs ranging from cold to thermal temperatures (9.0–59.3 °C) throughout the western United States. These springs have previously been identified to have inputs of deeply sourced fluids and many contain mantle-derived gases/substrates as documented by $^3He/^4He$ ratios (Crossey *et al.*, 2006, 2009). Like the more well-characterized deep-sea hydrothermal vents (Jannasch & Mottl, 1985), many of the springs we analyzed here are geochemically analogous with higher than ambient temperature, high CO_2, low dissolved oxygen, high total dissolved solids (TDS), and metal content that reflects deep fluid components and acquisition of solutes from rock. The springs, however, are distinct in that they represent an interface between groundwater, surface water, and underappreciated deeply sourced fluids that mix with shallow surface waters. In most cases, a visible hallmark of the unique geochemistry and geomicrobiology of these springs is the presence of travertine that is formed via degassing of CO_2 at spring vents. Although some of the springs analyzed here have been documented in previous analyses, this is the first report to consider a cross-regional and deep subsurface fluid mixing perspective in describing their microbial communities. Here, we test the hypothesis that these mesothermal to thermal carbonic springs provide unique environments for microbial communities where the geochemical setting is shaped primarily by solute and volatile movement from mixing with deeply sourced fluids.

We analyzed the microbial communities of these 28 springs using culture-independent techniques with two goals: (i) to describe the bacterial and archaeal communities of temperate to thermal springs in areas with endogenic fluid circulation and to (ii) investigate the role of physicochemical characteristics on the distribution of microbial taxa and community structure among these springs. Results indicated that microbial communities were largely disparate among the 28 springs. The taxonomic lineages present provided evidence that communities were shaped by variable mixing of shallowly circulated fluids with reduced, deeper sourced waters, as evidenced by genera typical of surficial waters in those springs with less evidence for endogenic fluid input and putatively chemotrophic populations in those springs with greater endogenic fluid input. Statistical analyses supported the hypothesis that endogenic fluid input corre-

lated with differences in microbial community membership. Additionally, although present as minor overall populations, novel lineages were present in some of the springs with higher endogenic input. These data motivate continued study of the complete spectrum of continental thermal springs, including mesothermal springs, for potential fundamental new insights into both the biodiversity of such systems and metabolic–geochemical linkages in these systems.

MATERIALS AND METHODS

Sample sites and collection

Thirty-one microbial samples were collected from 28 temperate to thermal springs in five geologic provinces in northern New Mexico/South-Central Colorado (Rio Grande Rift Valley, RGR), Colorado (Aspen Anomaly, AA), Arizona (Colorado Plateau, CP, and Transition Zone, TZ), and California (Death Valley, DV) in 2005 and 2006 (Fig. 1; Table 1). Planktonic communities were sampled by filtering spring source water (600–1800 mL) through Millipore Sterivex-GP filter (Bedford, MA; pore size 0.22 μm) using sterile 50-ml syringes. Water was filtered from spring sources to sample the microbial communities most associated with subsurface fluids. Sample filters were stored with equal volumes of Sucrose Lysis Buffer, pH 9.0 [SLB: 20 mM EDTA, 200 mM NaCl, 0.75 M sucrose, and 50 mM Tris–HCl (Giovannoni *et al.*, 1990)], and frozen or immediately extracted upon returning to the laboratory (within 3 days). Previous work has shown that DNA yield and biodiversity detected are greatest, but comparable when preserved in SLB at ambient temperature for up to 7 days, compared with SLB preserved samples frozen immediately in liquid nitrogen (Mitchell & Takacs-Vesbach, 2008).

Geochemistry

Spring water was sampled as described in Crossey *et al.* (2009). Water was filtered (aside from alkalinity samples to avoid degassing) and stored cold in the dark for up to 3 weeks. Water for cation analysis was acidified to <pH 2 with the use of concentrated nitric acid. Alkalinity was measured with titrations using standardized sulfuric acid using the end point titration method (Pearson, 1981). Anions were measured using ion chromatography on a Dionex 500× Ion Chromatograph. Major cations and trace elements were measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES; Perkin-Elmer, Waltham, MA, USA). Anions, cations, and trace elements are given in ppm, while arsenic was measured with a hydride sample introduction system and given in ppb. Temperature, pH, and conductivity were measured at the field site at the time of sampling. DIC (in moles C kg^{-1}) and equilibrium pCO_2 were modeled from major ion chemistry, alkalinity, and pH in PHREEQC (version 2, USGS, http://wwwbrr.cr.usgs.gov/projects/GWC_coupled/phreeqc/). While high-pCO_2 values are typical of endogenic waters (Crossey *et al.*, 2006), external CO_2 inputs into a spring, C_{ext}, can be distinguished from CO_2 derived from carbonate dissolution, C_{carb}, following methodology described previously (Chiodini *et al.*, 2004; Crossey *et al.*, 2009). C_{ext} can be further attributed to organically derived sources (C_{org}) or endogenic inputs (C_{endo}) using $\delta^{13}C$ values typical of either component (Crossey *et al.*, 2009). Carbon isotopic values that were originally reported in Karlstrom *et al.*, 2013 (measured from the gas phase CO_2 of waters) for a subset of the springs analyzed here ($n = 13$) were used to assess C_{endo} in our springs. $\delta^{13}C$ values are given as per mil relative to Pee Dee Belemnite (PDB) with a 1-sigma error of <0.2%. Higher helium isotope ratios ($^3He/^4He$) are also typical of springs with mantle-derived fluids and were obtained for a subset of the

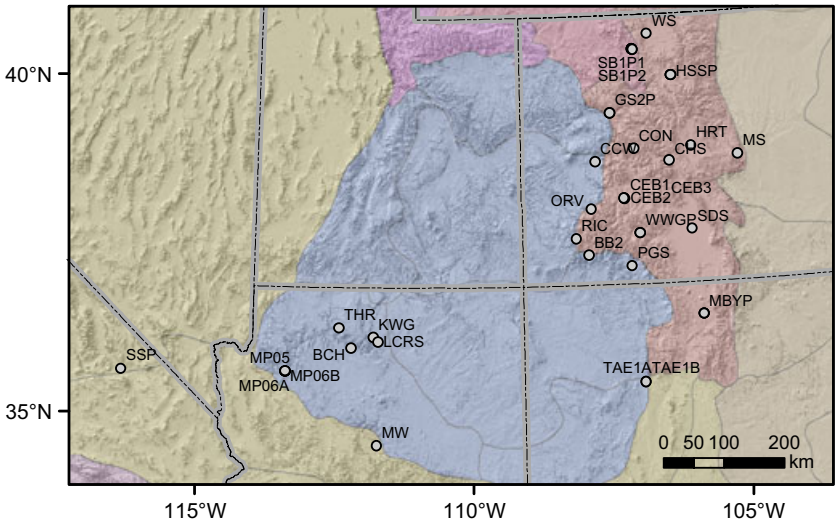

Fig. 1 Map of the western United States showing the sampling location for each spring. Regions are colored according to the physiographic provinces. From lower left to upper right: Pacific Mountain System (light pink), Great Basin/Death Valley (light yellow), Colorado Plateau (blue), Middle Rocky Mountains (dark pink), Wyoming Basin (light red), Southern Rocky Mountains/Aspen Anomaly (red).

Table 1 Site descriptions and microbial richness estimations

Geologic Province	Site Name (abbreviation)	T (°C)	pH	R*	Chao1	C (%)†
AA	Cebolla Hot Spring (CEB1)‡	41.0	6.25	16	19	93
	Cebolla Warm Spring (CEB2)	28.4	5.94	37	202	56
	Cebolla Cold Spring (CEB3)	9.0	5.48	10	38	89
	Colonel Chins Hot Well (CCW)	36.3	5.9	9	24	92
	Conundrum Hot Spring (CON)	40.0	7.92	38	89	72
	Glenwood Springs (GS2)	50.1	6.16	11	26	89
	Hartsel Hot Springs (HRT)	51.9	6.57	16	23	91
	Hot Sulfur Springs (HSS)	44.9	6.68	23	31	82
	Manitou Springs (MS)	14.7	6.12	10	11	93
	Orvis Hot Springs (ORV)	51.6	6.42	7	13	92
	Pagosa Springs (PGS)	53.0	6.42	14	37	82
	Pinkerton Spring (BB2)	32.7	6.14	28	85	72
	Rico Hot Spring (RIC)	43.2	6.27	25	43	81
	Steamboat Springs					
	Original pool (SB1P1)	26.3	6.45	26	41	83
	Original pool (SB1P2)			19	24	91
	Wagon Wheel Hot Springs (WWG)	53.7	6.63	4	7	96
	Walden Springs (WS)	39.6	6.25	9	24	91
CP	Boucher Creek (BCH)	18.7	7.65	16	71	85
	Kwagunt Spring (KWG)	28.0	6.94	48	154	37
	Sipapu Spring (LCRS)	26.8	6.3	20	34	86
	Thunder River (THR)	12.0	8.18	33	93	52
	Travertine Grotto					
	MudPit (MP05)§	30.2	7.23	23	276	0
	MudPit (MP06A)	30.8	7.31	12	27	92
	MudPit (MP06B)‡	30.8	7.31	36	334	05
DV	Saratoga Springs (SSP)	29.0	7.46	39	138	55
RGR	Cottonwood Hot Springs (CHS)	59.3	8.64	12	17	89
	Great Sand Dunes Swimming Pool (SDS)	41.7	8.44	6	7.5	88
	Manby Hot Springs (MBY)	37.9	7.91	7	10	92
	Tierra Amarilla Spring A (TAE1A)‡	24.5	6.18	24	214	57
	Tierra Amarilla Spring B (TAE1B)‡	24.4	6.24	15	30	88
TZ	Montezuma Well (MW)	24.2	6.59	6	7	98

*Richness as defined by measured OTU diversity.
†Coverage (as Good's coverage) as an estimation of sampling completeness.
‡Archaeal amplification.
§05, sampled in 2005; 06A & 06B sampled in 2006.

springs reported here (n = 16) from previous reports (Crossey *et al.*, 2009; Karlstrom *et al.*, 2013). He ratios (R_c) are reported after being normalized to that of air (R_a). Because He ratios were not available for every site, and testing the correlation between deeply sourced fluid and microbial communities is a goal of this study, geochemical proxies were assessed by linear regressions of the chemical parameters available for every sample and R_c/R_a. Data were both untransformed and log-transformed in linear regressions.

DNA extraction, amplification, and cloning

Nucleic acids were extracted using a variation in the CTAB method (Mitchell & Takacs-Vesbach, 2008). Reagents were added directly into the Sterivex filters. CTAB buffer (400 μL; 1% CTAB, 0.75 M NaCl, 50 mM Tris pH 8, 10 mM EDTA) and proteinase K (final concentration 100 μg mL^{-1}) were added to samples and incubated for 1 h at 60 °C. Sodium dodecyl sulfate was added to a final concentration of 2%, and samples were incubated for 1 h. DNA was extracted one time with an equal volume of phenol:chloroform:isoamyl alcohol (50:49:1) followed by two extractions with an equal volume of chloroform. DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate and two volumes of 95% ethanol, followed by overnight incubation at –20 °C. The samples were then centrifuged for 45 min (~21 000 × *g*), washed in 70% ethanol, and resuspended in 10 mM filter-sterilized Tris buffer pH 8.0. The 16S rRNA gene was amplified with two sets of primers: bacteria-specific primer 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and universal primer 1492R (5′- GGT TAC CTT GTT ACG ACT T-3′) (Lane, 1991) for bacterial-specific assays and 4F (5′ - TCC GGT TGA TCC TGC CRG – 3′) (Hershberger *et al.*, 1996) and 1492R for archaeal-specific assays. PCRs (50 μL) contained 12.5 mM each dNTP (BioLine USA, Inc., Taunton, MA, USA), 20 pmol of each forward and reverse primer (Invitrogen, Carlsbad, CA, USA), approximately 50 ng of

DNA, and either 5 μL 10× Buffer (with 15 mM $MgCl_2$) with 2.5 U Taq (Promega, Madison, WI, USA) or 10 μL 5× Colorless GoTaq Buffer (with 7.5 mM $MgCl_2$) with 2.5 U GoTaq (Promega). PCRs were incubated for 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 90 s at 72 °C with a final extension of 72 °C for 7 min. Negative PCRs for archaeal amplifications were subject to modification of reaction conditions including an increase in sample DNA and addition of betaine (commonly used as a PCR additive; Baskaran *et al.*, 1996) to confirm negative results. PCR amplicons were spin-purified using a DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA, USA) and ligated into pCR 2.1 TOPO vector using the TOPO-TA cloning kit.

Bacterial sequencing and sequence analysis

Cloned 16S rRNA inserts were sequenced with either 8F or M13F (5′-GTA AAA CGA CGG CCA G-3′) and M13R (5′- CAG GAA ACA GCT ATG AC-3′) primers. All vector sequence was removed, and sequence ends were trimmed to remove nucleotides with a PHRED value <20 using Codon Code Aligner software. Only forward sequences that had more than 300 PHRED20 bases were included in this study. Each clone library was checked for chimeric sequences by identifying suspect sequences with Mallard (Ashelford *et al.*, 2006) and comparing each suspect sequence to its nearest neighbor in Pintail (Ashelford *et al.*, 2005). Sequences identified as chimeric by both Mallard and Pintail were discarded. The remaining dataset (mean 16S rRNA gene length = 749 bp) was aligned and taxonomically classified in the mothur software package (Schloss *et al.*, 2009) using the Bayesian classifier with the Greengenes reference database and a 70% confidence cutoff for taxonomic binning. Mothur was also used to create a distance matrix and bin sequences into operational taxonomic units (OTUs) at ≥95% similarity level, which is commonly used as a bacterial genus-level cutoff (Schloss & Handelsman, 2004). Sample OTU richness was estimated in mothur by observed OTU richness and Chao1 estimation of terminal OTU richness. To estimate the completeness of sequencing sampling effort in describing richness, Good's coverage was calculated as $1 - n_1/N$, where n_1 = singleton OTUs and N = total sequence number per sample (Good, 1953).

Archaeal phylogenetic analysis

Archaeal community clone library sequences were produced and analyzed as described above for bacteria. Archaea were only detected in four samples despite varying PCR conditions and template concentration; therefore, the limited 16S rRNA gene clone library dataset was analyzed phylogenetically. Representative archaeal 16S rRNA gene clones were chosen for full-length sequencing by examining the phylogenetic distribution of fragments produced from clone library analysis. 16S rRNA gene fragments were parsimony-inserted into the Greengenes backbone tree (Hugenholtz, 2002), and phylogenetically unique clones were then sequenced fully in the forward and reverse directions (at least 2× coverage of the gene) with archaeal-specific and universal primers (Reysenbach & Pace, 1995) nearly covering the full-length 16S rRNA gene (~1400 bp). The nearly full-length representative sequences were aligned with the NAST alignment tool (Desantis *et al.*, 2006) and imported into ARB (Ludwig *et al.*, 2004). Tree topology was explored in ARB using parsimony, neighbor-joining, and maximum likelihood algorithms and various filters to mask out hypervariable regions. The 'Archaea' filter available in the Hugenholtz ARB database was used to mask hypervariable regions, and the final analysis was based on 1036 homologous characters. The final tree was produced in PAUP*, version 4.0b (Swofford, 2003) using maximum likelihood analysis with tree bisection-reconnection branch swapping, and a general time-reversible substitution model with a gamma-shaped distribution of 0.6904 and a 0.1954 proportion of invariant sites, as suggested by the Akaike Information Criterion test in modeltest, version 3.7 (Posada & Crandall, 1998). Bootstrap values were determined from 1000 replicate parsimony trees.

Statistical analysis of bacterial community composition

Statistics were all conducted in the R environment using the vegan and labdsv packages (Oksanen *et al.*, 2012; Roberts, 2013), unless otherwise noted. OTU abundances were used to measure community differences based on Bray-Curtis distances. The distribution of OTUs among samples was graphically assessed using an OTU network constructed with the qiime software package (v 1.5; Caporaso *et al.*, 2010) and visualized in Cytoscape (Smoot *et al.*, 2011). A non-metric multidimensional scaling (NMDS) ordination of the OTU distance matrix was used to assess community differences in an iterative process that resulted in an optimal configuration of samples in space that minimized stress. Ordination stress and dimensionality were used to assess the quality of the NMDS analysis. OTUs occurring in only one site were excluded from the dataset for this analysis. The ordination was used to test chemical and physical correlates with community structure by fitting all physical and chemical variables measured (Table S1) to the ordination. Because samples originating from the same spring had identical (or nearly identical) geochemistry, samples from the same spring (MudPit samples and Steamboat Spring Samples) were collapsed to a single sample for only this analysis to mitigate artificial correlation. Correlation strength and directionality are given for significant ($P \leq 0.05$) environ-

mental correlates by the length and direction of the arrows fit to the first two axes of the ordination. Correlative significance was assessed by a permutation test, wherein the data are randomly permuted iteratively to assess the probability of the original correlation arising by chance. Samples were additionally clustered by phylum-level abundances using hierarchical cluster analysis of Bray-Curtis distances to assess higher-level groupings of samples not apparent in the OTU-based clustering because of limited OTU sharing among samples (Fig. 5, Fig. S2). The significance of the cluster groups was tested using a permutational MANOVA test (using the adonis function in vegan), which tests the hypothesis that within-group distances are less than that of between-group distances. Phylum-level abundances and OTU abundances were tested against the groups using indicator species analysis to obtain taxonomic signatures of each group. Indicator species analysis tests whether particular groups of samples exhibit higher than expected fidelity and abundances of taxonomic groups (McCune & Grace, 2002). Differences in physicochemical parameters among groups were tested using a Kruskal–Wallis non-parametric analog of the one-way ANOVA test of between-group variances (Kruskal & Wallis, 1952). All physicochemical parameters that were available for all samples were used in this analysis. Because ANOVA only indicates significant deviations from the mean between at least two groups in a set, indicator analysis was used to test the hypotheses that the physicochemical parameters significant by ANOVA test were significant indicators of any one particular group. Box plots were also constructed to analyze differences in physicochemical parameters among groups. Permutational MANOVA was used to test the hypothesis that the communities were significantly correlated with the province of spring origin.

Nucleotide accession numbers

Near full-length archaeal 16S rRNA gene sequences were deposited in GenBank under the accession numbers KC588524–KC588533. Unique (<100% nucleotide similarity), partial bacterial 16S rRNA gene sequences were deposited in GenBank under the accession numbers KC711065–KC711713, KC711775–KC711917, KC712042–KC712129, KC712186–KC712416, KC712516–KC712982, KC713035–KC713141, KC713259–KC713468, and KC713516–KC713586.

RESULTS

Hydrogeochemistry

The temperature of the springs largely fell within our defined mesothermal range, with median values for our microbial community-defined groups ranging from 29 to 41.7 °C, and a total sampling range of 9–59.3 °C (Fig. S3). Major ion composition was variable across all springs (Fig. 2, Fig. S1), with most of the major hydrochemical facies (defined as >50% composition of each major anion/cation pair; Drever, 1998) being represented by at least one spring. Many of the springs were Na^+/K^+ dominated, and many were also dominated by HCO_3^-. Major ion concentrations ranged from two to three orders of magnitude difference in values (Table S1), which was also indicated by a wide range in salinities (as total dissolved solids, TDS). TDS also ranged several orders of magnitude across samples (Fig. 7, Fig. S1).

Higher TDS values are generally typical of endogenic waters, and samples also exhibited a wide range for geochemical markers of endogenic fluid input. R_c/R_a values (indicating the amount of mantle-derived ^3He) provide the best estimate of mantle-derived fluids, but were not available for every spring. However, the available R_c/R_a ratios were generally higher than the minimum threshold of 0.1 to infer the presence of mantle-derived fluids (Karlstrom *et al.*, 2013), with a range of 0.06–1.28 and an average of 0.37. High CO_2 is also an indicator of mantle-derived fluids, although the sources of CO_2 in waters can also be attributed to other, non-endogenic fluid sources. Using $\delta^{13}C$ values of CO_2 to assess the origin of the CO_2 in these waters yielded estimates of C derived from endogenic fluids (C_{endo}) ranging between 0 and 97.9%, with a mean of 73%. Excluding one site, Conundrum Hot Spring, the minimum percentage of DIC contributed from C_{endo} was 52.5% among the 13 springs with $\delta^{13}C$ data. However, because these values were also only available for a subset of our dataset, correlates to R_c/R_a were sought among the parameters that were measured for every spring. Of all 25 geochemical variables with data for nearly every sample, only log (Na) and the percent of DIC attributable to C_{ext} (amount of C not attributable to carbonate dissolution, but rather to other external sources) were significantly ($P \leq 0.05$) correlated with R_c/R_a ($r^2 = 0.2466$ and 0.2373, respectively). log pCO_2, although not significant at the 0.05 alpha level ($P = 0.055$), was nearly as strong of a correlate as log (Na) and % C_{ext}, with $r^2 = 0.2106$. In general, the five groups defined by microbial community analyses exhibited differential levels of markers for endogenic fluid input. For example, samples from groups D and E exhibited higher pCO_2, lower pH, higher TDS, higher levels of some trace metals, and higher SO_4^{2-} (Fig. 7, Fig. S3). In contrast, groups A, B, and C generally exhibited lower pCO_2, higher pH, lower TDS, lower SO_4^{2-}, and lower concentrations of trace metals.

Archaeal community composition

Archaeal 16S rRNA genes were detected in only four samples: Cebolla Hot Spring (CEB1), two springs at the Tierra Amarilla site (TAE1A and TAE1B), and at the MudPit site in the Travertine Grotto (MP06B). Of the

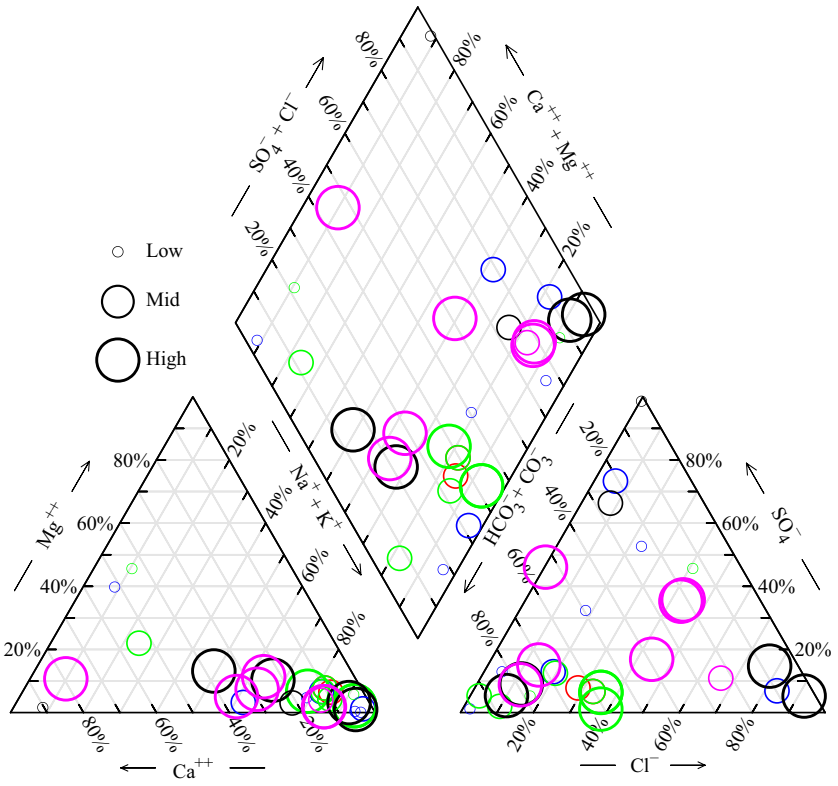

Fig. 2 Piper diagram illustrating major cation and anion compositions of spring waters. Cation and anion compositions are projected onto a diamond, where each sample's point is scaled to log pCO_2 as groups of low (−3.7 to −1.8) mid (−1.4 to −0.4) and high pCO_2 levels (−0.3 to 0.5). Samples are colored by groups from phylum-level differences in microbial communities as indicated in Fig. 4: A (red), B (blue), C (green), D (black), and E (pink).

283 total archaeal 16S rRNA gene sequences, 279 were classified as Thaumarchaeota and four as an unidentified division of the Euryarchaeota. Archaea from the Tierra Amarilla sites grouped only with *Nitrosopumilus* strains (Fig. 3). MP06B harbored several thaumarchaeal phylotypes including sequences closely related to mesophilic *Cenarchaeum* and uncultured 1.1A phylotypes, moderately thermophilic Nitrososphaerales, and an uncultured group of thaumarchaeotes including a hydrothermal fluid clone from the Mariana Trough (Papm3A65) that is affiliated with the marine benthic group of Archaea (La Cono *et al*., 2009). Cebolla Hot Spring also harbored thaumarchaeal phylotypes in the marine benthic group and a phylotype closely related to the 1.1b/Nitrososphaerales group. One Cebolla Hot Spring phylotype (CEB1_c09) was distantly related to other clones (≤87% 16S rRNA gene sequence identity to two clones from hydrothermal vents at the Southern Mariana Trough) and cultured Euryarchaeota (≤81% 16S rRNA gene sequence identity to the nearest *Methanothermus* spp. and *Methanothermobacter* spp. isolates by BLAST). Despite the limited number of clades represented by these libraries, each site harbored a different archaeal community. Only three of the nine genus-level OTUs (≥95% identity in 16S rRNA gene sequence) were found in more than one site. The two springs from the Tierra Amarilla site shared two OTUs affiliated with *Nitrosopumilus* with similar abundances of both OTUs in each site. Cebolla Hot Spring and the MudPit sample also shared an OTU with closest affiliation to *Nitrososphaera* (97% nucleotide identity by BLAST).

Bacterial community composition

Thirty-one 16S rRNA gene clone libraries were constructed from samples collected at 28 temperate spring sites, resulting in 1966 DNA sequences (24–85 16S rRNA genes/sample, mean = 63). Thirty-two established phyla and candidate divisions were identified in the springs, and those phyla/divisions with four or more OTU representatives are labeled in Fig. 4 (individual OTU identities are listed in Table S2). In addition, ~15% of the OTUs (~6% of the total 16S rRNA gene sequences) could not be classified at the phylum level. Four samples accounted for the majority of sequences (71% of all 16S rRNA gene sequences) that could not be classified into a known phylum: BB2 (14%), MP06B (9%), TAE1A (17%), and TAE1B (31%). While nearest GenBank matches were quite varied among the unclassified OTUs, many of the Tierra Amarilla sites' sequences were related to uncultured clones (87–96%, GenBank accession numbers JF320724) from Loihi seamount hydrothermal vents and Zetaproteobacteria isolates in the genus *Mariprofundus* (87–91%, HQ206653, JF317957, F493243). Proteobacteria (all five subdivisions) were the most prevalent phylum and

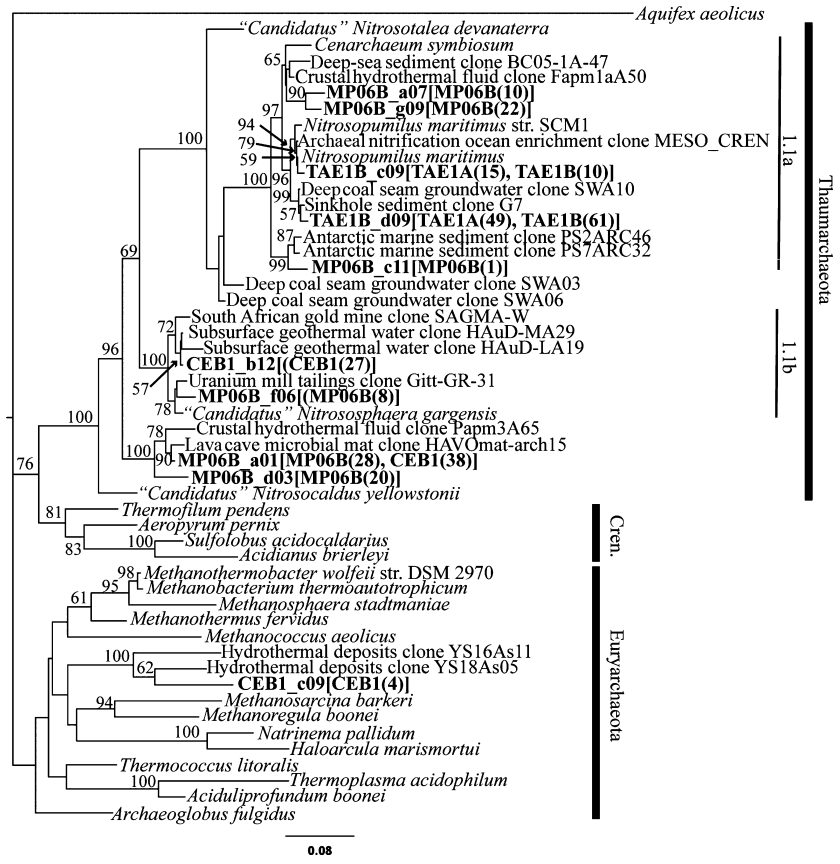

Fig. 3 Phylogeny of archaeal 16S rRNA gene sequences found in MP06B, CEB1, TAE1A, and TAE1B springs. Bold names indicate unique sequences obtained in this study with the number of clones mapping to each unique sequence in parentheses.

accounted for 81.2% of the total dataset. The γ-subdivision accounted for 51.8% of the total dataset, followed by Betaproteobacteria (17.0%), Alphaproteobacteria (10.4%), bacteria not able to be assigned to a phylum (unclassified; 6.3%), and Bacteroidetes (4.1%). In addition, Gammaproteobacteria were found at every site except Cebolla Hot Spring (CEB1).

Each spring generally had a unique OTU composition with few OTUs shared among springs. Of the total 441 genus-level OTUs, 375 were found in only one sample, and only 63 OTUs were shared by samples from different springs (Fig. 5). Observed richness was highly variable overall, with Chao1 estimates of richness ranging from 7 to 276 OTUs per sample (Table 1). However, sample coverage was generally high, with only nine samples having <80% estimated coverage of diversity (Table 1). Clustering samples by differences in phylum-level relative abundances revealed five distinct clusters of samples (groups A–E, Fig. 4). The grouping scheme was significant by per MANOVA test ($r^2 = 0.66$, $P < 0.001$). Each of the clusters was significantly associated with a bacterial phylum/subphylum ($P < 0.05$) with the exception of group A, which only contained two samples from the same site. Only five OTUs were significant indicators of any of the groups. Alphaproteobacteria were significantly associated with group B, along with two OTUs that had high relation to a *Sphingomonas melonis* strain (KF542913, 100%) and an uncultured bacterium associated with sugar beet roots (AB810826, 100%, clone SU_F12). Gammaproteobacteria and Chloroflexi were significant phylum-level indicators of groups C and D, respectively, but there were not any OTUs significantly associated with either group. The Betaproteobacteria subphylum was a significant indicator of group E, and two OTUs significantly associated with this group were most closely related to uncultured clones from an iron oxidizing biofilm (GenBank accession number FJ037618, 96% similarity in nucleotide sequence, clone: 070125-BRIC7-5) and a carbonate aquifer (KC437184, 98%, clone: 11D).

Correlation between microbial communities and environmental variables

The final NMS ordination was a three-dimensional solution with a stress value of 14.17. Although stress values <10 are preferred, values <20 are acceptable solutions and useful for ecological data (McCune & Grace, 2002). There were significant correlations ($P \leq 0.05$) between several chemical variables and the first two axes of the ordination (Fig. 6). Mg concentration, modeled equilibrium pCO2, and pH were similarly the strongest correlates of all variables ($r^2 = 0.3081$, 0.3007, and 0.3004, respectively). Tempera-

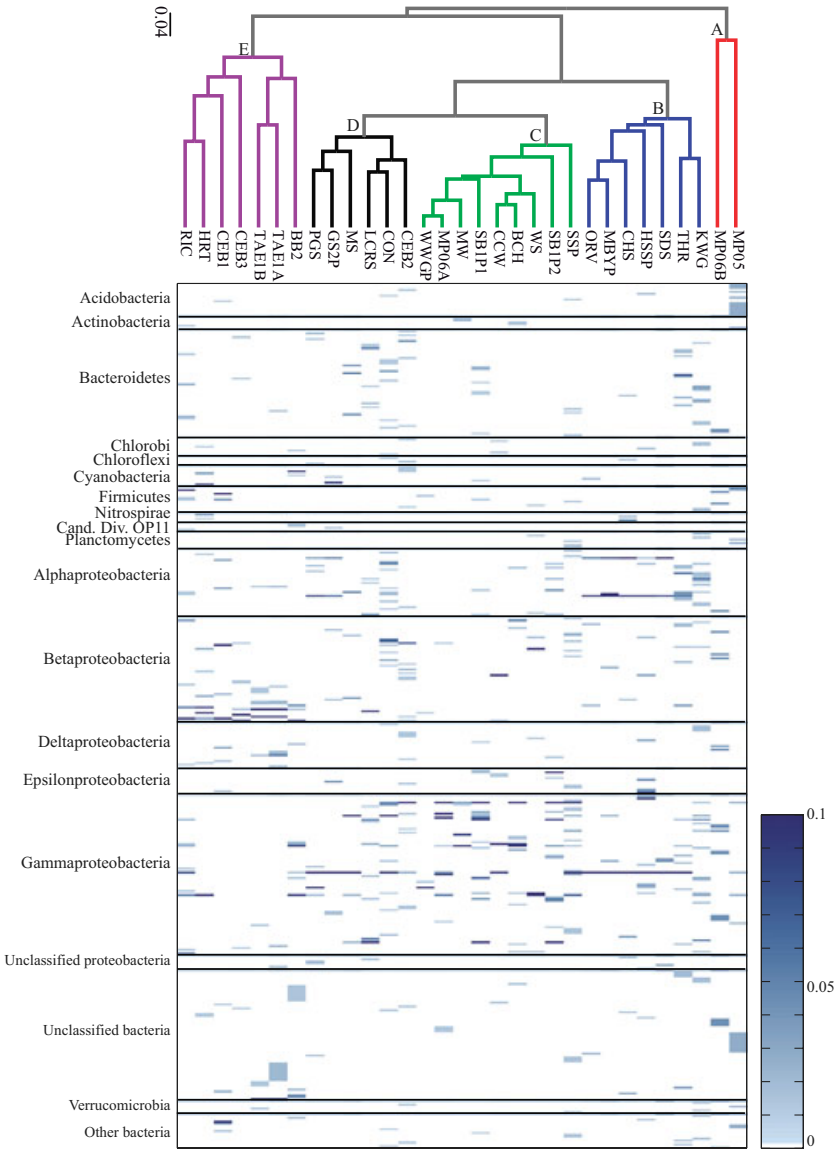

Fig. 4 Heatmap of 16S rRNA gene abundance in each of the samples ordered by sample clustering horizontally and taxonomic classification vertically. Dendrogram of sample clustering by phylum-level differences in community composition is shown above the heatmap with each cluster colored according to the group indicated at the node of each cluster. Each row in the heatmap represents a genus-level (≥95% similarity in 16S rRNA gene identity) OTU, and OTU color is scaled to the relative abundance within each sample. All taxa listed are phylum-level classifications except the subdivisions of Proteobacteria. Each OTU's classification and abundance across all groups is listed in Table S2.

ture was the next strongest correlate ($r^2 = 0.2846$) followed by F ($r^2 = 0.2568$), DIC_{total} ($r^2 = 0.2478$), C_{ext} ($r^2 = 0.2426$), and alkalinity as HCO_3^- ($r^2 = 0.2193$). Arsenic and lithium, while not significant at the 0.05 alpha level ($P = 0.06$ for both), were also moderately correlated with the ordination ($r^2 = 0.1955$ and 0.1919, respectively). Limited correlational analyses for a subset of samples where R_c/R_a and C_{endo} were measured suggested no correlation between community composition and either of the two values. However, these data were only available for 17 and 13 springs, respectively, which limited the power of the statistical analysis, as noted by the lack of significant correlation between any variables and community composition when these reduced datasets were used (data not shown).

Several physicochemical parameters were significantly different among at least two of the five groups from the phyla-level clusterings. Ca, Mg, K, alkalinity (as HCO_3^-), SO_4^{2-}, pH, TDS, Ba, Mn, Sr, pCO_2, DIC, and C_{ext} were all statistically significant ($P < 0.05$) between at least two groups. In general, box plot diagrams suggested that groups D and E exhibited different geochemistries than the other three groups, with generally higher values for Ca, Mg, K, alkalinity, SO_4^{2-}, TDS, Mn, Sr, pCO_2, DIC, and C_{ext} and lower values for pH and Ba (Fig. 7). However, indicator analysis suggested that only two of those geochemical parameters, pCO_2 and Mn, were significant indicators of any specific group–both being indicators of group E. Mn, however, appeared to be significantly abundant in group D, but to a greater extent in group E. Similarly, Fe was not detected in group A and at only one site in both groups B and C, but had median values of 0.03 and 0.15 ppm in groups D and

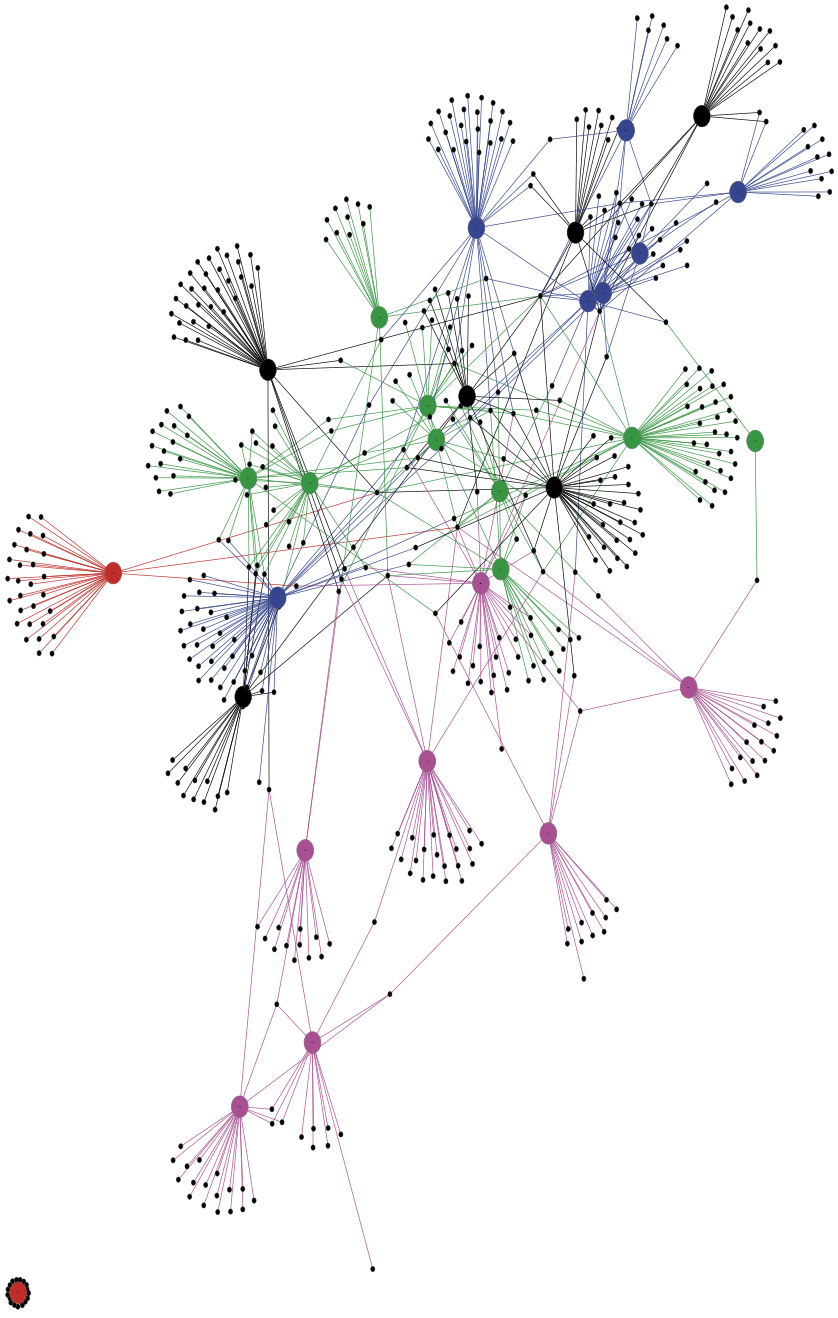

Fig. 5 OTU network of all sites, with samples as larger nodes and OTUs as smaller black nodes. Sample nodes and OTU edges are colored by groups based on phylum-level differences in microbial communities, as indicated in Fig. 4: A (red), B (blue), C (green), D (black), and E (pink). Sample MP05 did not share OTUs with any other sample and is demarcated as an isolated node in the bottom left corner.

E, respectively (Fig. S3). However, Fe was not significantly different among groups by ANOVA, nor was it significantly associated with any one group by indicator analysis. Geologic province of spring site was not significantly correlated with community composition ($P = 0.13$, $r^2 = 0.17$).

DISCUSSION

The microbial taxonomic assemblages and richness present among the 31 carbonic spring samples analyzed here varied considerably and suggested that many sites harbor distinct populations from one another, despite many originating from the Aspen Anomaly province. Although many of the springs contained geochemical markers of endogenic fluid input (much higher than atmospheric pCO_2, R_c/R_a values typical of mantle-derived fluid mixing), the degree of surface and deep fluid mixing with considerable heterogeneity in deep fluid endmember chemistry likely contributes to varied chemistry and microbial communities across the sites. The springs shared some bacterial taxonomic similarities with typical freshwater

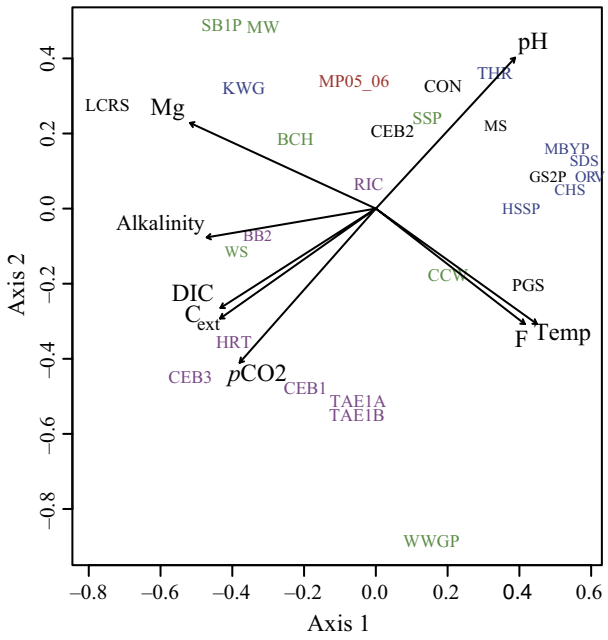

Fig. 6 NMS ordination plot of planktonic bacterial communities by genus-level OTU (≥95% similarity in 16S rRNA gene identity) composition. Only the first two axes are shown. Environmental correlates to ordination positions are given for significant ($P \leq 0.05$) correlations by arrows indicating the directionality and relative magnitude of the correlations to the plot. Sample names are colored by the groups they cluster into based on phylum-level community differences, as indicated in Fig. 4.

systems, but appear to be different overall. For example, lakes and rivers are often dominated by Betaproteobacteria, Actinobacteria, Bacteroidetes, and Alphaproteobacteria (Glockner *et al.*, 2000; Zwart *et al.*, 2002; Cottrell *et al.*, 2005; Oh *et al.*, 2011; Martinez-Garcia *et al.*, 2012). Betaproteobacteria were the second most abundant division among our samples, but they were subdominant to the Gammaproteobacteria and were found with other lineages not typical of freshwater bacterial communities, such as uncultured Zetaproteobacteria-like organisms and Epsilonproteobacteria. Further, top blast matches for Betaproteobacterial OTUs suggested close relations to environmental clones largely from anaerobic bioreactors, iron-rich biofilms, thermal and temperate springs, and anoxic freshwater sediments rather than typical freshwater lineages (Table S3). Bacteria that could not be classified into a described phylum made up a small percentage of the over-

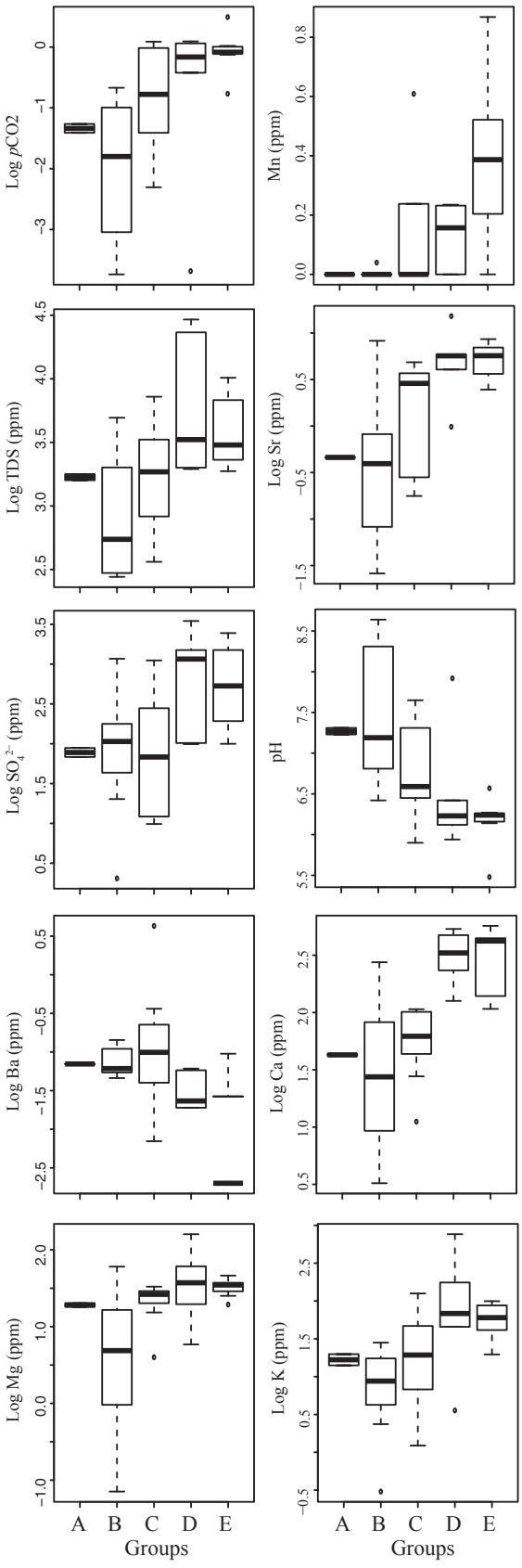

Fig. 7 Box plots of physicochemical parameters significantly different among the five phylum-level groups of our analysis. The median for each group is shown as a dark line in between quartiles. Range maximum and minimums are shown as whiskers, with outliers as open circles beyond these values. Although alkalinity, DIC, and C_{ext} were also significantly associated with OTU composition, these variables are largely redundant with log pCO_2 and are not represented here.

all community composition but provide evidence that there may be novel populations in those springs with high levels of endogenic fluid circulation. Bacteria unable to be assigned to phyla were distributed highly disproportionately, with four springs containing a large majority of these bacteria. Three of the four springs with greater abundance of unclassified bacteria (BB2, TAE1A, and TAE1B) belonged to group E, which was characterized as having the highest pCO_2 levels. In addition, Cebolla Hot Spring (CEB1; also in group E) contained a small population of Euryarchaea that were distantly related to clones from marine vents and potentially represent a previously undescribed archaeal lineage. Archaeal diversity, overall, was limited to only four springs with most Archaea classified as moderately thermophilic and mesophilic lineages within the Thaumarchaeota. While the metabolic diversity of Thaumarchaeota is an ongoing area of investigation, all cultured Thaumarchaea are ammonium oxidizers and are either autotrophic or mixotrophic (Konneke *et al.*, 2005; De La Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008; Lehtovirta-Morley *et al.*, 2011), suggesting a role in N cycling, and potentially primary production, within these spring communities.

Despite the distinctiveness of each spring bacterial community, OTU composition was correlated with environmental parameters known to structure communities in other systems, as well as parameters associated with endogenic fluid mixing. Temperature and pH, which were significantly correlated with community composition in our samples, can structure microbial communities in diverse habitats including thermal springs (Allewalt *et al.*, 2006; Miller *et al.*, 2009; Mitchell, 2009; Boyd *et al.*, 2010; Inskeep *et al.*, 2010), soils (Lauber *et al.*, 2009), and freshwater lakes (Lindstrom *et al.*, 2005). While temperature and pH are important microbial community structuring factors globally, in the springs described here, lowered pH is also mechanistically tied to increased endogenic fluid input (Crossey *et al.*, 2006). One of the strongest correlates to OTU community composition was modeled equilibrium pCO_2 (along with other equivalent measures of DIC). pCO_2 was also one of the best correlates to R_c/R_a, suggesting that pCO_2 was an adequate proxy for increased mantle-derived fluid input because R_c/R_a values were not available for every site. The correlation between pCO_2 and OTU composition, although moderate, suggests that increased input of deeply sourced fluids is structuring community composition. However, because correlational analysis can only suggest putative influences on community structure, we clustered the samples into groups based on phylum-level differences to more directly test the effects of physicochemical variables on community structure.

Several physicochemical parameters were significantly different among cluster groups by ANOVA test, and most suggested that the overall chemistry of groups D and E were different than that of groups A–C (Fig. 7). Many of the significant parameters suggested that group D and group E springs exhibited geochemistry more consistent with higher endogenic fluid input or less mixing with surficial circulated waters. For instance, group D and group E springs had high pCO_2 values coincident with lower pH, higher TDS, and higher levels of some trace metals like Mn, Fe, and Li (although Fe and Li were not significant by ANOVA), which are all indicators of endogenic fluid influence (Newell *et al.*, 2005; Crossey *et al.*, 2009; Williams *et al.*, 2013). Additionally, the higher levels of SO_4^{2-} and TDS in groups D and E may reflect the presence of reduced, saline groundwaters, likely impacted by increased endogenic fluid circulation, as is the case in some regions of the Colorado Plateau (Crossey *et al.*, 2009). Although indicator analysis only detected two OTUs associated with group E, some of the taxa that are present in this group suggest a linkage between the reduced fluids in these springs and the microbial communities. One of the OTU indicators of group E was associated with a carbonate aquifer, and the other, an unclassified Betaproteobacteria, was related to a clone detected in an iron oxidizing biofilm at a depth of 297 m in the Aspo hard rock tunnel observatory. Zetaproteobacteria-like bacteria were also detected in the Tierra Amarilla springs of group E, which are presently only known to oxidize Fe and are generally associated with the reduced fluids near warm marine vents (McAllister *et al.*, 2011). The presence of these Beta- and Zetaproteobacteria-like populations hints at the presence of Fe metabolizing, chemolithotrophic communities inhabiting some springs that are highly impacted by endogenic fluids. It should, however, be noted that the bacteria detected here were not of high (>97%) sequence relation to either the Beta- or Zetaproteobacteria entries in GenBank and further metabolic and phylogenetic analysis would be necessary to confidently assign their *in situ* activity.

In contrast, groups B and C exhibited geochemical and microbiological evidence of either less endogenic input or more mixing with surficial waters than groups D and E. Group B and C springs were from several provinces, but shared some microbial taxa. pCO_2 levels closer to atmospheric values (along with higher pH, lower metals, TDS and SO_4^{2-}) suggest that many of the springs from these groups are subject to either more mixing with surficial waters or less endogenic input overall than those springs of groups D and E. Both bacterial subdivisions that were indicators of groups B and C, the Alpha- and Gammaproteobacteria, respectively, are large and extremely metabolically diverse lineages. However, both significant OTU indicators of group B, a strain of *S. melonis* and a bacterium with high identity to a clone associated with the root of sugar beet (and classified as a *Methylobacterium* sp.), are ubiquitous soil- and freshwater-associated aerobic heterotrophs (Balkwill *et al.*, 2006; Green, 2006). Their wide-

spread presence in group B may indicate mixing of waters with shallow soil- or surface-associated waters. Although no OTUs were significantly associated with group C, six of the nine sites contained a Gammaproteobacterium classified as an *Aeromonas* sp. in 4–24% abundance. *Aeromonas*, like the Alphaproteobacteria associated with group B, are aerobic heterotrophs ubiquitously distributed in freshwater environments (Farmer III *et al.*, 2006). The presence of environmentally ubiquitous aerobic heterotrophs, in concert with the geochemical results for groups B and C, suggests a decreased role of endogenic input in the ecosystems of the springs in these groups. However, the presence of Delta- and Epsilonproteobacteria in many of these communities suggests that deeper sourced fluid mixing may also influence community composition. For example, the Epsilonproteobacteria orders such as the Sulfurovumales and Sulfuricurvales, which generally oxidize-reduced S species, like S^o and H_2S (Campbell *et al.*, 2006), were present in several sites (Steamboat Springs, Colonel Chins Hot Well, Saratoga Springs, and Hot Sulfur Springs). This suggests either that there are reduced S compounds available for metabolic use in the springs or that the populations are active in the subsurface but are being conveyed to the surface with the deep fluids. In either case, their presence, even at low population levels, hints at the presence of reduced fluids in the subsurface, which are likely influenced, to some degree, by endogenic fluid input.

CONCLUSIONS

In conclusion we found a wide diversity of bacteria and Archaea inhabiting mesothermal and slightly thermal artesian springs throughout five provinces in the western United States that are subject to differential inputs of deeply sourced fluids. Taxonomically, the spring communities were heterogeneous but overall exhibited community compositions atypical of freshwater systems. Despite the heterogeneity in microbial communities, our analyses suggest that springs influenced to a greater extent by deeply sourced, mantle-derived fluid harbor different (and potentially chemolithotrophic) communities, compared with less influenced springs. Novel phylogenetic lineages of both bacteria and Archaea, although both as minor populations and as highly disproportionately distributed, suggest spring environments highly influenced by endogenic fluids can provide opportunities for biodiversity discovery and a greater understanding of continental ecosystems that bridge reduced subsurface conditions and surficial oxidizing conditions.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Piper diagram illustrating major cation and anion composition of spring waters, scaled to TDS.

Fig. S2. Dendrogram of community differences based on OTU differences in samples.

Fig. S3. Boxplots of physicochemical parameters that were not significantly different between phylum-level defined groups.

Table S1. Excel file of geochemical data and site information for all springs. n.d. indicates not detected.

Table S2. Excel file with corresponding taxonomic information for heatmap in Fig. 4.

Table S3. Excel file with best BLAST hit matches of OTUs that were classified as Betaproteobacteria.