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RESEARCH ARTICLE

Ecological differentiation in planktonic and sediment-associated chemotrophic microbial populations in Yellowstone hot springs

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One sentence summary: The presence of minerals, such as elemental sulfur, that can support microbial metabolism promotes the ecological differentiation of sediment- and planktonic-associated microbial populations within Yellowstone National Park hot springs.

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ABSTRACT

Chemosynthetic sediment and planktonic community composition and sizes, aqueous geochemistry and sediment mineralogy were determined in 15 non-photosynthetic hot springs in Yellowstone National Park (YNP). These data were used to evaluate the hypothesis that differences in the availability of dissolved or mineral substrates in the bulk fluids or sediments within springs coincides with ecologically differentiated microbial communities and their populations. Planktonic and sediment-associated communities exhibited differing ecological characteristics including community sizes, evenness and richness. pH and temperature influenced microbial community composition among springs, but within-spring partitioning of taxa into sediment or planktonic communities was widespread, statistically supported ($P < 0.05$) and could be best explained by the inferred metabolic strategies of the partitioned taxa. Microaerophilic genera of the Aquificales predominated in many of the planktonic communities. In contrast, taxa capable of mineral-based metabolism such as S° oxidation/reduction or Fe-oxide reduction predominated in sediment communities. These results indicate that ecological differentiation within thermal spring habitats is common across a range of spring geochemistry and is influenced by the availability of dissolved nutrients and minerals that can be used in metabolism.

Keywords: Yellowstone hot springs; chemotroph; thermophiles; archaea; Aquificales; ecological differentiation

INTRODUCTION

Life in environments with temperatures that exceed the upper limit of photosynthesis (~73°C) is supported by chemical energy (Brock 1967; Boyd *et al.* 2010, 2012; Cox, Shock and Havig 2011; Hamilton *et al.* 2012). In volcanic hydrothermal ecosystems, chemical energy in the form of volatiles from magmatic degassing as well as in the form of solutes derived from water–rock interactions supports microbial communities (Amend and Shock 2001; Inskeep *et al.* 2005; Spear *et al.* 2005;

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Shock *et al.* 2010). Variations in the extent of subsurface water–rock interactions and magmatic degassing exert strong controls on the geochemistry of hot springs, which in turn shapes the taxonomic and functional composition of chemotrophic microbial communities (Amenabar, Urschel and Boyd 2015). For example, molecular analyses of sediment-associated communities inhabiting high-temperature hydrothermal environments that span a wide range of geochemical conditions reveal patterns in the distribution of organisms and their metabolic potential along geochemical gradients. In particular, gradients in pH and temperature appear to be the primary controls on the structure and composition of chemotrophic communities, with secondary controls including the availability of dissolved nutrients (Inskeep *et al.* 2005, 2013; Meyer-Dombard, Shock and Amend 2005; Swingley *et al.* 2012; Alsop, Boyd and Raymond 2014).

Hot spring sediments are heterogeneous in composition, both within a given hot spring (Langner *et al.* 2001; Macur *et al.* 2004; Hurwitz and Lowenstern 2014) and among hot spring systems (Inskeep *et al.* 2005; Huang *et al.* 2011; Wang *et al.* 2014). These include differences in sediment mineralogy as well as the availability of nutrients in sediment porewaters. In the latter case, microscale (mm or less) vertical gradients in the availability of key nutrients, in particular O_2 and H_2S, were observed in sediments in high-temperature, chemotrophic hot spring environments, likely due to microbial activity (Revsbech and Ward 1984; D'imperio *et al.* 2008; Bernstein *et al.* 2013). Moreover, hot spring sediments often comprise minerals that can serve as electron donors or acceptors for microbial life. These include elemental sulfur (Jackson *et al.* 2001; Boyd *et al.* 2007; Kamyshny *et al.* 2014), iron oxides (Kashefi *et al.* 2002; Inskeep *et al.* 2004; Wu *et al.* 2013) and iron-sulfides (Livo *et al.* 2007; Kozubal *et al.* 2008), among others. Thermophiles capable of reducing or oxidizing elemental sulfur have been shown to dominate hot spring microbial communities (Brock *et al.* 1972; Boyd *et al.* 2007; Boyd, Leavitt and Geesey 2009; Reysenbach *et al.* 2009; Takacs-Vesbach *et al.* 2013). Similar observations have been made for organisms capable of reducing iron oxides or oxidizing Fe (II) (Lovley, Holmes and Nevin 2004; Slobodkin 2005; Yoshida *et al.* 2006; Kozubal *et al.* 2008; Slobodkina *et al.* 2012) and/or manganese oxides (Boone *et al.* 1995; Greene, Patel and Sheehy 1997; Slobodkina *et al.* 2012). Together, these observations suggest that variation in the availability of dissolved and mineral-based nutrients both between springs and within springs are likely to (i) further influence the distribution and abundance of taxa within sediment-associated microbial communities and (ii) lead to their differentiation from free-living (i.e. planktonic) communities.

Ecologically differentiated planktonic and sediment-associated populations have been described in microbial communities inhabiting globally distributed marine and freshwater systems (Lozupone and Knight 2007; Zinger *et al.* 2011). More recent studies also suggest that planktonic- and sediment-associated populations in geothermal communities may be ecologically differentiated. For example, the composition of a planktonic community in Great Boiling Spring, Nevada was distinct from that associated with several sediment samples taken from the perimeter of the spring, in particular the abundance of a *Thermocrinis* phylotype in the planktonic phase (Cole *et al.* 2013). Likewise, sediment-associated communities sampled from hot springs in the Tengchong geothermal area (Yunnan Province, China) appeared to vary more in their taxonomic composition over a seasonal cycle than their planktonic counterparts (Wang *et al.* 2014). Other analyses of Tengchong area spring sediment- and water-associated microbial communities revealed varying extents of community-level differentiation between the two habitats, which may be partially explained by spring residence time (Hou *et al.* 2013). Regardless of the mechanisms leading to habitat differentiation, these studies support the notion that populations comprising planktonic and sediment-associated communities can be ecologically differentiated within thermal spring ecosystems both spatially and temporally. However, it is unclear if within-spring ecological differentiation is due to differences in the availability of dissolved or mineral-based nutrients or whether the level of differentiation between planktonic and sediment-associated communities and their populations is system specific.

In the present study, we hypothesized that populations comprising planktonic and sediment-associated communities are differentiated in Yellowstone National Park (YNP) thermal springs, in particular in chemosynthetic microbial communities that are dependent on chemical energy supplied by their local environment. Moreover, we hypothesized that the level of within-spring planktonic/sediment differentiation is secondary to primary effects imposed by overall geochemical regime on the distribution and abundance of taxa. However, we hypothesized that planktonic community composition will be more reflective of aqueous geochemistry than sediment-associated communities and that sediment-associated communities will be more reflective of mineralogy than planktonic communities, especially in springs containing solid phase minerals that can serve as electron donors or acceptors. To evaluate these interrelated hypotheses, we used Illumina multiplex paired-end tag sequencing to assess 16S rRNA gene community composition and quantitative PCR of 16S rRNA genes to estimate population sizes in sediment and planktonic chemosynthetic communities in 15 springs in YNP. These data were analyzed and interpreted within a multivariate statistical framework that integrated dissolved geochemical and solid phase sediment mineralogical data. The results reveal evidence for ecological differentiation among populations comprising sediment and planktonic chemotrophic communities and suggest that a primary driving force behind differentiation is the availability of dissolved and mineral substrates capable of supporting microbial metabolism.

MATERIALS AND METHODS

X-ray diffraction

X-ray diffraction (XRD) analyses were carried out using a Rigaku Rapid II X-ray diffraction system with a 2-D image plate (Mo $K\alpha$ radiation). Resultant 2-D patterns were converted to 1-D X-ray powder diffraction patterns using Rigaku's 2DP program. The samples were analyzed using reflection mode in order to detect non-crystalline silica phases. The results are presented in binary format for the presence or absence of the detected minerals for each sample.

Geochemical analyses

Spring temperature and pH were determined with a portable pH meter and temperature-compensated probe (WTW 3300i or 3110; WTW, Weilheim, Germany) that was calibrated daily with standardized buffer solutions. Conductivity was measured with a YSI model 30 meter (YSI, Yellow Springs, OH, USA). Dissolved oxygen, Fe (II), SiO_2 (aq) and total sulfide were determined colorimetrically in the field with a portable Hach spectrophotometer (model DR2400 or 2800) and Hach reagents

(Hach Company, Loveland, CO, USA). Dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) analyses were performed on filtered (0.2 μm) water samples stored in separate 40-mL amber glass vials. For DOC, the vials were heated in a muffle furnace at 500°C and spiked with 100 μL of 85% phosphoric acid, while DIC vials were soaked in a 10% nitric acid bath overnight, rinsed multiple times with deionized water and dried in a laminar flow hood. The vials were capped with Teflon-lined septa (DOC) or butyl rubber septa (DIC) and stored at 4°C until processing. Analysis was completed on an OI Analytical Wet Oxidation Total Organic Carbon analyzer that is coupled to a Thermo Delta Plus Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Grand Island, NY, USA), as described in St-Jean (2003). Either sodium persulfate (DOC) or phosphoric acid (DIC) was added to generate CO_2 from each carbon pool, which was then separated via gas chromatography and quantified by mass spectrometry. Calibration for concentrations was conducted by constructing calibration curves with aqueous solutions of potassium hydrogen phthalate (DOC) or sodium bicarbonate (DIC).

Sample collection and DNA extraction

Two, 1-L polycarbonate bottles were rinsed three times with spring water from each spring prior to filling of each bottle. Filled bottles were capped, and water was allowed to cool to ~50°C before filtering each liter through a pre-sterilized (gamma irradiated) 0.22-μm Sterivex filtration unit (EMD Millipore, Billerica, MA, USA). Two replicate filters were collected for each spring (except Perpetual Spouter). The total volume of spring water that was filtered was recorded to standardize data across the filter sample set. Filters were placed in sterile containers and frozen on dry ice for transport to the lab where they were kept at −80°C prior to DNA extraction. Sediments for molecular analyses were collected aseptically using a flame-sterilized spatula or spoon, placed in 1.5-mL centrifuge tubes, and immediately flash-frozen on dry ice for transport to the lab, where they were kept at −80°C for use in molecular analyses.

In the lab, filters were cut open in a UV-sterilized laminar flow hood, using a flame sterilized jewelry saw. Filters were then removed from their housing using a flame sterilized scalpel and were placed in sterile FastDNA SPIN for Soil Kit bead-beating tubes (MP Biomedicals, Santa Ana, CA, USA) using flame sterilized forceps. Two blank filter cartridges were subjected to the same treatment and were carried through the entire DNA extraction and PCR steps (described below) to assess contamination. DNA was extracted from two replicate filters and two replicate sediment aliquots using the FastDNA SPIN Kit for Soils, as previously described (Boyd *et al.* 2007). Equal volumes of each replicate extract were pooled and genomic DNA was quantified using the Qubit DNA Assay kit (Thermo Fisher Scientific, Waltham, Massachusetts) and a Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

16S rRNA gene amplification and sequencing

Around 4 ng of purified genomic DNA was subjected to polymerase chain reaction (PCR) amplification of 16S rRNA genes using universal three-domain primers 515F and 806R (Caporaso *et al.* 2011). PCR was performed in triplicate with the following cycling conditions: initial denaturation at 94°C (4 min), followed by 35 cycles of denaturation at 94°C (1 min), annealing at 55°C (1 min), primer extension at 72°C (1.5 min) and a final extension step at 72°C for 20 min. Reaction vessels contained 1.5-mM $MgCl_2$ (Thermo Fisher Scientific), 200 μM each deoxynucleotide triphosphate (Eppendorf, Hamburg, Germany), 0.5-μM forward and reverse primer (Integrated DNA Technologies, Coralville, Iowa), 0.2 mg ml^{-1} molecular-grade bovine serum albumin (Roche, Indianapolis, IN, USA) and 0.25 units native *Taq* DNA polymerase (Thermo Fisher Scientific) in a final reaction volume of 50 μL. Following verification of PCR in a 1% agarose gel, an equal volume of each triplicate reaction was pooled and purified using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) for use in sequencing (see below).

Purified 16S rRNA gene amplicons were submitted to MrDNA (Shallowater, TX) for barcoding and sequencing. Briefly, amplicons were barcoded using primers 515F/806R (forward primer barcoded) and the HotStarTaq Plus Master mix Kit (Qiagen, Valencia, CA) under the following conditions: 94°C for 3 min followed by 5 cycles of 94°C for 30 s; 53°C for 40 s and 72°C for 1 min; after which, a final elongation step at 72°C for 5 min was performed. Following confirmation of PCR amplification via electrophoresis in a 2% gel, Illumina adapters were added. Libraries were generated using the 2 × 300 MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA).

A total of 2.2 million 16S rRNA gene sequences were generated from the sediment and filtered-water PCR amplicons via multiplexed, paired-end Illumina MiSeq tag sequencing. Post-sequencing processing was performed with Mothur (Schloss *et al.* 2009) as previously described (Hamilton *et al.* 2013) after merging the paired reads. Briefly, libraries were trimmed to a maximum length of 250 bases, filtered and trimmed, using a defined start site and an empirically determined end site based on inclusion of 85% of the total sequences. Chimeras were identified and removed using UCHIME (Edgar *et al.* 2011) and operational taxonomic units (OTUs) were assigned at a sequence similarity of ≥97% using the nearest-neighbor method. The remaining sequences were randomly sub-sampled to 9533 16S rRNA gene sequences per sample and representative sequence for each OTU was classified using the RDP classifier (Wang *et al.* 2007) and the most recent Greengenes reference database (McDonald *et al.* 2012) as implemented in QIIME (version 1.9; Caporaso *et al.* 2010). Manual BLASTn searches were conducted against the NCBI nr/nt database with representative OTU sequences to obtain better taxonomic resolution for high-abundance OTUs. Raw untrimmed sequence, quality score files and the mapping data were deposited in the NCBI SRA database, under the SRA accession SRP070467.

16S rRNA gene quantitative PCR (qPCR)

qPCR was used to estimate the number of 16S rRNA gene templates per unit dry mass (sediments) or per unit volume (filtered spring water), following previously developed methods (Boyd *et al.* 2011). Two 16S rRNA gene clones, generated as previously described (Boyd *et al.* 2007), were used to create standard curves to relate template copy number to the threshold qPCR amplification signal. The abundance of 16S rRNA gene clones used in generating the standard curves varied by less than a factor of 1.0 and thus was averaged for use in calculating the average template abundances and standard deviation in template abundances from replicate qPCRs. A standard curve was generated over 6 orders of magnitude from 4.6×10^3 to 4.6×10^9 copies of template per assay (Pearson $R^2 = 0.983$, 0.988 for filters and sediments, respectively). qPCR assays were performed in a CFX Connect quantitative real-time PCR machine (Bio-Rad Laboratories, Hercules, California) in 0.5 ml optically clear PCR tubes (Bio-Rad Laboratories) using a SsoAdvanced Universal SYBR

Table 1. Location and selected spring aqueous geochemistry measurements.[a,b,c]

ID	Name	GPS Coordinates	pH	Temp. (°C)	Cond. (μS)	Total Sulfide (μM)	DO (μM)	DOC (ppm)
Ca	'Forest Springs Ca'	44.712476, −110.469261	1.8	42.5	7270	48.2	62.5	3.00
Sb	'Geyser Creek Sb'	44.690460, −110.729550	2.5	71.9	2903	2.0	25	0.50
Dr	'Dragon Spring'[c]	44.731880, −110.711083	3.0	81.2	4140	121.3	28.8	0.98
Hf	'Norris Hf'	44.733245, −110.709712	3.1	89.2	4203	7.6	24.4	0.74
Ga	'Norris Ga'	44.727624, −110.715587	3.2	83.3	4140	8.8	43.8	0.49
Fi	'Mud Volcano Fi'	44.610039, −110.439430	3.8	62.0	4260	nm	nm	0.96
Ci	Cinder Pool	44.732440, −110.709800	4.6	89.4	5920	nm	nm	nm
Ob	Obsidian Pool	44.610018, −110.438827	4.6	74.7	1468	2.3	31.9	2.60
Sy	'Sylvan Spring'[c]	44.698927, −110.768588	5.4	79.3	5120	12.8	32.5	0.69
Ev	Evening Primrose	44.699437, −110.767147	5.5	78.6	5250	7.3	26.3	0.69
Em	'Geyser Creek Em'	44.687503, −110.727545	6.8	81.7	5730	16.4	20.0	0.52
Pe	Perpetual Spouter	44.726573, −110.709139	7.1	86.4	6130	0.7	31.9	0.31
Ba	Bat Pool	44.687054, −110.728418	7.2	89.5	8060	75.3	11.9	0.45
Bi	'Bison Pool'[c]	44.569626, −110.865181	7.5	88.5	3625	6.4	14.4	0.41
St[d]	Steep Cone	44.566670, −110.86320	7.7	90.7	1490	nm	nm	nm

[a]nm = not measured.
[b]Spring names demarcated by quotations are unofficial spring designations.
[c]Unofficial spring names are identified by their geothermal region of origin or by their first published designation: 'Dragon Spring' (Macur *et al.* 2004), 'Sylvan Spring' (Meyer-Dombard, Shock and Amend 2005) and 'Bison Pool' (Meyer-Dombard, Shock and Amend 2005).
[d]pH, temperature and conductivity values obtained from YNP RCN database.

Green Supermix qPCR Kit (Bio-Rad Laboratories). qPCR cycling conditions were as follows: an initial denaturing step at 98°C for 30 s followed by 35 cycles of 98°C for 30 s, specific annealing and elongation temperature for 1 min, followed by a melt curve of 65°C –95°C in 5 s/0.5°C step increments to determine specificity of the qPCR assays. Negative control assays were performed in the absence of template DNA. Each assay was performed in triplicate and the reported template abundances are the average and standard deviation of triplicate reactions for each DNA extract.

Community ecology and statistical analyses

Measures of 16S rRNA gene richness and diversity (observed OTUs, Simpson's evenness and Shannon/Simpson diversity indices) were calculated in QIIME on the subsampled OTU table. Community distance matrices were constructed using the Bray–Curtis distance for the entire dataset (sediment and planktonic communities), as well as for sediment or planktonic communities individually. Statistical analyses were conducted in R (version 3.2), using the vegan package version 3.2 (Oksanen *et al.* 2015), except where otherwise noted. A non-metric multidimensional scaling ordination (NMDS) was produced from the Bray–Curtis distance matrix (sediment + planktonic communities), using the 'metaMDS' function. The 'envfit' function was used to assess the correlation of environmental variables to the community ordination. Euclidean distance matrices (generated with the 'vegdist' function) were constructed for XRD data and individual environmental parameters in order to compare to sediment and planktonic community matrices individually. A Euclidean distance matrix was also constructed for the aqueous geochemistry data (excluding temperature and pH), after normalizing each parameter to its total value range (on a 0–1 scale; using the 'decostand' function), so that the distance matrix was not disproportionately weighted by parameters with greater numerical magnitude. Mantel regressions were used to test the co-variance of distance matrices, and the 'adonis' function was used to test the significance of categorical groupings of samples within matrices. SIMPER analysis was employed to analyze the OTUs that mostly contributed to community variation between habitats of the same pH realm and within habitats at different pH realms. Pearson's linear *R* was used to measure correlation strength among biological and geochemical measurements. Before measuring linear correlations, data were $\log_{10}$ transformed when distributions significantly deviated ($P \leq 0.05$) from normality as suggested by a Shapiro-Wilkes normality test in the base R statistics package.

RESULTS

Site descriptions

Sediment and planktonic microbial communities from 15 springs were collected from across YNP in order to sample a broad range of geochemical conditions that support chemosynthetic microbial communities (Table S1, Supporting Information; Supplementary File 1, Supporting Information). Spring temperatures ranged from 42.5°C to 90.7°C and pH ranged from 1.8 to 7.7 (Table 1). Pearson linear regression indicated that several of the geochemical measurements were significantly ($P \leq 0.05$) correlated to pH and temperature. For example, DOC (pH: $R = -0.48$, temp: $R = -0.74$), DO (pH: $R = -0.65$, temp: $R = -0.80$) and Fe (II) (pH: $R = -0.58$, temp: $R = -0.84$) all exhibited strong correlations with pH and temperature (Table S2, Supporting Information).

Sediment mineralogical composition exhibited a significant correlation with spring pH (Mantel $R = 0.36$, $P \leq 0.01$). Clustering of springs by mineralogical composition revealed three primary groups (Fig. 1): (i) elemental sulfur (S^0) containing-springs that included the most acidic springs and the two weakly acidic springs, Evening Primrose and 'Sylvan Spring', (ii) tridymite and alkali-feldspar-containing springs that lacked detectable S^0 ($3 < pH < 5$) and (iii) opal and tridymite-containing circumneutral springs ($pH > 7$). 'Geyser Creek Em' (Em; pH 6.8) did not group with other springs and sediments in this spring comprised a mixture of minerals that were identified in several of the acidic springs (alkali-feldspar, kaolinite and cristobalite).

16S rRNA gene abundance and taxonomic richness/structure

The abundance of 16S rRNA genes in planktonic communities varied over 6 orders of magnitude whereas variation in the

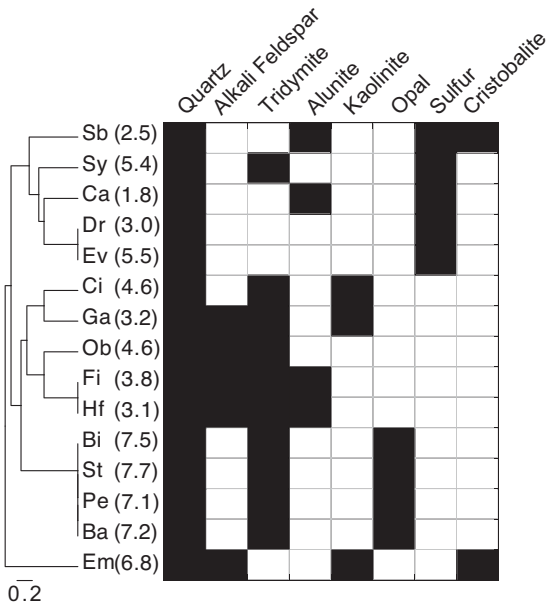

Figure 1. Sediment mineralogical distance dendrogram and corresponding mineralogical composition. UPGMA dendrogram of mineralogical compositional differences is shown on the left and spring pH is given next to each spring ID. The presence of each mineral constituent is indicated by a black rectangle. Scale shows Euclidean distance.

sediment communities spanned 4 orders of magnitude (Table S3, Supporting Information). Neither sediment nor planktonic 16S rRNA gene copy numbers exhibited a statistically significant linear correlation with any of the measured environmental parameters (Table S4, Supporting Information). However, the abundance of 16S rRNA genes in planktonic communities demonstrated a non-linear relationship with spring pH and temperature, with higher gene copies in springs with pH ~4–6 and with lower temperature (Table S3, Supporting Information; Fig. 2a).

16S rRNA gene OTU richness was generally higher in sediment communities when compared to planktonic communities and was not linearly correlated with any of the measured environmental parameters (Table S4, Supporting Information). However, OTU richness in planktonic communities exhibited a non-linear relationship with pH, with peaks in richness observed at the lowest and highest pH values (Table S3, Supporting Information; Fig. S1, Supporting Information). Planktonic community richness was significantly ($P \leq 0.05$) and inversely correlated to 16S rRNA gene copy abundance in hot spring waters ($R = -0.75$; Fig. 2b). Both Simpson and Shannon diversity indices exhibited similar relationships to pH and were both significantly correlated to the observed OTU richness ratio (Pearson $R = 0.61$ and 0.81, respectively, both $P \leq 0.05$) so were not included here for brevity. Simpson's evenness values for planktonic communities were also significantly and inversely correlated to 16S rRNA gene copy numbers in planktonic communities, and were lowest in the springs with the highest 16S rRNA gene copies ml^{-1} (Pearson $R = -0.52$, $P \leq 0.05$; Fig. 2c).

Overview of microbial community composition

A total of ~2.2 million paired-end Illumina MiSeq 16S rRNA gene sequences (9533 sequences per sample after subsampling, length ~250 bp), representing 3690 archaeal and bacterial OTUs, were recovered from sediment and planktonic communities sampled from 15 different springs. A total of 37 bacterial and three archaeal phyla (comprising 17 archaeal classes) were detected. The Aquificae, Crenarchaeota and Proteobacteria phyla constituted the majority of the sequences (32.1%, 21.3% and 21.2%, respectively), with the rest of the phyla each representing <10% of the total sequences. In addition to the three most-abundant phyla, several less abundant phyla comprised significant portions of individual communities or springs (Fig. 3). The five most-abundant OTUs (belonging to the genera *Thermocrinis, Pyrobaculum, Ralstonia, Sulfurihydrogenibium* and *Hydrogenobaculum*) represented 55.9% of the sequences.

While springs were selected for analysis on the basis of geochemical regimes that should suppress photosynthesis (e.g. Cox, Shock and Havig 2011; Boyd *et al.* 2012), three samples harbored subdominant bacterial and eukaryal (chloroplast) populations related to phototrophic organisms. An OTU classified as a *Phormidium* sp. (OTU024) was present in the planktonic community of Steep Cone (17% relative abundance), but no other OTUs within the Cyanobacteria, Chloroflexi or Chlorobi phyla were present in >~2% relative abundance of any sample. The presence of a *Phormidium*-like OTU in the planktonic community of Steep Cone is likely explained by exogenous input (e.g. wash-in from lower temperature mats nearby), as the sample site temperature (90.7°C), is well above the empirical upper temperature limit of light driven or photosynthetic activity in hot springs (Boyd *et al.* 2012). Moreover, the most closely related cultivated isolate to the *Phormidium*-like OTU, *Microcoleus chthonoplastes* (96% nt ID, Genbank accession: GQ402023.1), is widely distributed in mesic aquatic environments (Prufert-Bebout and Garcia-Pichel 1994), which also indicates an exogenous origin of this OTU.

Chloroplast sequences were filtered from the dataset to minimize the influence on community differences by exogenous sequences. However, analyses of sequences without filtering (data not shown) indicated that both the sediment and planktonic communities from 'Forest Springs Ca' contained appreciable abundances (20% and 41%, relative abundance, respectively) of 16S rRNA sequences affiliated with chloroplasts from the thermophilic red alga *Cyanidioschyzon merolae* str. DBV201. Because 'Forest Springs Ca' was the only spring community to contain putatively endogenous photosynthesizing Eukarya, the data presented in the analyses reported here have had the chloroplast sequence removed.

Community structure and habitat differentiation

Mantel tests were used to assess co-variance between community (sediment/planktonic) composition and variation in individual geochemical characteristics of springs. Sediment and planktonic communities significantly and strongly co-varied across the dataset (Mantel $R = 0.55$, $P \leq 0.05$). Mantel tests also indicated that sediment and planktonic community distances both significantly co-varied with pH ($R = 0.30$ for both, $P \leq 0.05$ for both) and spring temperature ($R = 0.25$ and 0.43 for sediments and aqueous communities, respectively, $P \leq 0.05$ for both). Neither sediment nor planktonic community distances significantly co-varied with other geochemical measurements or with sediment mineralogical composition (as assessed by X-ray diffraction).

An NMDS ordination of community compositional differences segregated samples by spring pH and temperature, and also segregated sediment and planktonic communities from the same spring (Fig. 4). The extent of the difference between sediment and planktonic community composition in individual springs was variable, with some springs not exhibiting any

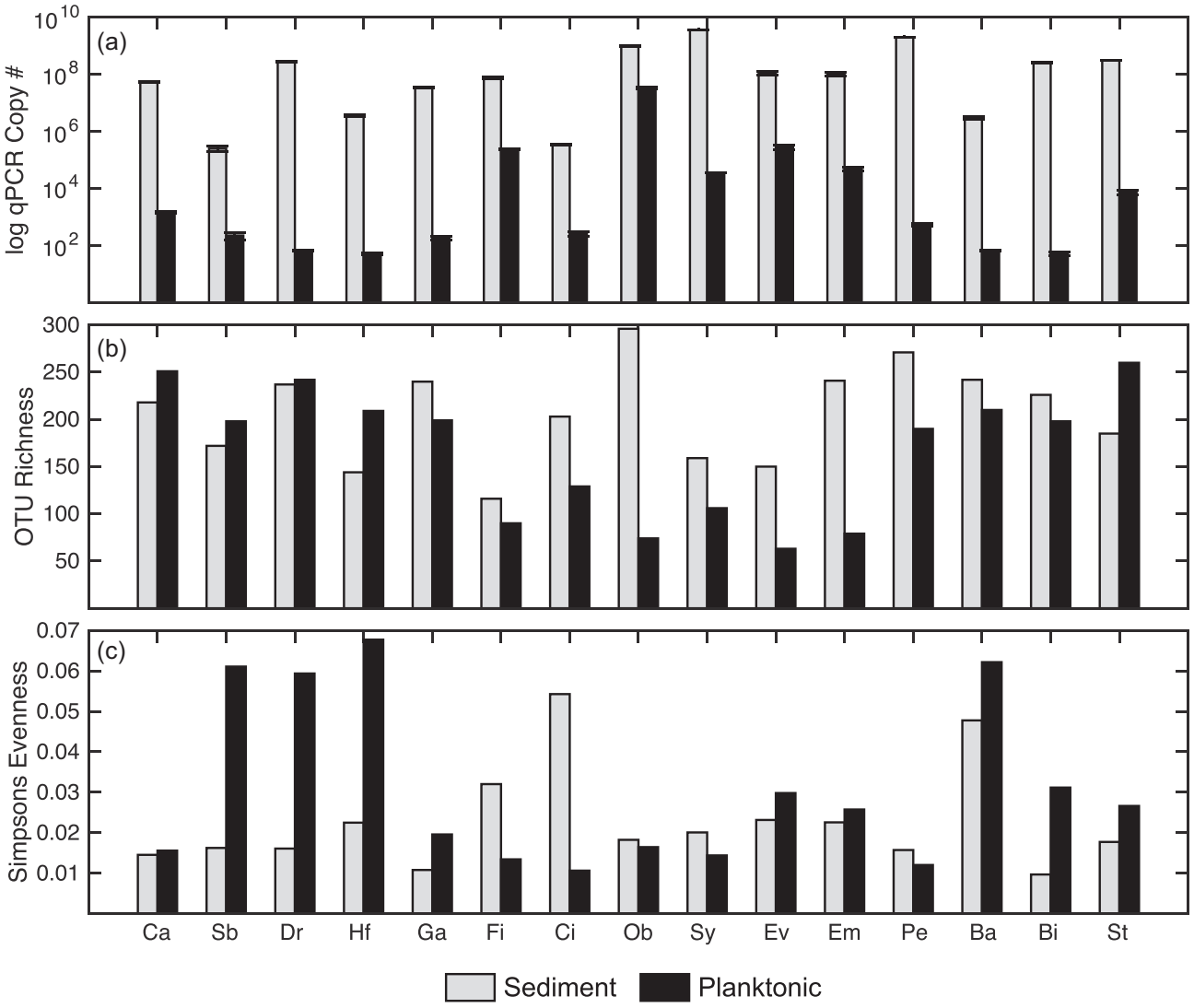

Figure 2. qPCR and community diversity measurements for each sample. (**a**) Sediment (gray) and planktonic (black) 16S rRNA gene copies g^{-1} and ml^{-1}, respectively. Standard error bars are overlaid for each bar. Springs are ordered from the lowest pH to the highest. (**b**) OTU richness and (**c**) community evenness for each spring sample, colored and ordered as in (a).

considerable difference in planktonic and sediment community composition and others exhibiting distances comparable to between-spring differences (Fig. 4). Similar to the results of the Mantel regression, both pH and temperature were significantly ($P \leq 0.05$) and strongly correlated to the community composition ordination ($R^2 = 0.54$ and 0.55, respectively). DOC ($R^2 = 0.51$) and SiO_2 (aq) ($R^2 = 0.47$) were also significantly correlated to the NMDS ordination. Spring site was a highly significant grouping for samples (adonis test, $R^2 = 0.65$ $P \leq 0.001$), whereas sample habitat (planktonic versus sediment) was not significantly correlated to community composition across the entire dataset (adonis test, $R^2 = 0.04$, $P \geq 0.05$).

Because pH and temperature appeared to exert such strong influence on community composition, habitat association was tested while also separating samples according to low pH and high pH. An adonis test of sample dissimilarities that were separated into two pH groups (with cutoffs at pH 4, 5 and 6) confirmed that pH 5 was the cutoff that minimized within-group habitat dissimilarity relative to between-group dissimilarity (pH 4: $R^2 = 0.15$, pH 5: $R^2 = 0.19$, pH 6: $R^2 = 0.08$, all $P \leq 0.05$). After accounting for pH, habitat association in low pH (<5) and high pH (>5) was a significant grouping ($R^2 = 0.26$, $P \leq 0.001$).

SIMPER, a statistical tool, was used to identify OTUs that mostly contributed to between-community distances in sediment and planktonic habitats in springs with pH < 5 and pH > 5. The five greatest contributors to habitat differences in springs with pH < 5 were OTUs classified as the genera *Pyrobaculum*, *Sulfurihydrogenibium* and *Hydrogenobaculum* (greater abundance in planktonic communities) and OTUs classified as *Ralstonia* and in the Thermoplasmatales order (greater abundance in sediments) (Fig. 5a). In the higher pH sites, *Thermocrinis* and *Pyrobaculum*-associated OTUs were in greater abundance in the planktonic communities whereas *Geothermobacterium*, an Aigarchaeota-affiliated OTU and *Ralstonia* were more abundant in sediment communities (Fig. 5b).

SIMPER-based comparisons of the same habitat (e.g. planktonic or sediment associated) but in different pH realms revealed evidence for pH-related habitat differentiation of OTUs (Fig. 5c and d). The *Thermocrinis*-affiliated OTU was found in high pH (>5) environments, regardless of habitat type (Fig. 5c and d).

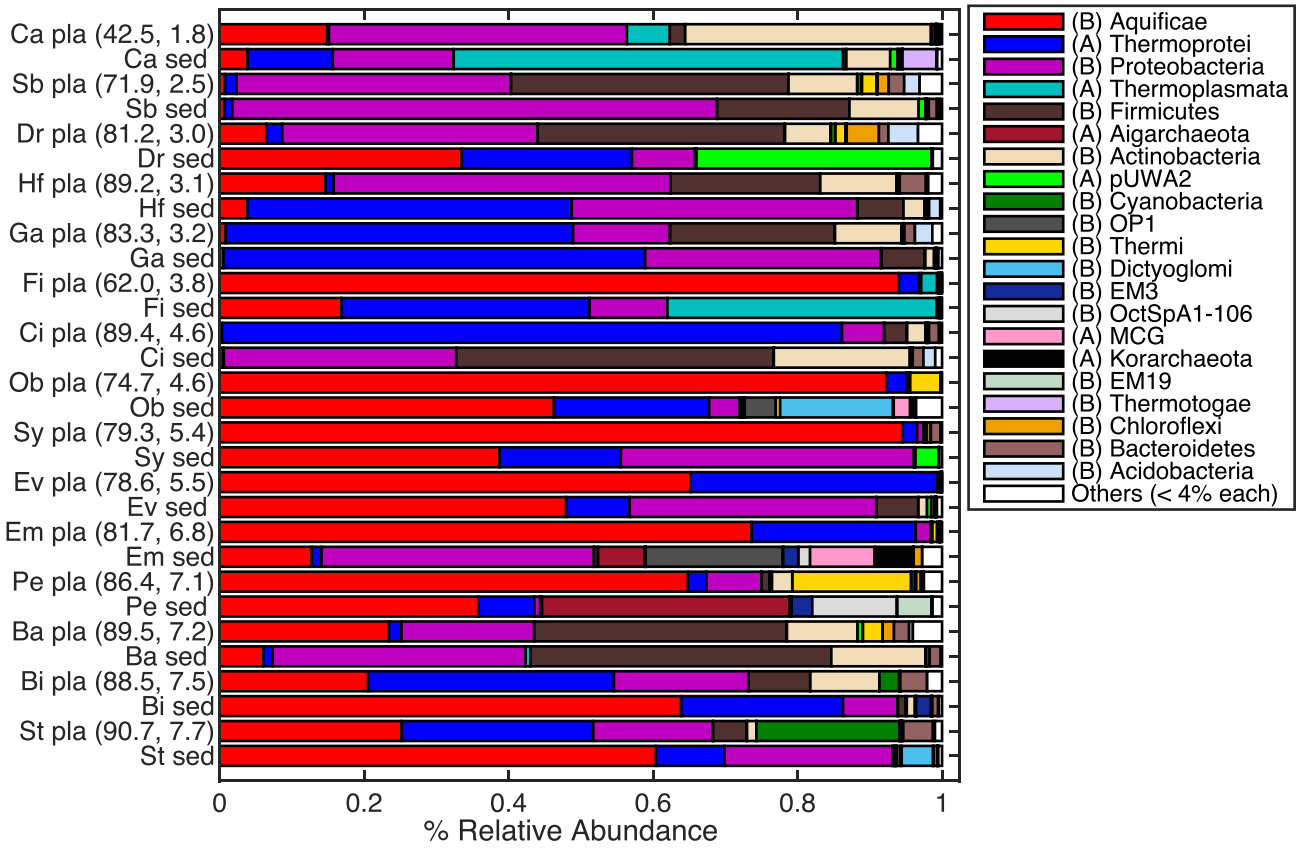

Figure 3. Community taxonomic composition at the phylum (Bacteria) or class (Archaea) level for each sample. Taxonomic groups are only shown for those with >4% relative abundance in at least one sample. Domain (Archaea (A) /Bacteria (B)) of taxonomic group is given in parentheses in legend. Springs are listed from the lowest to the highest pH with planktonic communities, 'pla', followed by sediment communities, 'sed', for each spring.

In contrast, a *Pyrobaculum*-affiliated OTU was more abundant in lower pH sediments than higher pH sediments (Fig. 5c), but relative abundances in planktonic communities were nearly identical in springs with low and high pH (Fig. 5d). Thermoplasmatales and *Geothermobacterium*-affiliated OTUs also contributed to pH-related differences in communities and were primarily partitioned into low and high pH sediment habitats, respectively (Fig. 5c). Finally, Aquificales-affiliated OTUs within the *Sulfurihydrogenibium* and *Hydrogenobaculum* genera were more abundant in lower pH springs and contributed to pH-related differences only in planktonic communities (Fig. 5d).

Sediment communities were predominantly composed of organisms that were inferred to be at least capable of, if not obligately dependent on, S° oxidation, S° reduction or Fe(III) reduction reactions for energy conservation (Table S5, Supporting Information). Predominant sediment community members were variable across the hot springs sampled, and their distribution was highly patchy. For instance, an OTU identified as the S° reducer *Caldisphaera draconis* str. 18U65 (100% nt identity) was only a large component of a single sediment community ('Mud Volcano Fi'; Table S5, Supporting Information). Likewise, the OTU belonging to the archaeal pUWA2 group (98% nt identity; putatively capable of S° reduction by genomic inference) was only detected in sediments sampled from a single spring ('Dragon'; Table S5, Supporting Information). The Thermoplasmatales-like OTU similarly only comprised a large abundance in two springs, 'Mud Volcano Fi' and 'Forest Springs Ca', but low 16S rRNA gene homology to known isolates or genomes (≤78%) precluded inference of putative metabolism for this euryarchaeote organism. An OTU, identified as the obligate Fe-oxide-reducing bacterium, *Geothermobacterium ferrireducens* str. FW-1a (100% nt identity), was an abundant member of sediment communities in slightly acidic to circumneutral springs (Evening Primrose, 'Sylvan Spring', Steep Cone and 'Geyser Creek Em'). Although OTUs classified as *Hydrogenobaculum* sp. Y04AAS1 (100% nt identity), *Thermocrinis ruber* (99% nt identity) and *Pyrobaculum yellowstonensis* WP30 (99% nt identity) were abundant members of sediment communities, and all are capable of S°-dependent metabolism, they were also abundant members of the corresponding planktonic communities. Lastly, an OTU with high identity to *Ralstonia* sp. NFACC01 (100%) was an abundant member in several sediment communities. Published metabolic information is not available for this isolate, but a survey of sulfur metabolism genes in the corresponding genome in the JGI-IMG database (IMG taxon ID: 2599185178), indicated the presence of a full suite of S° oxidation genes (sox system). A BLAST search of this *Ralstonia*-like OTU representative against the NCBI database indicated that it was closely related to *R. pickettii* (100% nt identity to accession KU220859.1, among other *Ralstonia* spp. isolates).

DISCUSSION

The analysis of 15 YNP thermal springs indicated that planktonic and sediment-associated microbial communities from a given spring typically have different taxonomic compositions and exhibit substantial differences in community structure, diversity and size. These observations indicate that taxa

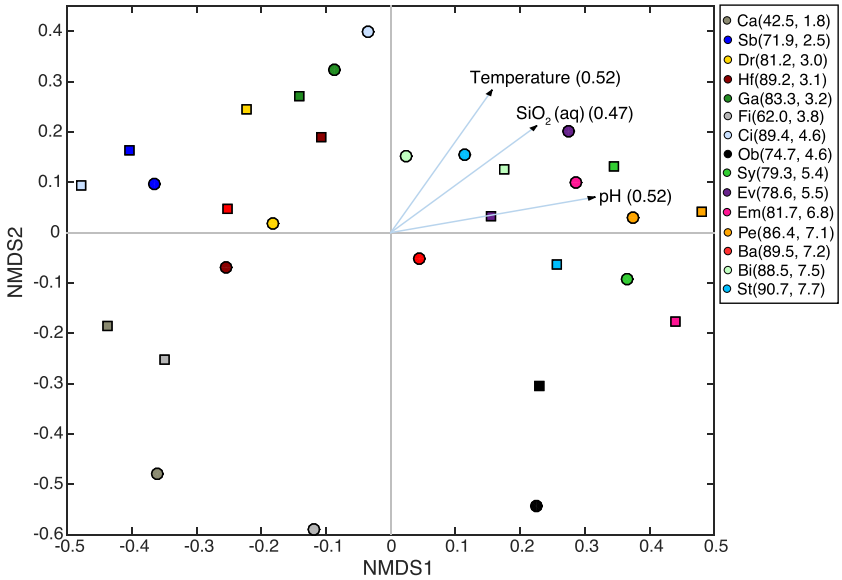

Figure 4. NMDS ordination plot showing community differences among samples and significantly correlated ($P \leq 0.05$) environmental parameters to the ordination. Planktonic communities are shown as circles and sediment communities as squares; each is colored by spring. Spring temperature and pH are given in the legend, respectively, next to the spring ID. Significantly correlated environmental parameters are given as arrows in the direction of highest correlation and with arrow length proportional to correlation strength (R^2 in parentheses). DOC, although also significantly correlated to community composition was also significantly correlated to temperature and pH and is omitted from this plot.

comprising planktonic and sediment-associated assemblages are differentiated by characteristics of their local habitat, and are consistent with observations made of global freshwater and marine ecosystems (Lozupone and Knight 2007; Zinger *et al.* 2011). The spring communities analyzed here were structured at a broad level by pH and temperature, which is consistent with previous studies of YNP spring microbial composition (Inskeep *et al.* 2005, 2013; Meyer-Dombard, Shock and Amend 2005; Boyd *et al.* 2010; Swingley *et al.* 2012; Boyd *et al.* 2013; Alsop, Boyd and Raymond 2014) and surveys of other geothermal systems (Xie *et al.* 2014). However, differentiation of taxa comprising sediment and planktonic communities within the same spring suggests that within-spring habitat heterogeneity can further partition hot spring diversity.

Sediment and planktonic communities from the same spring often exhibited substantially different community characteristics at the level of 16S rRNA gene abundances, diversity and evenness. 16S rRNA gene template abundances from qPCR assays, in general, should be treated qualitatively due to variation in gene copy numbers per genome in different taxa and PCR amplification biases, among other methodological biases (Bru, Martin-Laurent and Philipott 2008; May-Ping Lee *et al.* 2009). Moreover, direct comparisons between 16S rRNA gene abundances in association with sediments and volumes are difficult to make. Acknowledging these limitations, the abundance of 16S rRNA gene copies g^{-1} sediment was consistently greater (range = 1.5–6.7-fold; average = 4.3-fold difference in log transformed values) than the abundance of 16S rRNA gene copies ml^{-1} water. These results qualitatively indicate that hot spring sediments generally harbor considerably more abundant microbial communities than the water column of hot springs. While difficult to assess quantitatively, it is possible that the total biomass in the planktonic compartment of springs surpasses that of the sediments at a volumetric level, and thus, the water-associated microbial populations may contribute to biogeochemical cycling significantly. However, specific activities among the two habitats and more quantitative treatments of sediment and water volume (in total and compared to each other) are needed to assess the relative importance of each habitat to overall spring biogeochemical function. Importantly, planktonic 16S rRNA gene copy numbers are higher in circumneutral springs with pH ~4–7 when compared to more acidic and alkaline springs (Fig. 2a, top). Likewise, these same springs exhibited lower richness and evenness in planktonic communities (Fig. 2b, middle and bottom). Genera of the Aquificales (i.e. *Thermocrinis*, *Sulfurihydrogenibium* and *Hydrogenobaculum*) dominated the planktonic communities in these springs (Fig. S2, Supporting Information). Several of these springs were also vigorously outgassing (and thus undergoing mixing) at the time of sampling (Supplementary File 1, Supporting Information). The dominance of Aquificales, in these springs, is consistent with observations of their dominance in spring outflow filamentous communities that are exposed to increased O_2 ingassing due to water turbulence (discussed further below). These observations suggest that Aquificales and their specific physiological adaptations are partially responsible for the extent of habitat differentiation of taxa within pH ~4–7 springs.

Differentiation of planktonic and sediment communities may, in part, be explained by the metabolic attributes of organisms found in each habitat. For instance, members of the Aquificales order (*Thermocrinis*, *Hydrogenobaculum* and *Sulfurihydrogenibium* genera) were found to be ubiquitously distributed across springs (although individual genera followed pH gradients) and were most dominant in planktonic communities. Hot spring planktonic-associated communities would be exposed to higher concentrations of dissolved O_2 than sediments due to atmosphere-water gas exchange (Shock *et al.* 2010). The reliance of Aquificales genera present in YNP springs on microaerophilic-levels of O_2 for lithotrophy (Huber *et al.* 1998; Donahoe-Christiansen *et al.* 2004; Nakagawa *et al.* 2005) may explain their higher abundances in planktonic communities. This result is consistent with the dominance of Aquificales in high-temperature spring outflow channels across YNP, where dissolved O_2 is elevated due to ingassing from water

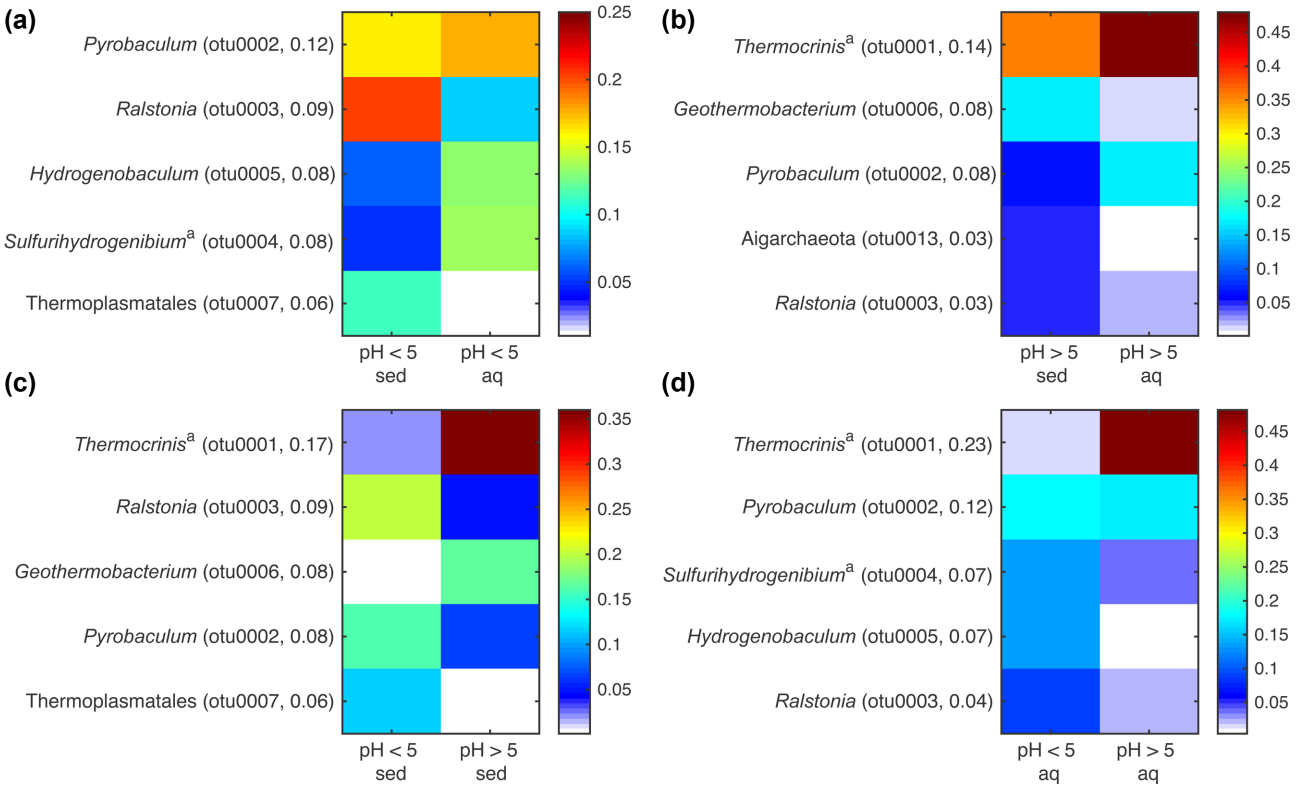

Figure 5. Relative abundance of OTUs that mostly contributed to sediment and aqueous community distance in pH < 5 and pH > 5 springs. The five OTUs that mostly contributed to community distances (by SIMPER analysis) are shown for each pairwise comparison. Taxonomic classification of each OTU is given on left ([a]Denotes manually annotated classification by BLAST search) followed by the OTU number and % contribution to distance in parentheses. Scale indicates each OTU's mean relative abundance in each category. Note that scales are not equivalent between plots to emphasize abundance differences for each comparison.

turbulence (Huber *et al.* 1998; Inskeep *et al.* 2005, 2013; Fouke 2011; Meyer-Dombard *et al.* 2011; Takacs-Vesbach *et al.* 2013) and is consistent with their dominance in the planktonic phase of other non-YNP thermal springs (Cole *et al.* 2013; Hou *et al.* 2013).

In contrast to planktonic communities, mineral-supported anaerobic respiratory metabolisms would be expected to be more prevalent in sediment communities, where O_2 is generally limited due to consumption by planktonic or sediment-associated populations (Huber *et al.* 1998; Donahoe-Christiansen *et al.* 2004; Nakagawa *et al.* 2005; Bernstein *et al.* 2013). Although we did not measure concentrations of O_2 in sediment porewaters, the low concentrations of dissolved O_2 in the water column (Table S1, Supporting Information) when coupled with the low solubility of O_2 at high temperatures, and the flux of gases leaving the fluids strongly suggest that O_2 is likely to be of limited supply as an electron acceptor in most of the hot spring sediments sampled here. The deposition of minerals like S^0 and Fe-oxides onto spring sediments is driven by the near-surface oxidation of reduced chemical species such as H_2S and Fe(II) (Inskeep *et al.* 2005; Nordstrom, Ball and McCleskey 2005; Shock *et al.* 2010). Most of the high-abundance taxa detected in the sediments have, previously, been shown to, or have the genomic potential for, mineral-based metabolisms (e.g. S^0 oxidation, S^0 reduction or Fe(III) reduction). For example, a Thermodesulfobacteriales OTU with 100% nt identity to *Geothermobacterium ferrireducens* str. FW-1 was an abundant member of sediments in springs with pH ~5.0–7.7, but was a minor component of planktonic communities. *G. ferrireducens* obligately reduces poorly crystalline Fe(III) oxides using only H_2 (Kashefi *et al.* 2002), and thus requires mineral Fe(III) oxides for anaerobic growth. Although Fe-oxides were not detected as a dominant mineral phase by XRD in the sediments analyzed here, the insensitivity of XRD in detecting minerals in <~3% abundance (as a conservative estimate), in conjunction with the overwhelming abundance of silicate minerals in these springs likely contributed to the lack of a detectable Fe-oxide signal. It is also possible that Fe(III) oxides are locked in interlayers of clays (e.g. kaolinite), thereby hindering their detection.

A *Ralstonia*-related OTU was a relatively high-abundance member of several acidic spring sediment communities, even though this genus is not commonly found in thermal environments. *Ralstonia* spp. (and specifically *R. picketti*) are ubiquitously distributed in soils, freshwater and other non-thermal environments (Stelzmueller *et al.* 2006), and it's therefore unclear if this OTU represents an active member of these acidic spring sediment communities, or is rather an indicator of soil or freshwater input into these systems. However, the discrete distribution of this OTU among primarily acidic spring sediment communities suggests that there is a mechanism, whether exogenous or endogenous, underlying its distribution among the springs sampled here. It is possible that increased weathering of rocks by acid springs allows for more input of exogenous material into the spring environment, which may lead to an increase in exogenous microbial phylotypes in acidic hot spring libraries.

Sediment communities were highly variable in their composition and dominant taxa exhibited a patchy distribution, but the inferred ability to reduce or oxidize S^0 was a consistent metabolic feature associated with organisms that

predominated in sediments, in particular those in acidic springs. Thus, the high abundance of various organisms (e.g. *Caldisphaera, Hydrogenobaculum, Pyrobaculum* and pUWA2-like Archaea) capable of either S° oxidation or reduction in acidic systems is consistent with sulfur-rich geochemistry, and the presence of S° in five of the springs as determined by X-ray diffraction. The dominant planktonic Aquificales OTUs were also found in high abundance in several sediment samples, and their widespread distribution can likely be explained by their metabolic versatility and capability of using organic carbon, S°, and other inorganic compounds as energy sources (Huber *et al.* 1998; Donahoe-Christiansen *et al.* 2004; Nakagawa *et al.* 2005; Reysenbach *et al.* 2009; Takacs-Vesbach *et al.* 2013). Further, the prevalence of microaerophilic Aquificales (e.g. *Hydrogenobaculum*, *Sulfurihydrogenibium* and *Thermocrinis*) and others (PUWA2-like Archaea) that are capable of oxidizing sulfide to the partial oxidation product, S°, coincides with the presence of sulfur flocs in those spring sediments (e.g. 'Dragon', 'Sylvan' and Evening Primrose), and suggests that there may be multidirectional community–mineralogical interactions.

Two springs, 'Mud Volcano Fi' and 'Forest Springs Ca' contained abundant populations of a euryarchaeote, which was ≤78% identical at the 16S rRNA gene level to any cultured isolates or sequenced genomes, which precludes metabolic or taxonomic inference despite classification as an unclassified 'Thermoplasmatales'. The considerable abundance of reduced chemical constituents including sulfide and Fe(II), in 'Forest Springs Ca' (Table 1, Supporting Information) and 'Mud Volcano Fi' (Alysia Cox, pers. comm), the presence of S° and the ability of some Thermoplasmatales to reduce S° (e.g. *Thermoplasma*; Segerer, Langworthy and Stetter 1998), is suggestive that this novel Euryarchaeote is able to respire S°. This taxon was only present in minor abundances (<6.0%) in the planktonic phase of both of these communities and thus, regardless of its true metabolism, provides further evidence for the role of sediment mineralogy or geochemistry in the partitioning of taxa into either planktonic or sediment habitats.

Although metabolic-niche partitioning appears to be a primary driving force of planktonic and sediment community differentiation, other additional mechanisms may be involved in differentiating community composition in hot springs. For example, it has been proposed that longer water residence times (and a consequent lack of mixing) promote the establishment of more mature planktonic communities and consequent differentiation from the sediment communities (Dodsworth, Hungate and Hedlund 2011). The relevance of this mechanism to the springs described here is unclear, as springs such as Obsidian Pool contained highly differentiated communities (Fig. S2, Supporting Information), despite vigorous outgassing at the time of sampling (Supplementary File 1, Supporting Information) that would effectively mix the planktonic communities. Moreover, while multiple springs without outflow channels, and thus longer water residence times (e.g. 'Geyser Creek Em', Evening Primrose and 'Sylvan Spring') contained highly differentiated communities, other springs with source outflows and thus shorter residence times also contained highly differentiated communities (e.g. 'Dragon Spring' and Obsidian Pool). These comparisons indicate that, while residence time may lead to population differentiation in some spring systems, it is contextually dependent on other mechanisms that also promote differentiation.

A temporal analysis of Tengchong hot springs also suggested that hot spring sediment communities are more dynamic in composition than their planktonic counterparts on a seasonal scale (Wang *et al.* 2014), which suggests that habitat differentiation may be occurring due to changes in sediment communities. Adding to these potential mechanisms, our data suggest that the planktonic communities (in size, composition and diversity) are spatially more variable than the sediment communities across YNP geochemical space. Further, supporting this assertion is that several OTUs closely related to mesophilic soil taxa (>97% 16S rRNA gene nt identity) were detected in the planktonic communities (particularly in acidic springs), which is likely reflective of transiently present, inactive populations from soil wash-in events, as has been suggested to be an important process in supporting heterotrophy in YNP springs (Schubotz *et al.* 2013, 2015; Urschel *et al.* 2015). Characterization of planktonic and sediment community compositional variability on shorter time scales (e.g. hours to days) could provide further evidence for the mechanisms that promote the differentiation of planktonic and sediment communities.

An alternative, but not mutually exclusive, explanation for our observations is that sediment physical characteristics may promote population differentiation from free-living planktonic counterparts through physical attachment, proliferation and the development of micro-scale niche heterogeneity. The physical and geochemical complexity of mesic sediments has been used to explain consistent observations of higher microbial abundances and diversity in such environments relative to planktonic communities (Torsvik, Øvreås and Thingstad 2002; Lozupone and Knight 2007; Zinger *et al.* 2011). In the context of the springs studied, here, sediment heterogeneity in addition to mineral availability, may promote population differentiation and increased sediment diversity relative to the planktonic communities. Additionally, although macroscopically visible biofilms or filaments were not sampled for the data presented, here, it is possible that microbial attachment to sediment or mineral surfaces may also promote population differentiation, increased diversity and the increased microbial densities that were observed in sediments. Microbial attachment to sediment or solid substrate surfaces has been documented in chemosynthetic hot springs (Jackson *et al.* 2001; Macur *et al.* 2004; Boyd, Leavitt and Geesey 2009), and is likely an additional contributing mechanism that adds to sediment population differentiation.

CONCLUSIONS

The results reported here expand our understanding of the extent and magnitude of habitat differentiation within thermal springs. Importantly, this study documents differentiation across major geochemical gradients and integrates compositional and population abundance data to document the characteristics of the environment that likely contributes to differentiation among taxa inhabiting thermal spring sediment and planktonic communities. The results suggest that the metabolic attributes of predominant thermal spring taxa reflect the partitioning of their presence and abundances into either sediment or planktonic spring habitats. In particular, mineral-based metabolic strategies appear to be prevalent among sediment community taxa, while taxa that require dissolved oxidants (such as O_2) for energy conservation were prevalent in the planktonic communities. Our results indicate that ecological differentiation within thermal spring habitats is common across a wide range of spring geochemical gradients and can be largely explained by the availability of dissolved nutrients and minerals used in microbial metabolism.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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