Cyanobacteria and Algae Meet at the Limits of their Habitat Ranges in Moderately Acidic Hot Springs

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Abstract

Microbial oxygenic photosynthesis in thermal habitats is thought to be performed by Bacteria in circumneutral to alkaline systems (pH > 6) and by Eukarya in acidic systems (pH < 3), yet the predominant oxygenic phototrophs in thermal environments with pH values intermediate to these extremes have received little attention. Sequencing of 16S and 18S rRNA genes was performed on samples from twelve hot springs in Yellowstone National Park (Wyoming, USA) with pH values from 3.0 to 5.5, revealing that Cyanobacteria of the genus *Chlorogloeopsis* and algae of the genus *Cyanidioschyzon* (phylum Rhodophyta) coexisted in ten of these springs. Cyanobacteria were detected at pH values as low as 3.0, challenging the paradigm of Cyanobacteria being excluded below pH values of 4.0. Cyanobacterial 16S rRNA genes were more abundant than rhodophyte 18S rRNA genes by up to 7 orders of magnitude, with rhodophyte template abundance approaching that of Cyanobacteria only at the most acidic sites. Light-driven carbon fixation was observed at two sites where chlorophyll *a* was detected but not at two other sites where chlorophyll *a* was not detected. Collectively, these observations suggest that many of the rhodophyte 18S rRNA gene sequences were from inactive cells. Fluctuations in the supply of meteoric water likely contributes to physicochemical variability in these springs, leading to transitions in photosynthetic community composition. Spatial, but perhaps not temporal, overlap in the habitat ranges of bacterial and eukaryal oxygenic phototrophs indicates that the notion of a sharp transition between these lineages with respect to pH is unwarranted.

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essoar.10507341.1.docx available at https://authorea.com/users/530141/articles/600569cyanobacteria-and-algae-meet-at-the-limits-of-their-habitat-ranges-in-moderately-acidichot-springs Cyanobacteria and Algae Meet at the Limits of their Habitat Ranges in Moderately Acidic Hot Springs

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KEY POINTS

Gene sequences affiliated with Cyanobacteria and sequences affiliated with algae were both identified in ten mildly acidic hot springs

Cyanobacterial sequences were much more abundant than algal sequences, and algae appeared to be inactive in most of the springs when sampled

The springs are derived from meteoric water and are prone to changes in pH and temperature that affect the activity of microbial populations

ABSTRACT

Microbial oxygenic photosynthesis in thermal habitats is thought to be performed by Bacteria in circumneutral to alkaline systems (pH > 6) and by Eukarva in acidic systems (pH < 3), yet the predominant oxygenic phototrophs in thermal environments with pH values intermediate to these extremes have received little attention. Sequencing of 16S and 18S rRNA genes was performed on samples from twelve hot springs in Yellowstone National Park (Wyoming, USA) with pH values from 3.0 to 5.5, revealing that Cyanobacteria of the genus Chlorogloeopsis and algae of the genus Cyanidioschyzon (phylum Rhodophyta) coexisted in ten of these springs. Cyanobacteria were detected at pH values as low as 3.0, challenging the paradigm of Cyanobacteria being excluded below pH values of 4.0. Cyanobacterial 16S rRNA genes were more abundant than rhodophyte 18S rRNA genes by up to 7 orders of magnitude, with rhodophyte template abundance approaching that of Cyanobacteria only at the most acidic sites. Light-driven carbon fixation was observed at two sites where chlorophyll a was detected but not at two other sites where chlorophyll a was not detected. Collectively, these observations suggest that many of the rhodophyte 18S rRNA gene sequences were from inactive cells. Fluctuations in the supply of meteoric water likely contributes to physicochemical variability in these springs, leading to transitions in photosynthetic community composition. Spatial, but perhaps not temporal, overlap in the habitat ranges of bacterial and eukaryal oxygenic phototrophs indicates that the notion of a sharp transition between these lineages with respect to pH is unwarranted.

PLAIN LANGUAGE SUMMARY

Photosynthesis evolved in the Bacteria and was transferred to the Eukarya. Photosynthetic microbes from each domain have different habitat ranges with respect to temperature and pH, the limits of which may be reached in geothermal environments. Cyanobacteria are thought to be excluded from acidic hot spring environments where algae are the dominant photosynthetic microbes, whereas Cyanobacteria are predominant in neutral to basic hot spring environments. We characterized photosynthetic microbes in Yellowstone hot springs with intermediate pH values (3–6). Cyanobacteria and algae coexisted in ten of the twelve hot springs studied, but Cyanobacteria were more abundant by 1-7 orders of magnitude. Observations including photosynthetic carbon uptake, pigment (carotenoid and chlorophyll) distributions, and culturing collectively suggested that algae were inactive in these hot springs at the time of sampling in the summer. These hot spring waters are derived from rainwater and snowmelt, with inputs of gases from the hydrothermal system leading to their moderately acidic pH and moderate temperatures. As a result, the springs are chemically dilute and susceptible to changes in pH and temperature caused by fluctuations in fluid supplies that may lead to the springs crossing habitat range boundaries of Cyanobacteria or algae on seasonal or other timescales.

KEYWORDS

pH, Yellowstone, hot spring, pigment, Chlorogloeopsis, Cyanidioschyzon

INTRODUCTION

Microbial photosynthesis dominates primary production in most aquatic environments. To date, photosynthetic organisms have been detected among a limited number of bacterial and eukaryal taxa, but have yet to be identified within the Archaea (Blankenship, 2014). In aquatic environments, bacterial and eukarval microbes performing oxygenic photosynthesis commonly coexist in the same habitat. For example, diatoms often occur together with Cyanobacteria (*i.e.*, *Prochlorococcus* and *Synechococcus*) in oligotrophic ocean regions (Biller et al., 2015). In contrast, terrestrial hot springs span temperatures and chemical conditions, such as pH, that cross the boundaries of the habitat ranges for specific groups of oxygenic phototrophs, making them ideal habitats to assess how transitions between lineages occur with respect to their physicochemical environment. The distribution of hot springs with respect to pH exhibits a bimodal distribution (Figure 1), which has been demonstrated in Yellowstone National Park (YNP) and elsewhere (Brock, 1971; 1978; Nordstrom et al., 2009; Amenabar et al., 2015). This distribution is a consequence of two prevailing buffering systems, with alkaline hot springs being bicarbonate buffered and acidic systems being sulfuric acid buffered. As detailed below, the distribution of bacterial and eukaryal lineages of oxygenic phototrophs in hot springs has been suggested to generally track with this bimodal distribution in spring pH, with Cyanobacteria dominating circumneutral to alkaline (pH > 6) springs and eukaryotic algae dominating acidic (pH < 3) springs.

Recent efforts to catalog the distribution of photosynthetic microbes with respect to extremes of physical and chemical conditions in YNP hot spring habitats have confirmed an upper temperature limit for photosynthesis of $\sim 73^{\circ}$ C that is reached in alkaline environments, while acidic environments exhibit a lower temperature maximum of ~56°C (Boyd et al., 2010; 2012; Cox et al., 2011; Hamilton et al., 2012). The upper temperature limit in alkaline locations is reached only by certain strains of Cyanobacteria of the genus Leptococcus (Brock, 1967; Brock and Brock, 1968; Meeks and Castenholz, 1971; Brock, 1978); until recently these strains were classified as constituents of the genus Synechococcus (Walter et al., 2017; Salazar et al., 2020). Leptococcus isolates from alkaline hot springs exhibit several phylotypes, each with highly specialized temperature niches (Peary and Castenholz, 1964; Allewalt et al., 2006; Becraft et al., 2011). At cooler temperatures, which are encountered farther downstream in outflow channels of alkaline hot springs, phototrophic diversity generally increases (Podar et al., 2020) and other bacterial phototrophs flourish, including other Cyanobacteria such as Mastigocladus laminosus (Miller et al., 2006; Miller et al., 2009), Calothrix spp. (Jahnke et al., 2004; Smythe et al., 2016), and Leptolyngbya spp. (Reves et al., 2013), and anoxygenic phototrophic bacteria (Madigan, 2003) including the Firmicute Heliobacterium modesticaldum (Kimble et al., 1995; Stevenson et al., 1997), the Acidobacterium Chloracidobacterium thermophilum (Bryant et al., 2007; Tank and Bryant, 2015), and filamentous anoxygenic phototrophs such as Chloroflexus aurantiacus (Bauld and Brock, 1973; Pierson and Castenholz, 1974; Brock, 1978) and Roseiflexus sp. (Boomer et al. 2002). Molecular surveys suggest that *Roseiflexus* sp. can predominate over *C. aurantiacus* in a variety of hot spring systems (van der Meer et al., 2010; Hamilton et al., 2019).

In contrast to alkaline systems, photosynthetic communities in acidic environments are dominated by eukaryal phototrophs. About fifty years ago, Doemel and Brock surveyed acidic hot springs extensively and concluded that one species of red algae (phylum Rhodophyta), Cyanidium caldarium, was the only photosynthetic organism present above 40°C at locations with pH values below 4 (Doemel, 1970; Doemel and Brock, 1970; 1971; Brock, 1978). This conclusion is based on the absence of other algae growing above 40°C, together with observations of an apparent lack of Cyanobacteria below pH values of 4 in both thermal and non-thermal environments (Brock, 1973). Below 40°C a variety of other algae are also commonly present, including strains of Chlorella, Chlamydomonas, Euglena, Zygogonium, and diatoms (Doemel and Brock, 1971; Boyd et al., 2009). With the advent of molecular biology and its application to hot spring environments, it is now evident that the organism studied by Doemel and Brock in acidic, aquatic environments was Cyanidioschyzon merolae of the order Cyanidiales within the phylum Rhodophyta (Toplin et al., 2008), which has been reported to be the only species of this algal lineage found in aquatic environments (Skorupa et al., 2013). The upper temperature limit for these algae, and thus for photosynthesis in general in acidic conditions, is 56°C (Doemel and Brock, 1970).

This dichotomous view of microbial oxygenic phototrophs in hydrothermal en-

vironments - that algae dominate acidic springs and Cyanobacteria dominate circumneutral and alkaline springs - has been left largely untested in the halfcentury since the observations of Brock and colleagues. Cyanobacteria have been observed under moderately acidic, thermal conditions at pH values as low as 4 (Brock, 1973), while *Cyanidioschyzon* isolates from YNP can grow at pH values approaching 5, above which no growth is observed (Doemel and Brock, 1971). Based on these observations, the habitat ranges for algae and Cyanobacteria in thermal environments overlap with respect to pH. Indeed, Brock observed both Cyanobacteria and algae present at pH values between 4 and 5 in a mixing zone created when the effluent from alkaline springs (Clearwater Springs) meets the acidic Obsidian Creek in YNP (Brock, 1973; 1978). Nevertheless, studies to date have not yielded adequate data to further investigate the potential coexistence of algal and cyanobacterial photosynthesis in thermal environments, in spite of several of these studies including sample locations within the pH range 4–5. While Doemel (1970) noted Cyanobacteria in locations where *Cyanidioschuzon* were absent based on microscopic observation, it is quite possible Cyanobacteria could have gone unnoticed, especially at sites where algae were predominant. Later studies focusing on *Cyanidiales* relied on sequencing of 18S rRNA and RuBisCO large subunit (rbcL) genes of cultures (Toplin et al., 2008) or amplified and sequenced rbcL genes from natural samples using *Cyanidiales*-specific primers (Skorupa et al., 2013). Hamilton et al. (2012) examined the habitat range of chlorophototrophs by characterizing the distribution and diversity of dark-operative protochlorophyllide oxidoreductase subunit L (chlL), which is involved in (bacterio)chlorophyll biosynthesis (Chew and Bryant, 2007). However, this gene is not present in *Cyanidioschyzon* and it was therefore not possible to determine the distribution and abundance of these algae using *ChlL*. The composition of phototrophic communities in YNP hot springs has also been examined using metagenomic techniques or amplification of ribosomal genes, but most studies either exclusively examined circumneutral and alkaline sites or did not investigate Eukarya (Ross et al., 2012; Swingley et al., 2012; Klatt et al., 2013; Jiang and Takacs-Vesbach, 2017; Hamilton et al., 2019; Bennett et al. 2020). In contrast, Schuler et al. (2017) recovered algal sequences along with those associated with Cyanobacteria from hot spring locations with pH values of ~ 6 , but these were affiliated with Chlorophyta rather than with Rhodophyta.

A compilation of reported observations of algae and Cyanobacteria at temperatures above 40°C is depicted in Figure 2 with respect to the pH and temperature of the sample locations. It can be seen that separate observations of algae and Cyanobacteria have been made in springs with pH values between 4 and 5, yet the data in this pH range are sparse, exacerbated by the rarity of thermal features within this pH range (Brock, 1971; 1978; Nordstrom *et al.*, 2009; Amenabar *et al.*, 2015), as illustrated in Figure 1. Therefore, in this study hot springs in YNP were identified that fall within a pH range of approximately 3 to 6 with temperatures conducive to photosynthesis (see Figure 2). These springs and the microbial mats that inhabit them were targeted for detailed investigations of the physical and chemical constraints that shape the habitat range of bacterial and eukaryal oxygenic phototrophs, including the source of fluids that source these springs to inform hypotheses regarding their susceptibility to temporal (e.g., seasonal) variability. Sequencing of 16S and 18S ribosomal RNA genes (targeting Bacteria and Eukarya, respectively) was integrated with analyses of pigments, arguably the most obvious examples of gene expression by phototrophic organisms, to evaluate the composition of phototrophic microbial communities and offer insights into the metabolic state (*i.e.*, likely active or inactive) of specific phototrophic populations at the time of sampling. Several sites with pH values below 4 were included to further examine the transition from putatively algae-only photosynthetic systems to those also likely to contain Cyanobacteria, as there is a growing body of observations of Cyanobacteria in acidic, low temperature environments (Steinberg et al., 1998; Hamilton et al., 2012; Hao et al., 2012; Urbieta et al., 2015; Hamilton et al., 2019) that challenge the paradigm of Cyanobacteria being excluded below a pH of 4 (Brock, 1973). Ultimately, the underlying hydrological and geochemical processes yielding environments of intermediate pH, in combination with competition for fulfilling similar metabolic niche space, may have facilitated niche differentiation between these two lineages of oxygenic phototrophs at the edges of their habitat ranges.

METHODS

Sample collection. Samples for DNA, pigment, and elemental analysis were collected with sterilized spatulas or forceps, placed in sterile specimen containers, and then aliquoted to cryovials that were immediately frozen in the field by storing them in insulated containers with dry ice. Samples designated for DNA extraction contained 0.8 mL of sucrose lysis buffer (Mitchell and Takacs-Vesbach, 2008). Upon return from the field each day, DNA and pigment samples were transferred to dry shippers previously charged with liquid nitrogen (approximately -150°C) and transferred to a -80°C freezer upon return to the laboratory for storage until sample processing. Samples for elemental analysis were stored at -20°C at the end of each sampling day and maintained at that temperature until analysis.

Water sampling and field measurements were conducted using previously described methods (Shock *et al.*, 2010; Leong *et al.*, 2021) as closely as possible to the site of and immediately prior to biological sample collection. Measurements of pH were performed with a WTW pH meter (model 3300i or 3110) and temperature-compensated WTW probes constructed with a gel electrolyte that were calibrated daily at ambient temperature with buffered pH solutions. Corrected pH values were calculated based on charge balance as described in the Supplementary Materials. Specific conductivity (*i.e.*, conductivity normalized to 25°C) and temperature were measured using a YSI model 30 meter. Dissolved oxygen and total dissolved sulfide were determined colorimetrically (Hach HRDO and methylene blue methods, respectively) in the field using a Hach 2400 or 2800 portable spectrophotometer and Hach reagents on unfiltered water samples immediately upon collection. Hach spectrophotometers and reagents were also employed for the measurement of aqueous silica (silicomolybdate method) using 0.2 micron-filtered water samples, described below.

Water samples for laboratory analyses were filtered with a series of Supor (Pall Corporation) filters to 0.2 microns and preserved in bottles designated for various analyses. Samples for stable isotope determinations of water were collected in 30 mL Qorpak square bottles with polymer-lined caps to ensure gas-tight storage and sealed with no headspace. The bottles were rinsed with deionized water and dried prior to use; water isotope samples were stored at room temperature $(\sim 23^{\circ}C)$ until analysis. Samples for ion chromatography (2 per site) were filtered into 30 mL high-density polyethylene (HDPE) bottles that had been soaked and rinsed with multiple aliquots of deionized water, frozen at -20°C at the end of each sampling day, and kept frozen (-20°C) until analysis. Samples for dissolved inorganic carbon (DIC) were sealed with no headspace in acid-washed 40 mL amber glass vials with black butyl rubber septa and stored at 4°C upon return to the laboratory and until analysis. The filtering apparatus consisted of a 140 mL plastic syringe and caulking gun to facilitate filtering. Sample water was collected in 1 L HDPE bottles and loaded into the syringe via plastic tubing and a 3-way stopcock. The entire apparatus was rinsed with three aliquots of sample prior to filtering into sample bottles.

Geochemical analyses. Concentrations of major anions (F⁻, Cl⁻, SO₄²⁻, NO₃⁻) and major cations (Li⁺, Na⁺, K⁺, Ca²⁺, Mg²⁺, NH₄⁺) were determined on separate Dionex DX-600 ion chromatography systems using suppressed conductivity detection as described in detail in the Supporting Information. Analyses of DIC were conducted using an OI Analytical Wet Oxidation Total Organic Carbon analyzer coupled to a Thermo Delta Plus Advantage mass spectrometer as previously described (Havig *et al.*, 2011) based on the methods of St-Jean (2003). Stable isotopic ratios of water were determined on a Los Gatos Research water isotope analyzer (model DLT-100) that employs off-axis integrated cavity output spectroscopy as previously described (Meyer-Dombard *et al.*, 2015), with drift accounted for according to van Geldern and Barth (2012).

DNA extraction and ribosomal gene sequencing. Samples preserved for DNA extraction were thawed and mat material (~0.5 g) was transferred aseptically to Lysing Matrix E tubes supplied with the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). Extraction was performed according to the manufacturer's instructions except that 250 μ L of the phosphate buffer was replaced by 250 μ L of tris-buffered phenol (pH 8; Sigma-Aldrich, St. Louis, MO, USA), which is a slight modification of previously described procedures (Boyd *et al.*, 2007). Extracted DNA was quantified using the PicoGreen dsDNA assay kit (Invitrogen, Carlsbad, CA, USA) and a Mx3005P QPCR System (Agilent Technologies, Inc., Santa Clara, CA, USA) operating in quantitative plate read mode with the FAM filter set (492 nm excitation; 516 nm emission).

Bacterial 16S rRNA and eukaryal 18S rRNA genes were amplified via polymerase chain reaction (PCR) from ~5 ng of genomic DNA using primers 1100F/1492R (annealing temperature of 55°C) and A7F/570R (annealing temperature of 42°C), respectively, as previously described (Hamilton *et al.*,

2013). All 14 DNA extracts yielded eukaryal 18S rRNA gene amplicons while 13 of the 14 DNA extracts (exception being RS5-2012) yielded bacterial 16S rRNA gene amplicons. Amplicons were purified using the Promega Wizard PCR purification system (Madison, WI), quantified via the Qubit DNA Assay kit (Life Technologies, Grand Island, NY) and a Qubit 2.0 Fluorometer (Life Technologies), and sequenced using an Ion Personal Genome Machine (Life Technologies).

Post-sequencing processing was performed with Mothur (ver. 1.25.1; Schloss et al., 2009) as previously described (Hamilton et al., 2013). Briefly, primers and adapters were removed from the raw sequences, the sequences were trimmed based on a PHRED score of > 25, and the raw bacterial 16S rRNA and eukarval 18S rRNA gene libraries were trimmed to a maximum length of 215 and 195 bases, respectively. Remaining sequences were subjected to a filtering step using the quality scores file to remove sequences with anomalous base calls. Unique sequences were aligned using the SILVA database (Quast et al., 2014) and sequences were trimmed using a defined start and end site based on inclusion of 75% of the total sequences; those that started before or after these defined positions were removed without further consideration. The resulting unique sequences were pre-clustered to remove amplification and sequencing errors and chimeras were identified and removed using UCHIME (Edgar et al., 2011). Operational taxonomic units (OTUs) were assigned at a sequence similarity of 95% (Eukarya) or 97% (Bacteria) using the furthest-neighbor method. The remaining sequences were randomly sub-sampled in order to normalize the total number of sequences in each library. This processing resulted in a total normalized library size of 861 bacterial rRNA gene sequences and 3307 eukarval 18S rRNA gene sequences for each amplicon pool that was sequenced. Sequences were classified using the Bayesian classifier (Wang et al., 2007) and the RDP database (Cole et al., 2013), with manual verification using BLASTn (https://blast.ncbi.nlm.nih.gov). Raw untrimmed sequence and quality score files along with a mapping file have been deposited in the NCBI SRA database under the accession number SRR2147823.

Inorganic carbon uptake assays. Total DIC uptake was assessed using slight modifications to methods described previously (Boyd *et al.*, 2009), with procedures detailed in the Supporting Information. Briefly, sealed microcosms containing a slurry of mat material and hot spring water were injected with 5.0 µCi (10 µM final concentration) of radiolabeled sodium bicarbonate (NaH¹⁴CO₃). Triplicate microcosms were wrapped in foil (dark) and triplicate microcosms were allowed access to light (light). A separate series of triplicate light and dark assays was amended with HgCl₂ to a final concentration of 500 µM as a killed control. Microcosms were incubated in the hot spring for < 60 minutes and then frozen on dry ice. In the lab, microcosms were thawed, acidified, and filtered; the filters were then dried and their radioactivity measured via liquid scintillation counting. Rates of carbon assimilation based on the ¹⁴C tracer were calculated by multiplying the uptake of ¹⁴C-labeled substrate by the total effective concentration of the substrate (¹⁴C-labeled substrate + native substrate)

using an isotopic discrimination factor of 1.06, following the approach of Lizotte *et al.* (1996). Rates measured in the light and dark treatments were corrected by subtracting the activity measured in the respective killed controls.

Pigment analyses. Pigments were analyzed by high performance liquid chromatography-diode array detection-mass spectrometry (HPLC-DAD-MS) using procedures described in detail in the Supporting Information. To summarize, samples were allowed to thaw and transferred to Lysing Matrix A tubes (MP Biomedicals), centrifuged to remove excess water, and then subjected to ballistic bead beating with multiple aliquots of 7:2 acetone:methanol (v/v). Aliquots (50 µL) of the pooled extracts were then injected onto a YMC Carotenoid C-30 reverse phase column (3 x 250 mm x 5 µm particle size) and eluted using a solvent gradient of methanol-methyl tert-butyl ether-water modified from that described by Sander et al. (1994) using a Thermo Surveyor HPLC system. Chromatograms were collected at 360, 475, and 665 nm using the photodiode array to track carotenoids and chlorophylls. The column eluate was then directed into a Thermo Quantum Discovery MAX triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) probe operated in positive ion scanning mode with ionization parameters based on those of van Breemen et al. (2012).

Pigment analyte peaks in the diode array chromatograms were assigned tentative identifications by synergistic comparison of visible absorption spectra and molecular ions obtained from the mass spectra. These data are reconciled with respect to the retention time of the analyte peaks and knowledge of the elution profile for common carotenoids on the C30 stationary phase (Sander et al., 1994). In the case of carotenoids, the spectral fine structure, specifically the %III/II value and the presence or absence of a *cis*-peak, are particularly important for making identifications (Britton, 1995). In the case of *cis*-isomers, the relative height of the *cis*-peak is especially useful at distinguishing isomers (Muller *et* al., 1997). Semi-quantitative comparison for specific pigments was performed using integrated peak areas from the diode array chromatograms and normalized per gram dry mass of the sample after extraction and total solvent volume used. Quantitative results for chlorophyll a and -carotene were obtained using response factors at 665 nm and 475 nm, respectively, determined using a series of pigment solutions prepared using 7:2 acetone:methanol and authentic samples of each pigment obtained commercially (Sigma-Aldrich). As a proxy for the amount of biomass present in each sample, these results were then further normalized per gram N using the nitrogen content of the samples designated for elemental analysis (reported in Table S1), which were processed and analyzed for total carbon and nitrogen as previously described (Havig et al., 2011).

Enrichment culturing and microscopy. A mineral salts medium for enrichment of oxygenic phototrophs was designed using the geochemical data for RN1-2012. Inoculum was collected at RN1 on July 13th, 2013 aseptically as described above, placed into 15 mL Falcon tubes, and stored at ambient temperature in the dark. The base medium was prepared with the composition

described in Table S2, adjusted to a pH of 4.5 with 3.6 M sulfuric acid, and autoclaved. Medium (15 mL) was added aseptically to 18 x 150 mm crimptop tubes (anaerobic-style tubes; sterilized at 160°C overnight), inoculated with mat material (~1 g), and crimp sealed with autoclaved butyl rubber septa. An aliquot (0.5 mL) of the headspace (air) was removed and replaced with 0.5 mL of carbon dioxide (~2% final CO₂ headspace concentration). Tubes were incubated in a clear glass-covered water bath at 46 ± 2°C under incandescent lighting (18 µmol photons s⁻¹ m⁻² in the 400–700 nm range).

Microscopy of cultures was performed on a Zeiss Axioplan 2 imaging microscope equipped with a Zeiss X-cite 120 fluorescence light source and an AxioCam HRc camera. Filaments suspended in medium were removed aseptically with a sterile 1 mL plastic syringe and needle and were prepared for microscopy as wet mounts. Cells were observed and imaged using a 100x oil immersion objective lens. Autofluorescence of phycocyanin was observed using Zeiss filter set 14 (excitation 510–560 nm; emission > 590 nm).

Quantitative PCR. Quantitative PCR was performed with the SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA, USA) according to the manufacturer's protocol, and assayed on a CFX Connect detection system (BioRad) using the aforementioned primers. Reactions were performed in triplicate, with 500 nM forward and reverse primer, in a final reaction volume of 20 L using the following cycling conditions: initial denaturation at $98^{\circ}C$ (0.5 minute), followed by 35 cycles of denaturation at $98^{\circ}C$ (0.5 minute), annealing and elongation at specified temperature (1 minute), and eventually a melt curve of $65-95^{\circ}C$ in $0.5^{\circ}C$ steps at 5 seconds/step. Control reactions contained no template DNA. Plasmids containing inserts of each amplicon were used as standards as prepared and reported previously (Hamilton *et al.*, 2013).

RESULTS AND DISCUSSION

Study sites. Moderately acidic hot springs with visibly pigmented biofilms were identified within the Rabbit Creek thermal area of the Midway Geyser Basin and adjacent to Imperial and Spray Geysers in the southwestern portion of the Lower Geyser Basin in YNP. Twelve sites were studied between July 2011 and July 2012; two locations were sampled in both years to begin to evaluate temporal variability in their geochemistry and microbial communities. When sampled, the sites had moderately to mildly acidic pH values (3.0-5.5) and moderate temperatures (49–70°C) as reported in Table 1 (for reference, neutral pH decreases from 6.63 to 6.35 over the temperature range 50-75°C; Bandura and Lvov, 2006). The conductivities ranged from 59 to 914 µS/cm (Table S3). indicating that the springs are dilute in comparison to other YNP hot springs (see Amenabar et al., 2015). The springs range in size from ~20 cm to several meters in diameter and are generally closely surrounded by grasses and other terrestrial vegetation. Sampling dates and GPS coordinates for each site are reported in Table S3 and photographs of each site appear in the Supporting Information.

Geochemistry of moderately acidic hot springs. Concentrations of major solutes for the 14 hot spring samples are reported in Table 1. The sulfate and chloride concentrations appear in Figure 3, along with data for local meteoric water and for hot springs and other waters from throughout the YNP hydrothermal system. Many of the study sites are dilute with respect to these solutes and appear in the center-left portion of the plot; only some low-temperature surface waters from Secret Valley are more dilute than those of this study (McCleskey et al., 2009). Evaporation of approximately 80% of the local meteoric fluid (0.7 log units) could lead to the observed chloride compositions of many of the moderately acidic springs, which are among the lowest encountered in YNP hot springs. However, evaporation cannot explain the intermediate sulfate concentrations observed. Thus, a hydrothermal vapor-phase component containing hydrogen sulfide is invoked from which most of the sulfate is derived via sulfide oxidation (Fournier, 2005; Nordstrom et al., 2009), with the observed chloride concentrations representing a combination of evaporation of the meteoric water component and minor amounts of shallow water-rock reactions. Ratios of chloride to fluoride are also particularly low in many of the study sites (Table 1), likely due to a vapor-phase contribution of hydrogen fluoride to these hot spring fluids (Deng et al., 2011). Being derived from meteoric water and hydrothermal vapor, these hot springs can be classified as MG-type hot springs (meteoric plus gas) using the categorization of Nordstrom et al. (2009), as are many other acidic hot springs in YNP. Yet, these sites must be considered extreme cases of this type with large meteoric components, as the chloride concentrations for many of the springs are below 1 mg/L (< 28 M), sulfate is often below 100 mg/L (< 1 mM), and they are less acidic than the majority of YNP springs in this category. These springs are similar in major solute composition to the gas-rich, high-temperature Smokejumper Hot Springs (Lindsay et al., 2019).

Several spring waters were appreciably higher in chloride concentration than the others, indicating additional fluid components from sources in addition to meteoric water and hydrothermal vapor. The study sites are all in close proximity to alkaline hot springs, suggesting the possibility that mixing of the deeply sourced hydrothermal fluid feeding the alkaline springs is contributing small amounts of chloride and other solutes to the moderately acidic springs. This deep hydrothermal fluid is theorized to have 310–400 mg/kg chloride, with subsurface processes possibly changing chloride concentrations before mixing (Fournier, 2005). It is likely that mixing occurs in the shallow subsurface where the plumbing systems of proximal springs are partially connected. For example, site FF1 is approximately 100 meters from an alkaline hot spring, while site IG3 is only about 5 meters from the main source of Spray Geyser (pH = 8.0). Given their proximity, solutes from Spray Geyser may also reach IG3 via aerosol deposition. In contrast, mixing with fluids enriched in chloride at site RN3 is possibly a surface process, as this small pool is adjacent to the outflow channel of a circumneutral hot spring with a pH of ~ 6 . Nevertheless, these springs are more dilute than some moderately acidic springs described elsewhere in YNP (Macur et al., 2013; Colman et al., 2016; Jiang and Takacs-Vesbach, 2017; Payne et al., 2019).

The stable isotopic compositions (2 H and 18 O) of the hot spring waters in this study are depicted in Figure 4A. The data trend away from the local meteoric water line (Kharaka *et al.*, 2002; Holloway *et al.*, 2011) with a slope of approximately 3, typical of other hot springs experiencing non-equilibrium evaporation (Nordstrom *et al.*, 2009). The 2 H and 18 O values correlate with sulfate concentrations (Figure 4B; only 2 H shown), though no analogous correlation exists for chloride concentrations. One interpretation of this correlation with sulfate is that the isotopic composition depends most strongly on the amount of hydrothermal vapor-phase input, because the mixing of hot gas with cold, oxygenated meteoric water at or near the surface both generates sulfate and provides heat that drives evaporation. This correlation offers additional evidence for mixtures of meteoric water and hydrothermal vapor leading to moderately acidic hot springs waters. Site IG3 does not follow this trend, indicating that a different mechanism is required to yield the observed sulfate concentration, which greatly exceeds that of the other samples (see below).

Ribosomal gene sequences. Sequencing of 16S rRNA gene amplicons from the 14 microbial mat samples revealed that 11 contained OTUs affiliated with Cyanobacteria (Figure 5A). In these samples, Cyanobacteria represented from < 1% to 85% of the total sequences, nearly all of which were most closely related (88-100% sequence similarity; most abundant OTUs 99-100% sequence similarity) to *Chlorogloeopsis* sp. Greenland 5, an isolate from an alkaline, high-salinity hot spring in Greenland (Roeselers et al., 2007). Observations and reports of successful culturing of *Chlorogloeopsis* morphotypes have been briefly reported for springs with pH values of ~4–5.5 in other areas of YNP (Castenholz, 1978; 1996) and *Chlorogloeopsis* was also the dominant cyanobacterial phylotype in DNA sequences of stromatolite samples obtained from a hot spring with a pH of ~6 in YNP (Berelson et al., 2011; Schuler et al. 2017). Site RS1 also contained a 16S rRNA gene OTU affiliated with the genus Synechococcus (i.e., Leptococcus) representing 15% of the cyanobacterial sequences (9% of the total sequences) in that sample. The sample associated with RS5 in 2012 is not shown in Figure 5, since the amount of DNA obtained from this sample was quite low $(2.5 \text{ ng/}\mu\text{L})$, and the extract failed to yield 16S rRNA gene amplicons (but did vield 18S rRNA gene amplicons, see below). Anoxygenic phototrophs were also detected in most samples, including two samples having sequences associated with Chloracidobacterium thermophilum, as discussed in the Supporting Information.

Sequencing of eukaryal 18S gene amplicons revealed the presence of sequences affiliated with the algal phylum Rhodophyta in 13 of the 14 samples, including the 11 samples that also contained Cyanobacteria (Figure 5B). Nearly all rhodophyte OTUs were most closely related (97–100% sequence similarity) to Cyanidioschyzon merolae 10D, which other studies have identified as the most common Cyanidiales phylotype in acidic, aquatic environments at YNP (Toplin et al., 2008; Skorupa et al., 2012). The relative abundance of Cyanidioschyzon sequences was quite variable, ranging from < 1% to nearly 100% of 18S rRNA gene sequences in these samples. Surprisingly, very few chloroplast se-

quences were detected in the 16S rRNA gene libraries and only in IG2 were they associated with Cyanidioschyzon. Two samples with pH values below 4 (FF1, IG1) vielded sequences associated with *Cyanidioschuzon* but vielded no cyanobacterial sequences. Several algal mats dominated by *Cyanidioschyzon* in YNP with pH values of ~ 3 have received considerable attention including those at Nymph Creek (Ferris et al., 2005; Boyd et al., 2012) and Dragon Spring (Lehr et al., 2007; Boyd et al., 2012), thus this study extends sequencing observations of Cyanidioschyzon populations to higher pH. Green algae (Chlorophyta) were also detected at 8 of the sites, representing < 1-35% of the 18S rRNA gene sequences, rivaling *Cyanidioschyzon* in abundance in some samples. Most chlorophyte OTUs were affiliated with the *Chlamydomonadales*, the *Chaetophorales*, the Trebouxiophyceae, and the Chlorellales. One sample not shown in Figure 5 (RS5-2012), which failed to yield any 16S rRNA gene amplicons, only yielded 18S rRNA gene sequences affiliated with *Ranunculales*, an order of angiosperms, rather than with microbial eukaryotes. Sequences associated with fungi and other non-phototrophic microbial eukaryotes are discussed in the Supporting Information.

Carbon fixation assays. To demonstrate active photosynthesis by the microbial communities inhabiting hot springs within this pH range, dissolved inorganic carbon (DIC) fixation assays were completed at 4 of the study sites at the time of sampling in the presence and absence of light. As shown in Figure 6, greater DIC uptake rates were observed in the light than in the dark in microcosms from RS1 and RS2, indicating light-driven carbon uptake (photoautotrophy) was occurring at the time samples were collected from these springs. In contrast, rates of DIC uptake were low in experiments conducted with mat biomass collected from FF1 and RS5-2012 and the rates were not different between light and dark treatments, signifying that light-driven carbon uptake was not occurring when these springs were sampled.

Photoautotrophs employ DIC both as a carbon source and an electron acceptor, but do so differently depending on the speciation of the DIC. All sample-site pH values were lower than the pK_a for carbonic acid, implying that DIC concentrations (Table S1) are dominated by aqueous carbon dioxide, as indicated by speciation calculations. At the highest pH values encountered in this study, ratios of aqueous carbon dioxide to bicarbonate approach unity, but at lower pH, aqueous carbon dioxide dominates by up to 3 orders of magnitude. While it has been suggested that aqueous carbon dioxide may be less bioavailable than bicarbonate, particularly for bacterial phototrophs (Hamilton et al., 2012; 2019), it seems unlikely that populations could sustain significant biomass using only bicarbonate at the acidic sites in this study. Indeed, the substrate eventually fixed by RuBisCO in the Calvin cycle (*i.e.*, the reductive pentose phosphate cycle) is carbon dioxide (Berg, 2011), and under acidic conditions Cyanidioschyzon appears to rely on free membrane diffusion of carbon dioxide which undergoes an intracellular accumulation mechanism (Zenvirth et al., 1985). Cyanobacteria in this pH regime may also primarily employ aqueous carbon dioxide as an inorganic carbon source for photoautotrophy. Nevertheless, it is possible that

the ability to more effectively utilize one substrate or the other may affect niche differentiation among different groups of microbial photoautotrophs in intermediate pH environments.

Pigment compositions of biofilms. The major chlorophyll type in all samples was chlorophyll a, the major chlorophyll found in Cyanobacteria and Rhodophyta. Every sample also had significant quantities of compounds related to chlorophyll a, including chlorophyll a' (epimer at the 13^2 carbon, using IUPAC numbering), pheophytin a and its 13^2 derivatives, including its epimer (pheophytin a'), allomer, and pyrolyzed product (pyropheophytin a), and pheophorbide a as depicted in Figure 7A. The presence or absence of chlorophyllide a, the magnesium-containing analogue of pheophorbide a, was more ambiguous, as analyte peaks with retention times and absorbance spectra consistent with this compound were often present, but none yielded mass spectra expected for chlorophyllide a. In contrast to the other sites, samples FF1 and RS5-2012 did not yield any intact chlorophyll a; the major chlorophyll molecules present were pheophorbide a and pyropheophytin a, respectively, with smaller amounts of pheophytin a and other derivatives. It follows that most of the chlorophyll present at these sites had been demetallated and further degraded and likely was not present in active cells at the time of sampling.

Evidence for low amounts of chlorophylls possessing a chlorophyll b chromophore, including pheophytin b, was found in three of the sites (FF1, IG1, IG2). Chlorophyll b is found in the Chlorophyta as well as in land plants, both of which also synthesize chlorophyll a (Blankenship, 2014). There was no evidence of intact chlorophyll b, so it is unlikely that these compounds were derived from active cells. While Chlorophyta 18S rRNA gene sequences were present in these samples, sequence abundances were very low in FF1 and IG1 and no pigment peaks exhibiting a chlorophyll b chromophore were found in samples from sites with the highest Chlorophyta abundance. Thus, the observed chlorophyll b derivatives do not correlate with Chlorophyta sequence abundance and the observed chlorophyll b derivatives may be derived largely from exogenous plant matter. Compounds related to bacteriochlorophyll a, including Zn-bacteriochlorophyll a, were detected in some samples and are discussed in the Supporting Information.

Carotenoids are present in photosynthetic microbes as accessory light-harvesting pigments and for UV-protection (Blankenship, 2014). The relative abundances of major carotenoids in the fourteen samples are shown in Figure 7B. The common carotenoids -carotene and zeaxanthin ((3R,3'R)-, -carotene-3,3'-diol) were present in all samples and are among the most abundant carotenoids. Both carotenoids are known to be present in cultures of *C. merolae* (Cunningham *et al.*, 2007) and *Chlorogloeopsis fritschii* (Evans and Britton, 1983), close relatives of the dominant phototrophs in these springs. The majority of the samples exhibited a relatively constant zeaxanthin: -carotene ratio of ~0.3, while five samples, including the three most acidic samples, deviate from this trend with greater relative amounts of zeaxanthin (Figure 8). This deviation may indicate that

C. merolae synthesizes zeaxanthin to a greater extent than does Chlorogloeopsis and are more abundant in these samples. Sample RS5-2012 also exhibited a slightly larger ratio, but the amounts of both carotenoids in this sample were the lowest of those analyzed (Table S5) and there is no evidence of microbial phototrophs at this site in 2012 in the sequencing or carbon uptake results.

-cryptoxanthin, a mono-hydroxylated analog of zeaxanthin and its putative biosynthetic precursor, was present at two sites (IG1, IG2) where Cyanobacteria were either absent or not very abundant. While -cryptoxanthin is known to occur as a minor carotenoid in both C. merolae (Cunningham et al., 2007) and *Chlorogloeopsis fritschii* (Evans and Britton, 1983), its occurrence at only IG1 and IG2 leads to the hypothesis that -cryptoxanthin is associated with algae in these springs, rather than Cyanobacteria. Detectable abundances of cryptoxanthin are consistent with larger relative amounts of zeaxanthin at these acidic sites and active biosynthesis of zeaxanthin. As a corollary, the lack of cryptoxanthin at the other sites where C. merolae sequences were observed likely indicates that algae were inactive at those sites when sampled. In particular, cryptoxanthin was absent at site FF1, where the only putative phototroph was C. merolae yet the chlorophyll composition and lack of light-driven carbon fixation indicate that photosynthesis was not occurring under the conditions observed at this site. This observation confirms the relationship of inactive algae and a lack of -cryptoxanthin and therefore supports the idea that -cryptoxanthin may be a sensitive indicator of active C. merolae populations.

The ketocarotenoid echinenone (, -carotene-4-one) was abundant at nearly all sample sites where cyanobacterial 16S rRNA gene sequences were found, with the exception of IG2, where cyanobacterial OTU abundances were relatively low. Echinenone was also absent from pigment analyses from sites without Cyanobacteria, suggesting this carotenoid is largely derived from the *Chlorogloeopsis* spp. present at these sample sites and not from *C. merolae*. Smaller amounts of canthaxanthin, a carotenoid similar to echinenone where both rings possess 4-keto groups, were present in the same samples, suggesting canthaxanthin is also associated with the Cyanobacteria at these sites. Indeed, echinenone is a major carotenoid in *C. fritschii* cells, with traces of canthaxanthin present as well (Evans and Britton, 1983). The lack of echinenone and canthaxanthin at IG2 may indicate that Cyanobacteria were not active there when sampled.

Enrichment culturing and microscopy. The geochemical data for RN1-2012 were used to design a mineral salts medium for enrichment of oxygenic phototrophs from this site. Incubation of the enrichment in the light at 46°C resulted in the growth of green filaments at the headspace/water interface. Photomicrographs of these filaments after 27 days of incubation are shown in Figure 9. Fluorescence microscopy under green light demonstrated the presence of the protein-bound accessory pigment phycocyanin throughout each vegetative cell (Figure 9c,d). Terminal heterocysts lacking phycocyanin were also evident on one of the filaments (Figure 9a,c). These observations are consistent with microscopic observation of *Chlorogloeopsis* from other hot springs (Castenholz, 1978;

1996). No evidence for unicellular, phycocyanin-containing *Cyanidioschyzon* was observed in the culture samples. Successful enrichment of *Cyanobacteria* and a lack of enrichment of *Cyanidioschyzon* from RN1 offer further support for *Chlorogloeopsis* being active photosynthetic members of the microbial communities of moderately acidic hot springs.

Abundances of Cyanobacteria and algae. To estimate and compare the abundances of Cyanobacteria and rhodophyte algae within the photosynthetic communities of each spring, quantitative PCR was performed on samples for which sufficient DNA was obtained (Table S8). For these samples, the ratio of 16S to 18S rRNA gene templates was used to correct the ratio of cyanobacterial to rhodophyte OTUs, as shown in Figure 10. Since the genomes of both Chlorogloeopsis fritschii 9212 (Dagan et al., 2013) and C. merolae 10D (Matsuzaki et al., 2004), which represent nearly all the cyanobacterial and rhodophyte OTUs, respectively, each indicate three copies of ribosomal genes within the genome, no correction for copy number per cell was employed. Cyanobacteria were more abundant than Rhodophyta in all sites analyzed and Rhodophyta only approached the abundance of Cyanobacteria in the two most acidic sites examined (IG2, IG3). Yet, even in these sites, Cyanobacteria were more abundant by at least an order of magnitude. Cyanobacteria in the other sites exceeded Rhodophyta by 3 to 7 orders of magnitude. These results further substantiate indications from the combination of pigment, carbon uptake, and sequence data indicating that *Cyanidioschyzon* were not significant contributors to light-driven primary production at most sites when these springs were studied.

Dynamics of moderately acidic hot spring photosynthetic communities. As described above, hot springs with moderately acidic pH values are dilute and poorly buffered, which is itself a consequence of the derivation of these hot spring fluids largely from meteoric water, a major contributor to hydrothermal fluids in YNP (Hurwitz and Lowenstern, 2014). It is likely that temporal fluctuations in meteoric water availability are extensive, with the greatest contributions of meteoric water occurring during the winter and spring due to snowmelt. Such fluctuations would change the relative contributions of meteoric water and hydrothermal vapor to the hot spring, overwhelming the limited buffering capacity of the fluid and changing the pH. Moreover, as the vaporphase component increases, additional heat may be added to the system, especially at times of low meteoric water input, resulting in a higher steady-state temperature that can drive additional evaporation, causing lower water levels. It follows that temporal fluctuations in the chemical and physical characteristics of such hot springs may be reflected in the temporal dynamics of the composition and activity of the microbial communities they host, as has been observed in other hot spring systems (Macur et al., 2004; Lacup et al., 2007; Schubotz et al., 2013; Briggs et al., 2014; Wang et al. 2014; Colman et al., 2021).

Observations of these study sites over several years offer preliminary insights into the dynamics of moderately acidic hot springs. Of particular interest is site IG3, a small (~20 cm diameter) pool near Spray Geyser. This spring is presumably closely related to the "small pool near Spray Geyser" studied by Doemel over fifty years ago which had the highest pH reported for an aquatic environment hosting Cyanidioschyzon (Doemel, 1970). Modern observations of this site include pH measurements as high as 4.8 (unpublished data), yet at the time of sampling the pH was 2.98. A hailstorm occurred just prior to sampling, which increased the volume of the hot spring and allowed for collection of a complete geochemical sample set. Nevertheless, the aqueous geochemistry indicates the hot spring fluid differs considerably from meteoric water, with the highest concentrations of dissolved solutes (except for oxygen, chloride, and silica) of all samples in this study. In particular, the anomalously high nitrate concentration (221 M) suggests a significant contribution from soil or sediment pore water where nitrate may be produced via microbial nitrification, a process for which there is growing evidence in geothermal environments (Dodsworth et al., 2011). Given the shallow water depth (~ 2 cm), it is possible that the sediments were disturbed during sampling. Alternatively, it is possible that the hydrology of this hot spring is such that recharge of meteoric water during a rain event leads to an increased flow into the hot spring from the surrounding or underlying sediments that become saturated. Extensive evaporation due to its especially small volume and shallow depth likely caused the observed enrichments of the major solutes, which may accumulate in sediment pore water.

Two of the study sites were sampled in both 2011 and 2012. In 2011, site RS5 yielded sequencing and pigment results, as well as biofilm carbon and nitrogen contents (Table S1), that were comparable to the other sites in the area, while in 2012 the abundances of dissolved oxygen, pigments, and biofilm carbon and nitrogen were substantially lower. No intact chlorophyll a was observed, nor was light-driven carbon fixation, indicating that photosynthesis was not occurring at the site in 2012. While carbon uptake was not assessed in 2011, the presence of chlorophyll a and differences in the other data above suggest an active photosynthetic population at this time. Most notably, the temperature increased from 56.8°C in 2011 to 70.3°C in 2012, which likely exceeded the upper temperature limit of the phototrophs observed in 2011. The water level in the spring was noticeably lower in 2012 and the chloride concentration had more than doubled, consistent with a change in the extent of evaporation relative to the fluid supply into the hot spring, though with no change in the water isotopic ratios (Table S3). The sulfate concentration was lower in 2012 and the pH had increased, suggesting a decrease in the vapor-phase component of the hot spring fluid compared to 2011. These observations suggest that the heat flux and temperature of moderately acidic hot springs may not be simply correlated to vapor-phase input as hypothesized above, but rather could indicate that hydrothermal vapor may to some extent serve as an indirect heat source without contributing sulfate or other gas-derived solutes. The observed balance between spring fluid flux and evaporative loss is therefore controlled by a complex and dynamic interplay among the local water table, the meteoric water supply, and the flow of the vapor-phase gases.

In contrast to site RS5, site RN1 had putatively active photosynthetic populations in both years of sampling and the water chemistry had not changed appreciably during the year between sampling events (Figure S2). The temperature had increased from 51.6°C in 2011 to 57.4°C in 2012, yet in spite of the temperature increase, *Cyanidioschuzon* represented a larger fraction of the eukaryotic sequences in 2012 than in 2011. Chlorophyta sequences were also observed in 2012 when the temperature was higher, but not in 2011. Nevertheless, the absence of -cryptoxanthin suggests Cyanidioschyzon were not active during either sampling. The temperature in 2012 exceeds the known upper temperature limit for these algae (56°C; Doemel and Brock, 1970), further substantiating that these algae were inactive. Nevertheless, the increase in the relative abundance of sequences associated with Cyanidioschyzon and the appearance of Chlorophyta suggest that these algae may have been active members of the photosynthetic community at some point during the intervening time between sampling, which includes periods of cooler ambient temperatures and surficial snow input. In the headwaters of the aforementioned Obsidian Creek, diatoms were the dominant algae during periods of snowmelt runoff, but transitioned to Cyanidioschyzon as snowmelt declined and the runoff of acidic hot springs became the major fluid source, resulting in higher temperatures and lower pH that apparently favored Cyanidioschyzon (Doemel, 1970). Here, a similar seasonal transition could be occurring with opposite effect, where the decline in snowmelt input in the summer could have led to an increase in temperature that exceeded the limits of the Cyanidioschyzon population but were amenable to Chlorogloeopsis. Culture assays inoculated with samples collected in July 2013 resulted in the enrichment of Chlorogloeopsis but apparently not Cyanidioschyzon.

Activity of photosynthetic populations. Ribosomal gene sequencing indicated the presence of DNA associated with oxygenic phototrophs from both the Bacteria and the Eukarya in many of the moderately acidic hot springs studied here, and the preponderance of the other data enables some conclusions about the activity of these photosynthetic populations. The observation of cryptoxanthin at only two sites suggests that these may be the only sites where Cyanidioschyzon were active when sampling occurred. Site FF1 is particularly noteworthy in that no light-driven carbon fixation was observed, demonstrating that the algae here were photosynthetically inactive or were heterotrophic, as Cyanidioschyzon is known to be capable of organic carbon uptake (Doemel and Brock, 1971; Sato and Moriyama, 2007). Furthermore, very few chloroplast sequences were identified in the 16S rRNA gene libraries, suggesting that algal chloroplasts had undergone degradation, as illustrated by the observations of chlorophyll degradation products. The temperature at FF1 as well as those at IG1, RN1-2012, RN3, RS3, and RS5-2011 exceeded 56°C when sampled, which is the known upper temperature limit for *Cyanidioschyzon*. Curiously, cryptoxanthin was observed at IG1, suggesting algae were active in spite of the elevated temperature. Perhaps the temperature had only recently, or briefly, exceeded the upper temperature limit of the algae and this biosynthetic intermediate was still present.

In addition to temperature, pH, sulfide, and light can constrain the activity of Cyanidioschyzon populations. Sulfide concentrations were relatively low at each of the sample sites and within the documented sulfide concentration range of YNP phototrophs (Cox et al., 2011). Nevertheless, sulfide could at times disfavor algal populations, as algae in acidic hot spring environments were found to be less tolerant of sulfide than thermophilic Cyanobacteria (Boyd et al., 2012). The upper pH limit for Cyanidioschyzon exists between a pH value of 4 and 5 (Doemel and Brock, 1971), thus pH seems to favor Cyanobacteria at many of the sites. The pH for samples RS1, RS3, RS4, and RS5-2012 all exceeded 5, suggesting that the algae were inactive when these sites were sampled, as the low relative abundances of algae, low zeaxanthin: -carotene ratios, and undetectable -cryptoxanthin in these samples also suggest. It was observed that Cyanidioschyzon isolates can lower the ambient pH in culture toward their optimum pH (Ascione et al., 1966; Doemel and Brock, 1971; Lowell and Castenholz, 2013), which was hypothesized to offer advantages in certain natural settings (Lowell and Castenholz, 2013). Moderately acidic springs are poorly buffered, often small in size, and likely have low fluid fluxes, making them habitats where microbially mediated pH shifts may occur in nature. However, indications of inactive algae at many of the sites do not support the utility of this phenomenon at these sites, which if feasible would offer *Cyanidioschyzon* a competitive advantage over Cyanobacteria. Finally, UV irradiation associated with full sunlight during the long photoperiods of the summer has been associated with Cyanidioschyzon mat decline observed at Dragon Spring in Yellowstone (Lehr et al., 2007). Though many of the springs studied here likely experience greater shading than does Dragon Spring, it is possible that light intensity during the summer could be a factor leading to the apparent lack of algal activity in the springs at the time of sampling.

Cyanobacteria were present at many of the sites and were likely responsible for photosynthetic activity at these sites except those where algae were hypothesized to be active (IG1 and IG2). In contrast to Cyanidioschyzon, Cyanobacteria are not likely to be constrained by an upper pH limit at these sites, and it has been suggested that the upper temperature limit for the *Chlorogloeopsis* spp. present in YNP is near 63°C (Kallas and Castenholz, 1982; Castenholz, 1996), indicating temperature may also favor Cyanobacteria at the physicochemical conditions observed during sampling. Additionally, the presence of Cyanobacteria below pH values of 4 at several sites further challenges the validity of this lower pH limit for Cyanobacteria. In spite of reports of a sharp lower pH limit for *Chlorogloeopsis* spp. of 4.5 (Kallas and Castenholz, 1982), these Cyanobacteria overwhelmingly outnumbered Cyanidioschyzon at site RS5 in 2011 when the pH was 3.82. Intriguingly, Cyanobacteria exceeded Cyanidioschyzon by over an order of magnitude at IG2 and IG3, both of which had pH values well below 4 (3.33 and 2.98, respectively). While Cyanobacteria may not have been active at IG2 given the lack of detectable echinenone and canthaxanthin, IG3 offers an opportunity to assess the extent to which Cyanobacteria exhibit metabolic activity below a pH of 4.

Though detected in many of the samples, the upper temperature limit for Chlorophyta is likely close to 42° C, which is the upper temperature limit for a thermotolerant strain of *Chlorella* (Sorokin, 1967). A significant decrease in photosynthetic activity by *Chlamydomonas reinhardtii* occurs from 45–47°C that is likely insufficient for growth (Tanaka *et al.*, 2000), whereas a temperature of 39°C is apparently lethal to *Chlamydomonas acidophila* (Gerloff-Elias *et al.*, 2006). Since the ambient spring temperature exceeded 47°C in each location yielding Chlorophyta sequences, Chlorophyta may have been inactive at the time of sampling and may instead be relics of a time when conditions were different, possibly earlier in the year when the temperature was cooler or when the water level was lower and they established populations on the moist walls of the hot spring. The inactivity of these populations is supported by the lack of detection of intact chlorophyll *b*.

CONCLUSIONS

The collective observations presented here illustrate spatial overlap in the habitat range of Cyanobacteria of the genus *Chlorogloeopsis* and algae of the genus Cyanidioschyzon. However, it is unclear to what extent active populations overlap temporally in hot springs. The data offer snapshots of these photosynthetic communities and the geochemical conditions in which they live, both of which appear to be undergoing continuous and dynamic change. For example, site RS5 harbored a putatively active photosynthetic community at the beginning of this study, but neither sequences associated with microbial phototrophs nor light-driven carbon uptake were observed one year later when the temperature was notably higher, apparently exceeding the habitat range boundaries of *Chlorogloeopsis* and *Cyanidioschyzon*. More broadly, the ubiquity of *Cyanidioschyzon* sequences across the hot springs sampled raises fundamental questions concerning the role these populations have in these communities, given the numerical dominance of Cyanobacteria. Cyanidioschyzon sequences were observed at site FF1, but these were not associated with photosynthetically active cells and the temperature was in excess of the upper temperature limit for *Cyanidioschyzon*. These observations suggest that conditions in this spring were amenable to growth of these algae in the recent past, but an increase in temperature brought about by apparent changes in the supplies of meteoric water and hydrothermal vapor led to the physicochemical conditions of the spring moving outside of the habitat range of this organism. It follows that the Cyani*dioschyzon* sequences from many of the other springs examined here may also be remnants of populations that were previously active when physicochemical conditions such as temperature and pH were different, whereas the conditions observed at the time of sampling in the summer characterize niche space occupied by Chlorogloeopsis. Input of snowmelt during the winter and spring, as well as lower ambient temperatures, may lead to lower hot spring temperatures and yield favorable conditions for *Cyanidioschyzon* that do not persist into the summer. However, the limited temporal observations included here illustrate that the perceived fluctuations in the supplies of meteoric water and hydrothermal gases are likely much more complex than simple seasonal transitions.

Acidic hot springs arise from the oxidation of sulfur species to sulfuric acid by oxygen, which only became possible after oxygenic photosynthesis by Cyanobacteria became widespread (Colman *et al.*, 2018). The evolution of algae is also traceable to Cyanobacteria via the evolution of the chloroplast through endosymbiosis (Rodriguez-Ezpeleta et al., 2005), which is hypothesized to have facilitated the development of acidophilic algae such as *Cyanidioschyzon* by shielding the photosynthetic apparatus from extracellular chemical conditions with cytosol (Brock, 1973). Thus, Cyanobacteria are intrinsically rooted in the evolution both of acidic habitats and the photosynthetic microbes that inhabit them, but the transition from habitats dominated by photosynthetic microbes of one domain to those dominated by the other across pH remains inadequately described, particularly for thermal environments that exist at habitat range boundaries. While there are limited observations of the coexistence of algae and Cyanobacteria in hot spring environments, it was thought that a sharp lower limit for Cyanobacteria exists at a pH value of 4 (Brock, 1973; 1978). However, the presence of gene sequences and carotenoids associated with Cyanobacteria in springs studied here with pH values well below 4 suggests an underappreciated role for Cyanobacteria in these acidic habitats. Moreover, the chemically dilute waters of moderately acidic hot springs are poorly buffered against pH changes that may exceed the tolerances of either Cyanobacteria or algae on a variety of timescales. Ultimately, there appears to be spatial overlap in the habitat ranges of algae and Cyanobacteria and key attributes defining these ranges such as temperature and pH are temporally dynamic, resulting in a blurry transition between eukaryotic and prokaryotic oxygenic photosynthesis with respect to pH rather than a sharp demarcation.

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Sample	pH $^{\rm a}$	Temp.	O_2	$\Sigma H_2 S$	SiO_2	F-	Cl-	$\mathrm{SO_4}^{2\text{-}}$	NO_3^-	$\mathrm{NH_4}^+$	Li^+	Na^+	K^+
		°C	μΜ	μΜ	mM	μΜ	μΜ	μΜ	μΜ	μΜ	μМ	μΜ	μМ
FF1	3.28	64.4	22	1.7	2.4	45	150	1488	0.5	119	23.7	1663	442
IG1	4.00	57.0	$(31)^{b}$	(0.5)	(4.9)	13	10	1255	0.3	98	49.4	1944	252
IG2	3.33	48.8	56	0.1	5.7	44	30	1218	0.1	5.4	27.8	1214	380
IG3	2.98	53.6	84	5.5	2.7	291	378	5089	221	426	117	3902	1567
RN1-2011	4.62	57.4	50	1.6	4.3	22	15	195	3.8	19	3.0	234	131
RN1-2012	5.02	51.6	113	0.1	3.2	27	15	197	0.6	11	2.8	260	135
RN2	4.55	55.5	(113)	(0.5)	4.6	18	14	276	3.1	25	1.5	325	174
RN3	4.02	59.8	59	1.2	3.2	89	685	638	0.4	54	46.2	1431	288
RS1	5.07	54.0	41	0.6	4.1	84	28	601	0.2	11	2.7	747	445

 Table 1. Major geochemical measurements and temperatures of the 14 samples in this study.

Sample	pH $^{\rm a}$	Temp.	O_2	$\Sigma H_2 S$	SiO_2	F-	Cl-	$\mathrm{SO_4}^{2\text{-}}$	NO_3^-	$\mathrm{NH_4}^+$	Li^+	Na^+	\mathbf{K}^+
RS2	4.30	49.3	138	5.1	4.4	76	12	533	0.3	7.4	3.9	625	321
RS3	5.42	60.8	34	(3.7)	4.8	93	13	476	1.3	28	1.6	844	260
RS4	5.54	51.0	72	1.1	4.0	101	16	275	1.7	4.1	1.3	603	257
RS5-2011	3.82	56.8	109	0.5	3.6	74	15	470	1.1	19	1.5	479	256
RS5-2012	5.13	70.3	25	2.4	4.0	66	39	379	1.1	12	1.8	562	284

 $^{\rm a}$ Corrected pH, see Supporting Information. $^{\rm b}$ Data in parentheses were estimated from measurements at the site from other years.



Figure 1. Abundance of thermal features in Yellowstone National Park with respect to the pH of their waters. Data were obtained from a survey (n = 7693) of Yellowstone hot springs (www.rcn.montana.edu). Springs are binned every 0.5 pH units and plotted at the midpoint of each pH interval.



Figure 2. Observations of the presence of Cyanobacteria (filled circles) and algae (open symbols) in thermal aquatic environments with respect to *in situ* temperature and pH compiled from the literature (Doemel, 1970; Brock, 1973; Papke *et al.*, 2003; Toplin *et al.*, 2008; Boomer *et al.*, 2009; Meyer-Dombard *et al.*, 2011; Hamilton *et al.*, 2012; Loiacono *et al.*, 2012; Klatt *et al.*, 2013; Schubotz *et al.*, 2013; Skorupa *et al.*, 2013; Schuler *et al.*, 2017; Hamilton *et al.*, 2019; Bennett *et al.*, 2020). For algae, circles indicate sequencing-based confirmation of their presence while triangles indicate confirmation via microscopy or culturing; all data indicating the presence of Cyanobacteria are from sequencing-based analyses. Red crosses denote samples from this study. Empirical 'fringe' curves for Cyanobacteria (dashed line) and algae (dotted line) are drawn that encompass the data. The Cyanobacteria curve is not extended below a pH of 4

due to insufficient data from previous studies to define limits for Cyanobacteria below this pH. The algae fringe is drawn as a line and not extended above a pH of 4.5 owing to the lack of data from previous studies for algae from thermal environments above this pH. In cases where multiple observations were made of the same system, such as in outflow channels, only the highest temperature observation is plotted. Similarly, the highest temperature is plotted when a temperature range is reported for a particular sample location.



Figure 3. Distribution of sulfate and chloride compositions of waters from the Yellowstone National Park (YNP) hydrothermal system. The 14 samples included in this study (red crosses) are plotted along with analyses of YNP hot springs, geysers, and hydrothermally influenced surface waters derived from United States Geological Survey (USGS) open-file reports (filled circles) for
samples taken throughout YNP from 2001–2013 (McCleskey *et al.*, 2004; Ball *et al.*, 2006; Ball *et al.*, 2010; McCleskey *et al.*, 2014). The composition of local meteoric water (open circle) is shown at the mean concentrations of sulfate and chloride reported by the National Trend Network of the National Atmospheric Deposition Program for 2012 analyses of precipitation at Tower Falls, YNP (http://nadp.sws.uiuc.edu/data/ntn/); the evaporation trend for this water is plotted across the diagram as a dashed line.



Figure 4. (A) The isotopic composition of hydrogen (2 H) in hot spring waters relative to the isotopic composition of oxygen (18 O) in the same waters. The 14 samples in this study are plotted as red crosses along with data derived from USGS reports (open circles) for samples taken throughout YNP from 2001– 2013 (McCleskey *et al.*, 2004; Ball *et al.*, 2006; Ball *et al.*, 2010; McCleskey *et al.*, 2014). The local meteoric water line is plotted for reference (Kharaka *et al.*, 2002; Holloway *et al.*, 2011). (B) Correlation of the isotopic composition of hydrogen (2 H) of the 14 hot spring waters in this study with their sulfate concentrations. Site IG3 is indicated with an arrow plotted at its 2 H value, but its sulfate value is off scale. The least-squares regression line is shown (R² = 0.82); IG3 is not included in the regression.





Figure 5. Phylum-level taxonomic composition of bacterial 16S (A) and eukaryal 18S (B) rRNA gene sequences. Data are expressed in percent of total abundance of reads and arranged left to right by increasing pH of the hot spring waters where samples were collected for analysis. Operational taxonomic units (OTUs) representing less than 0.1% of total OTUs are not included. Sequence data for sample RS5-2012 are not shown as no bacterial 16S rRNA gene am-

plicons were obtained and the only eukaryal 18S rRNA gene sequences were affiliated with Streptophyta. Though not visible in this depiction, Cyanobacteria are present in IG2 (0.23% relative abundance) and Rhodophyta are present in RS4 (0.03% relative abundance).



Figure 6. Rates of dissolved inorganic carbon (DIC) incorporation into biomass observed under light and dark conditions for four of the study sites measured using a radiolabeled inorganic carbon tracer. The left scale applies to RS1 and RS2, while the scale on the right applies to RS5 and FF1. Rates were measured in July 2012. Rates were corrected by subtracting the activity measured in corresponding light or dark killed-control microcosms.



Figure 7. Relative abundances of chlorophyll a and its derivatives (A) and major carotenoids (B) in mat samples collected from hot springs in Yellowstone National Park, as determined from integrated peak areas in chromatograms obtained at 665 nm (A) and 475 nm (B) using a diode array detector. Samples are arranged from left to right by increasing pH of the hot spring waters where samples were collected for analysis. As an approximation, the relative abundance of

pheophytin and its derivatives (*i.e.*, all magnesium-free chlorophyll derivatives) were corrected by multiplying by the ratio of molar absorptivities of the Q_y absorption maxima for chlorophyll *a* to pheophytin *a* reported by Kobayashi *et al.* (2006) to reflect the weaker absorbance of demetallated chlorophylls. Similarly, the carotenoid relative abundances were corrected by multiplying by the ratio of the molar absorptivity for -carotene to that of each carotenoid using the molar absorptivities reported by Britton (1995) using light petroleum as solvent.



Figure 8. The mole ratio of the carotenoid zeaxanthin to the carotenoid - carotene as a function of hot spring pH, using data reported in Table S7. Se-

lected samples are discussed in the text.



Figure 9. Photomicrographs of filaments from an enrichment culture inoculated with material collected from site RN1 on July $13^{\rm th}$, 2013 under bright field (a,b) and green (c,d) light. Scale bars in each image are 20 µm.



Figure 10. Ratio of the abundance of 16S rRNA genes affiliated with Cyanobacteria to the abundance of 18S rRNA genes affiliated with Rhodophyta (plotted on a logarithmic scale) as a function of pH of the sample locations. The ratio was calculated using the template abundances of 16S and 18S rRNA genes (Table S8) multiplied by the relative abundance of OTUs affiliated with each taxonomic group (*i.e.*, those affiliated with Cyanobacteria and those affiliated with Rhodophyta). Sites are labeled above each point.

Supporting Information

To Accompany

Cyanobacteria and Algae Meet at the Limits of their Habitat Ranges in Moderately Acidic Hot Springs

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Procedures

1. Ion chromatography. Major ions were quantified using two Dionex DX-600 ion chromatography systems operated by Chromeleon software (version 6.8). The anion system employs a potassium hydroxide eluent generator, a carbonate removal device, and AS11-HC/AG11-HC columns. The hydroxide concentration of the eluent is held isocratically at 5 mM for 5 minutes, followed by a non-linear (Chromeleon curve 8) hydroxide concentration gradient to 55 mM applied over 31 minutes, after which the column is reequilibrated at 5 mM hydroxide for 10 minutes before the next sample injection. The eluent flow rate is held constant at 1.0 mL/minute. The cation system is equipped with CS-16 and CG-16 columns and cations are eluted isocratically with 19 mM methanesulfonic acid (MSA) at 0.5 mL/minute. Samples for cations were acidified with 6 N MSA to approximately 19 mM final concentration. Both systems are plumbed with an external source of deionized water for suppressor regeneration to improve the signal-to-noise ratio of the analyses and suppressor currents were 137 mA and 28 mA for anions and cations, respectively. Samples were delivered to the instruments from 5 mL vials via AS-40 autosamplers (2 injections per vial) onto 100 µL and 75 µL sample loops for anions and cations, respectively. Quantification is achieved externally via calibration curves constructed from a series of dilutions of mixed-ion standards (Environmental Express, Charleston, SC, USA). Quantification accuracy is verified daily by analysis of an independent mixed ion standard (Thermo Scientific, Waltham, MA, USA). Uncertainties in reported ion concentrations are estimated to be \pm 5%.

2. Correction of pH and speciation. The major ion and DIC data were used to calculate the speciation of inorganic carbon and assess charge balance in each sample with the geochemical speciation code EQ3/6 (Wolery and Jarek, 2003) using activity coefficients calculated with an extended Debye-Hückel equation and equilibrium constants derived from the revised Helgeson-

Kirkham-Flowers equation of state (Shock *et al.*, 1997; Sverjensky *et al.*, 1997). Two of the samples (RN1-2011 and RS4) exhibited charge imbalances (expressed as percent of the mean charge, as defined by Nordstrom *et al.*, 2009) in excess of 20%, which is believed to indicate faulty pH probes in use at the time of sampling. Since hydrogen ions are nearly at the same order of magnitude concentration as the major solutes in these samples, the pH measurements were corrected to achieve charge balance, as indicated in Table S1. These corrected values are believed to be closer to the actual pH of each hot spring at the time of sampling than the pH value determined in the field. The speciation of inorganic carbon was calculated using the corrected pH values.

3. Carbon uptake assays. Microcosms were prepared in pre-sterilized, N₂-purged 24 mL serum bottles. Ten mL of water was sampled directly from the spring source and added to each serum bottle using a syringe and needle. Mat samples were collected aseptically using a sterile spatula and placed in 50 mL falcon tubes. Twenty mL of spring water was added to each tube and each was shaken vigorously to create a homogenized slurry. One mL of this slurry was added to each serum bottle. The gas phase of all microcosms was equalized to atmospheric pressure using a sterile needle prior to injection of 5.0 μ Ci (10 μ M final concentration) of radiolabeled sodium bicarbonate (NaH¹⁴CO₃). Light, dark (foil-wrapped), and killed (500 μ M HgCl₂ final concentration) experimental treatments were conducted in triplicate. All microcosms were placed in a sealed bag (secondary containment) and incubated in the source of the spring for < 60 minutes.

In the laboratory, sealed microcosm assays were thawed at room temperature for approximately 2 hours, uncapped, and acidified to pH \sim 2 by injection of 1.0 mL of 1 N HCl to volatize unreacted CO₂. After acidification, microcosms were allowed to equilibrate for an additional 2 hours. Acidified samples were filtered onto white 0.22 µm polycarbonate membranes,

washed with 5 mL of sterile deionized water, and dried overnight at 80°C. Dried filters were placed in scintillation vials and overlain with 10 ml of CytoScint ES[™] liquid scintillation fluid. Radioactivity associated with each of the samples was measured on a Beckman LS 6500 liquid scintillation counter (Beckman Coulter, Inc., Indianapolis, IN, USA).

4. Pigment extraction and analyses. Samples (~1 g) preserved for analysis of pigments were thawed, transferred aseptically to Lysing Matrix A tubes (MP Biomedicals, Irvine, CA, USA), and centrifuged at 21000 x g for 5 minutes at 4°C. After removal of the supernatant, 250 μ L of 7:2 acetone:methanol (v/v) that had been stored over 4 Å molecular sieves (Sigma-Aldrich, St. Louis, MO, USA) was added and the samples were subjected to ballistic bead beating (FastPrep 24; 6.5 speed, 40 s). The samples were subsequently centrifuged and the organic supernatant was transferred to a microcentrifuge tube. Additional aliquots of 7:2 acetone:methanol and pure methanol were homogenized with the solid sample by additional rounds of bead beating and subsequently collected by centrifugation, whereupon the supernatants were pooled. This process was continued until the supernatant became clear and no obvious signs of methanol-soluble pigments remained in the solid sample, typically requiring ~ 1.5 mL of total solvent. The pooled supernatants were centrifuged and the top 500 µL were transferred to Teflon-sealed amber autosampler vials (Agilent Technologies, Inc., Santa Clara, CA, USA) for analysis. To minimize pigment degradation, all manipulations were conducted in a cold room (4 °C) without direct lighting and the samples were transported on ice in a closed container.

Pigment samples were analyzed immediately after extraction via high pressure liquid chromatography (HPLC) equipped with a photodiode array absorbance detector (Thermo Surveyor) coupled with atmospheric pressure chemical ionization mass spectrometry (Thermo Quantum Discovery MAX triple-quadrupole) operating in positive ion, single quadrupole scanning mode from 200 to 1500 m/z with a scan rate of 1 Hz. Parameters for mass spectrometry were based on those employed by van Breemen *et al.* (2012), specifically a corona discharge of 8 μ A, vaporizer temperature of 350°C, and capillary temperature of 300°C. The diode array detector monitored signal intensity at 360, 475, and 665 nm and collected spectra from 325–800 nm at 5 Hz. Samples were injected via a 50 μ L sample loop onto a YMC Carotenoid C-30 reverse phase column (3 x 250 mm). HPLC conditions were modified from those described by Sander *et al.* (1994). The solvent system was initially isocratic at 81:15:4 methanol:methyl *tert*-butyl ether:water for 30 minutes, followed by a linear gradient to 6:90:4 methanol:methyl *tert*-butyl ether:water at 90 minutes. The solvent system was then returned to initial conditions over 5 minutes and held isocratically for 15 minutes to re-equilibrate the column for the next sample.

Insights into Other Microbial Populations in Moderately Acidic Hot Springs

1. Anoxygenic phototrophs. Ribosomal gene sequences affiliated with putative anoxygenic phototrophs were observed in most samples. Two sites (RS1 and RS4) yielded 16S rRNA gene sequences most closely associated with *Chloracidobacterium thermophilum* (13% and 2% of 16S rRNA gene OTUs, respectively), an organism first cultivated from an alkaline hot spring and the only known phototrophic member of the Acidobacteria (Bryant *et al.*, 2007; Tank and Bryant, 2015). Sequence similarity ranged from 86–99%, yet the most abundant OTU associated with *C. thermophilum* in both sites is 99% identical to the cultured representative. *C. thermophilum* is a microaerophilic photoheterotroph (Tank and Bryant, 2015a,b), as its genome lacks key genes for known carbon fixation pathways (Garcia Costas *et al.*, 2012b). Most samples also contained sequences of the aerobic anoxygenic phototrophic bacteria (AAPB) *Acidiphilium* and *Acidisphaera*, which have previously been detected by ribosomal gene sequencing of other

acidic Yellowstone hot spring mats (Macur *et al.* 2004; Hamamura *et al.*, 2005; Hamilton *et al.*, 2019). The AAPB can be characterized as aerobic heterotrophs that synthesize bacteriochlorophylls and reaction centers for light harvesting to supplement energetic requirements (Yurkov and Beatty, 1998; Rathgeber *et al.* 2004) and are important constituents of marine phytoplankton (Eiler, 2006). Energy gained via photophosphorylation enables greater partitioning of organic carbon to biomass (anabolism) than to respiration, which is advantageous when organic carbon is limited (Hauruseu and Koblížek, 2012). *Acidiphilium* sequences were detected in five samples (FF1, IG1, RS1, RS2, RS4), comprising 0.1–0.5% of the 16S rRNA gene OTUs in these samples. Sequences affiliated with *Acidisphaera* were more widespread, detected in all samples except RS1, RS3, and RS5-2012, ranging in abundance from 0.1–1.4% of the 16S rRNA gene OTUs. The sequences are most closely related to isolates from acid mine drainage environments, including *Acidiphilium* sp. CCP3 (100% similarity; Hallberg *et al.* 2006), *Acidiphilium* sp. NO-13 (94–100% similarity; Johnson *et al.* 2001), and *Acidisphaera* MS-Y2 (90–96% similarity; Okamura *et al.* 2015).

Chromatographic pigment analyses for several samples (FF1, RS1, RS2, RS4) yielded peaks thought to represent small amounts of bacteriochlorophyll *a* or its derivatives, based on absorbance spectra associated with these peaks exhibiting Q_y (*i.e.*, longest-wavelength absorption) maxima of >750 nm. One such peak was identified in two mat extracts (RS1, RS4) with a retention time very close to that of bacteriochlorophyll *a* in a pigment extract of *Rhodobacter sphaeroides*, but maxima in the absorption spectrum of the peak were slightly blue-shifted relative to that derived from *R. sphaeroides*, and no molecular ion corresponding to bacteriochlorophyll *a* was observed in the mass spectrum. Instead, the mass spectrum corresponding to this peak in each sample showed a molecular ion (M+H) at 951 m/z with predominant M+H+2 and M+H+4 peaks (Figure S1), which is consistent with bacteriochlorophyll a containing zinc instead of magnesium as the central metal. Zinc-bacteriochlorophyll *a* is the primary chlorophyll in *Acidiphilium* spp. (Wakao et al., 1996; Hiraishi and Shimada, 2001), which is thought to afford greater acid tolerance to the organism due to its retarded pheophytinization rates relative to those of Mgbacteriochlorophyll a (Kobayashi et al., 1998). Ribosomal gene sequences associated with Acidiphilium were detected in these two samples, in addition to three others where Znbacteriochlorophyll a was not detected. The epimer of Zn-bacteriochlorophyll a is found in the reaction centers of C. thermophilum (Tsukatani et al., 2012; He et al., 2019), sequences of which were found only in samples from the two sites where Zn-bacteriochlorophyll a was detected. Therefore, this pigment could alternatively be associated with C. thermophilum, as it is not possible to distinguish between the two epimers with the present data. Magnesium-bacteriochlorophyll a was not detected in any sample, which is the primary chlorophyll of Acidisphaera (Hiraishi et al., 2000), despite Acidisphaera being more widely distributed and often at larger relative abundances than Acidiphilium. Bacteriochlorophyll a was previously observed in pigment extracts from other hot springs in YNP with pH values in the range of 3-6 (Hamilton et al., 2012), yet mass spectra for these analytes were not collected to assess the central metal. Bacteriopheophytin a was detected in pigment extracts from 3 sites (FF1, RS1, RS2), which likely is the primary degradation product of both Zn- and Mg-bacteriochlorophyll a.

In contrast to the observation of bacteriochlorophyll *a* derivatives, no bacteriochlorophyll *c* homologues were detected in any of the samples. *C. thermophilum* employs a variety of bacteriochlorophyll *c* structures as the major chlorosome antenna pigments (Garcia Costas *et al.*, 2012a) so it is surprising that none were identified in samples where *C. thermophilum* sequences were found. While the similar absorbance spectra of bacteriochlorophyll *c* and chlorophyll *a* make

identifying bacteriochlorophyll *c* compounds more ambiguous, a search of unassigned analyte peaks from sample RS1, where *C. thermophilum* was most abundant, did not offer strong evidence for bacteriochlorophyll *c* homologues in the mass spectra. Given the complexity of the mixture of bacteriochlorophyll *c* species produced by *C. thermophilum*, perhaps each individual homologue is below detection in these samples.

Some preliminary assessments can be made regarding the potential for active anoxygenic phototrophs, all of which putatively grow photoheterotrophically, in the moderately acidic hot springs studied herein. C. thermophilum was identified at only two sites, though these sequences represented significant relative abundances at these locations. Since the presence of bacteriochlorophyll c homologues specific to this organism could not be confirmed, it is difficult to assess whether C. thermophilum was active in these springs at the time of sampling. Though originally isolated from an alkaline hot spring outflow, C. thermophilum sequences were also recovered from an acidic, lower temperature spring in YNP (Hamilton et al., 2012), so these phototrophs do not appear to be limited to alkaline habitats. The detection of Znbacteriochlorophyll a at two sites might indicate the Acidiphilium sequences detected at those sites represented active populations, yet the weak signals of this pigment in samples from these sites do not lend themselves to the conclusion that the absence of this pigment indicates a lack of activity by Acidiphilium populations observed at other sites. The activity of Acidisphaera is also inconclusive in light of the sporadic detection of bacteriochlorophyll a chromophores, such as bacteriopheophytin *a*, that might be derived from these cells.

2. Fungi and other eukaryotes. Fungi represented a significant proportion of the 18S rRNA gene OTUs in most samples, with the exception of the three sites near Imperial Geyser. The diversity of the fungal OTUs is large, encompassing 6 of the 7 recognized phyla of fungi.

Thermotolerant fungi are thought to be more abundant in lower pH environments, and likely represent the most thermotolerant Eukarya, having a temperature maximum of ~60°C (Tansey and Brock, 1972; Brock, 1978). Though the Ascomycote Ochroconis (i.e., Dactylaria) has been reported in acidic hot springs at temperatures consistent with those of this study (Tansey and Brock, 1973), OTUs associated with this organism were only detected at RS3 in very low abundance. Other 18S rRNA gene sequences are affiliated with protists, arthropods, and land plants; these sequences in many cases may represent exogenous surface input of biomass rather than indigenous members of the hot spring community. Fungi and protists were also observed in hot spring samples from Lassen Volcanic National Park, California, again with uncertainty regarding to what extent they represent autochthonous organisms (Brown and Wolfe, 2006). Nevertheless, putatively indigenous amoebae have been reported in Nymph Creek and other locations in YNP at somewhat lower temperatures ($\leq 40^{\circ}$ C) than those of this study (Sheehan *et* al., 2003; Amaral-Zettler, 2013). A large (n = 160) study of thermal springs in New Zealand revealed extensive protist diversity across the planktonic samples, and the observation that communities in springs with higher temperatures (50-65°C) were different from those of lower temperature springs is suggestive of indigenous organisms inhabiting springs within this temperature range (Oliverio et al., 2018).



Figure S1. Mass spectrum of zinc-bacteriochlorophyll *a*. A) Uncorrected partial mass spectrum of the pigment extract for sample RS4 subjected to LC-MS analysis averaged over the retention time where light absorbance in the 700–800 nm region was observed in the diode array spectrum, which is indicative of bacteriochlorophylls. B) Predicted profile mass spectrum for $C_{55}H_{75}N_4O_6Zn^+$, the molecular formula for the putative protonated molecular ion (M+H) of zinc-bacteriochlorophyll *a*. The mass spectrum was calculated with a resolution of 0.7 Da at full width at half maximum using Thermo Xcalibur software. The high relative abundances and distribution pattern of the isotopic peaks are in large part attributed to the multiple stable isotopes of zinc; the predominance of the M+H+2 and M+H+4 peaks in particular is attributed to the significant natural abundances of ⁶⁶Zn and ⁶⁸Zn, respectively.



Figure S2. Comparison of major solutes at site RN1 for 2011 and 2012 samples. Corrected H⁺ concentrations are shown.





Figure S3. Typical chromatograms recorded at 475 nm (A) and 665 nm (B) with major peaks numbered (sample RS1 shown) as well as baseline-corrected diode array spectra for selected major pigments. The chromatogram and peak number are indicated in the upper left of each spectrum. Peak A7 is hypothesized to be lycopene (spectrum and data not shown).



Figure S4. Baseline-corrected diode-array spectra of other detected chlorophylls. The sample from which each spectrum arises is indicated in the upper right corner.



Figure S5. Structures of carotenoids discussed in this study arranged according to putative biosynthetic pathways.

Sample	Biofilm	SD ^a	Biofilm	SD	Biofilm	DIC	SD	CO ₂	HCO ₃ -	CO ₂ /HCO ₃ ⁻
	wt. % C	wt. % C	wt. % N	wt. % N	C:N ^b	μΜ	μΜ	μΜ	μΜ	
FF1	3.37	0.01	0.439	0.001	8.95	573	4	572	0.63	910
IG1	3.15	0.06	0.45	0.01	8.2	537	ND ^c	534	3.1	170
IG2	6.02	0.02	0.707	0.002	9.93	1830	30	1830	2.2	830
IG3	4.97	0.06	0.859	0.005	6.74	92	3	92	0.05	1800
RN1-2011	2.87	0.01	0.545	0.001	6.14	<i>140</i> ^d	30	140	3.3	42
RN1-2012	6.9	0.1	1.35	0.04	5.9	73	4	69	4.0	17
RN2	1.08	0.02	0.189	0.003	6.66	180	40	180	3.6	50
RN3	0.66	0.01	0.106	0.001	7.3	83	5	83	0.49	170
RS1	1.4	0.2	0.22	0.03	7.4	1120	20	1050	71	15
RS2	1.57	0.01	0.2080	0.0008	8.80	680	20	680	7.6	89
RS3	2.89	0.06	0.300	0.003	11.2	1000	5	869	130	6.7
RS4	6.1	0.1	0.596	0.008	12	1708	2	1430	280	5.1
RS5-2011	9.8	0.3	1.27	0.05	9.0	1320	20	1310	4.9	270
RS5-2012	0.29	0.01	0.038	0.003	8.9	2060	ND	1920	140	14

Table S1. Carbon and nitrogen content of biofilms and dissolved inorganic carbon (DIC) speciation in hot spring waters.

^a Standard deviation. ^b mol:mol ratio. ^c Not determined; only a single analysis could be completed. ^d DIC data in italics were below the lowest calibration standard.

Table S2. Composition	of enrichment	medium.
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Component	mg/L
Main Solution	
ammonium sulfate	1.39
sodium nitrate	0.32
potassium sulfate	4.96
magnesium sulfate heptahydrate	0.49
sodium sulfate	15.30
calcium chloride	0.94
sodium fluoride	0.92
Trace A Solution (1 mL/L in final media)	
disodium EDTA	130.28
potassium phosphate monobasic	51.71
ferrous sulfate heptahydrate	75.07
manganese (II) chloride tetrahydrate	96.98
boric acid	80.38
zinc sulfate heptahydrate	89.13
Trace B Solution (0.1 mL/L in final media)	
copper (II) sulfate pentahydrate	4.00
sodium molybdate dihydrate	30.24
vanadium (IV) oxide sulfate hydrate	5.71
cobalt (II) chloride hexahydrate	1.93
chromium (III) chloride hexahydrate	1.13
nickel (II) chloride hexahydrate	5.71
sodium selenate	1.89
sodium tungstate dihydrate	2.34

Sample	Sample ID ^a	Easting ^b	Northing	Field pH	Corrected pH ^c	% charge imbalance	Specific Conductivity (µS/cm)	δ ¹⁸ O vs. VSMOW (‰)	δ ² H vs. VSMOW (‰)
FF1	120720KF	513469	4929044	3.30	3.28	-1.02	462	-4.8	-108.3
IG1	110918R	509797	4930939	3.78	4.00	3.55	365	-8.5	-123.2
IG2	120722TE	509794	4930931	3.13	3.33	13.6	606	-5.4	-111.0
IG3	120722TK	510064	4930948	3.26	2.98	-9.24	914	-14.1	-127.4
RN1-2011	110708C	515110	4929721	3.94	4.62	22.0	69.5	-14.9	-137.7
RN1-2012	120718TJ	515110	4929721	4.91	5.02	0.874	96.7	-15.3	-138.2
RN2	110708D	515110	4929727	4.64	4.55	-1.10	59.0	-12.7	-133.2
RN3	120718TL	515045	4929810	4.43	4.02	-4.12	351.8	-11.7	-131.0
RS1	120713TI	515138	4928593	4.80	5.07	3.09	274.4	-9.1	-124.0
RS2	120713TL	515125	4928566	4.50	4.30	-2.49	171.4	-9.3	-123.4
RS3	110710Y	515146	4928521	4.87	5.42	9.18	155.3	-12.9	-127.8
RS4	110710D	515145	4928523	4.24	5.54	39.9	117.1	-15.3	-139.8
RS5-2011	110720F1	515129	4928550	3.80	3.82	0.916	165.8	-13.5	-134.2
RS5-2012	120713TJ	515129	4928550	4.52	5.13	14.4	224.3	-13.3	-134.8

Table S3. Sample dates, locations, charge balance results, conductivities, and isotopic ratios of water.

^a Sample IDs begin in YYMMDD format. ^b UTM coordinates, all in zone 12T, using the WGS84 datum. ^c Field pH corrected to achieve charge balance (see procedures above).

Assignment	chl. a	chl. <i>a</i> '	phe. a	phe. a'	phe. <i>a</i> allomer	pyrophe. a	phede. a
Peak number ^b	B2	B3	B6	B5	B4	B7	B1
Soret band (nm)	431	432	409	409	409	410	408
Qy band (nm)	665	665	666	666	666	666	665
molecular ion (m/z)	893.4	893.5	871.5	871.5	887.5	813.5	593.2
FF1	nd ^c	nd	20674	2898	3872	10418	28521
IG1	30452	396	1214	174	231	157	6180
IG2	62118	2475	1766	325	nd	572	3328
IG3	90981	1707	35160	5301	5152	586	2388
RN1-2011	12749	144	528	65	817	1894	nd
RN1-2012	93304	1479	2747	515	2494	286	nd
RN2	100392	3310	33731	6256	22130	15701	3863
RN3	223066	2857	14790	2512	9688	5654	nd
RS1	144631	4956	9389	2041	3534	3000	4786
RS2	41007	1158	7702	1435	5461	3388	nd
RS3	73171	1806	16080	2516	15697	3344	2303
RS4	83014	980	3615	385	1265	172	nd
RS5-2011	73321	1193	7088	1303	1259	1288	3115
RS5-2012	nd	nd	446	199	307	999	nd

Table S4. Semi-quantitative abundance data for chlorophyll *a* and derivatives.^a

^a Data from observation at 665 nm. ^b Figure S3. ^c Not detected. Abbreviations: chl, chlorophyll; phe, pheophytin; pyrophe, pyropheophytin; phede, pheophorbide.

assignment	β-carotene	β-crypto- xanthin	zeaxanthin	echinenone	cantha- xanthin	13-Z-β- carotene	9-Z-β- carotene
peak number ^b	A5	not shown ^c	A1	A3	A2	A4	A6
absorbance maxima							
(nm)	452, 478	452, 478	451, 477	465	477	338, 444, 470	448, 474
% III/II	22	28	31	undefined ^d	undefined ^d	4	25
molecular ion (m/z)	537.4	553.4	569.5	551.4	565.4	537.4	537.4
FF1	4457	nd ^e	7468	nd	nd	nd	1646
IG1	4371	1263	15303	nd	nd	621	1353
IG2	9709	3226	43092	nd	nd	1704	2986
IG3	12971	nd	11147	9416	3857	1774	3540
RN1-2011	4683	nd	990	1668	384	nd	1421
RN1-2012	21792	nd	8497	8198	2711	3464	5803
RN2	25869	nd	9258	21075	5877	nd	9186
RN3	52709	nd	13335	15340	4993	nd	14022
RS1	119548	nd	27570	9347	2336	26504	44259
RS2	6530	nd	2329	2404	424	nd	2236
RS3	7704	nd	3675	8885	17671	1037	2580
RS4	10565	nd	4573	4073	593	1433	3149
RS5-2011	17659	nd	5357	7845	1618	1856	4395
RS5-2012	398	nd	394	408	nd	nd	208

Table S5. Major carotenoid semi-quantitative abundance data.^a

^a Data from observation at 475 nm. ^b Figure S3. ^c Not present in Figure S3; retention time is 54 minutes. ^d Only one absorbance maximum, so value undefined. ^e Not detected.

Assignment	Zn- bacterio- chlorophyll <i>a</i>	bacterio- pheophytin a	chloro- phyllide b	pheophytin b	pheophytin <i>b</i> allomer	chlorophyll d ^d	pyro- pheophytin d ^d
Retention time (min.)	16	32	18	61	53	24	71
Soret band (nm)	356	359	436	436	435	443	424
Q_y band (nm) ^b	761	746	652	653	652	697	692
molecular ion (m/z)	951.4	889.6	628.5	885.6	901.4	895.7	815.5
FF1	nd ^c	416	nd	1774	310	nd	nd
IG1	nd	nd	166	338	nd	nd	nd
IG2	nd	nd	339	231	nd	nd	nd
IG3	nd	nd	nd	nd	nd	nd	nd
RN1-2011	nd	nd	nd	nd	nd	160	nd
RN1-2012	nd	nd	nd	nd	nd	nd	nd
RN2	nd	nd	nd	nd	nd	1207	nd
RN3	nd	nd	nd	nd	nd	nd	464
RS1	152	986	nd	nd	nd	36	nd
RS2	nd	1265	nd	nd	nd	nd	631
RS3	nd	nd	nd	nd	nd	nd	nd
RS4	385	nd	nd	nd	nd	nd	nd
RS5-2011	nd	nd	nd	nd	nd	nd	nd
RS5-2012	nd	nd	nd	nd	nd	nd	nd

Table S6. Semi-quantitative abundance data for other chlorophylls and their derivatives.^a

^a Data from observation at 360 nm. ^b Longest-wavelength absorption maximum. ^c Not detected. ^d It is likely that chlorophyll d and pyropheophytin d are derived from enzymatic oxidation of chlorophyll a and pyropheophytin a during extraction (*e.g.*, Kadowaki *et al.* 2005), though it is possible that chlorophyll d is biosynthesized by *Chlorogloeopsis* sp., which *C. fritschii* is known to do under natural light (Airs *et al.*, 2014), albeit concomitantly with chlorophyll f which was not detected here.

	chlorophyll a	β-carotene	zeaxanthin ^a	β-cryptoxanthin ^a
FF1	nd ^b	2.2	3.5	nd
IG1	16.7	2.2	7.2	0.6
IG2	34.0	4.8	20.2	1.6
IG3	49.7	6.4	5.2	nd
RN1-2011	7.0	2.3	0.5	nd
RN1-2012	51.0	10.8	4.0	nd
RN2	54.9	12.8	4.3	nd
RN3	122.0	26.2	6.2	nd
RS1	79.1	59.3	12.9	nd
RS2	22.4	3.2	1.1	nd
RS3	40.0	3.8	1.7	nd
RS4	45.4	5.2	2.1	nd
RS5-2011	40.1	8.8	2.5	nd
RS5-2012	nd	0.2	0.2	nd

Table S7. Quantitative abundances for selected pigments expressed as μ mol/g N.

^a Quantified using the response factor for β -carotene and not corrected for slight differences in molar absorptivity. ^b Not detected.

	Bacterial 16S rDNA		Eukaryal 18S rDNA	
	copies/ng DNA	uncertainty ^a	copies/ng DNA	uncertainty ^a
IG1	9 x 10 ⁶	3 x 10 ⁶	2.5 x 10 ⁵	0.4 x 10 ⁵
IG2	5.8 x 10 ⁵	0.6 x 10 ⁵	8 x 10 ¹	3 x 10 ¹
IG3	4 x 10 ⁵	1 x 10 ⁵	4.6 x 10 ³	$0.6 \ge 10^3$
RN1-2012	1.2 x 10 ⁶	0.2 x 10 ⁶	4	1
RS1	7.7 x 10 ⁵	0.3 x 10 ⁵	2.3 x 10 ²	$0.3 \ge 10^2$
RS2	5 x 10 ⁵	1 x 10 ⁵	5	1
RS4	4.0 x 10 ⁵	0.6 x 10 ⁵	1.31 x 10 ²	1
RS5-2011	1.41 x 10 ⁶	0.02 x 10 ⁶	1.4 x 10 ¹	1

Table S8. Abundance of bacterial 16S rRNA and eukaryal 18S rRNA gene templates as determined by quantitative PCR.

^a Uncertainty is the standard deviation of three replicate qPCR assays.

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APPENDIX A: Relative abundances, affiliations, and percent identities of the best BLASTn query for the five most abundant 16S rRNA gene OTUs (left) and 18S rRNA gene OTUs (right) in each of the fourteen samples in this study.

				F	=1						
Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance		
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)		
Hydrogenobaculum sp. Y04ANC1	Aquificae	Aquificales	97	46	Cryptococcus albidus	Bastidiomycota	Filobasidiales	100	39		
Desulfotomaculum kuznetsovii DSM 6115	Firmicutes	Clostridiales	93	9	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	18		
Thermoanaerobacterium	Firmicutes	Thermoanaerobacterales	99	9	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	11		
thermosaccharolyticum DSM 571											
Desulfoglaeba sp. Lake	Proteobacteria	Syntrophobacterales	89	6	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	7		
Ktedonobacteria bacterium Hsw-67	Chloroflexi	Ktedonobacterales	94	5	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	5		
IG1											
Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance		
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)		
Thermodesulforhabdus norvegica strain A8444	Proteobacteria	Syntrophobacterales	90	23	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	35		
Ktedonobacteria bacterium Hsw-67	Chloroflexi	Ktedonobacterales	94	19	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	22		
Hydrogenobaculum sp. SN	Aquificae	Aquificales	100	9	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	14		
Acidobacteria bacterium WSF1-34	Acidobacteria	Gp2	97	5	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	8		
Desulfotomaculum thermobenzoicum	Firmicutes	Clostridiales	92	4	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	7		
IG2											
Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance		
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)		
Ktedonobacteria bacterium Hsw-67	Chloroflexi	Ktedonobacterales	94	52	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	26		
Ktedonobacteria bacterium Hsw-67	Chloroflexi	Ktedonobacterales	93	10	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	16		
Thiomonas sp. 6C	Proteobacteria	Burkholderiales	99	6	Chlamydomonadaceae sp. RT1n14cul	Chlorophyta	Chlamydomonadales	99	10		
Hydrogenobaculum sp. SN	Aquificae	Aquificales	100	5	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	9		
Chloroflexi bacterium T81	Chloroflexi	unclassified	87	3	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	5		
				IC	33						
Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance		
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)		
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	100	48	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	32		
Ktedonobacteria bacterium Hsw-67	Chloroflexi	Ktedonobacterales	94	16	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	24		
Acidobacteria bacterium IGE-010	Acidobacteria	unclassified	91	5	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	14		
Ktedonobacteria bacterium Hsw-67	Chloroflexi	Ktedonobacterales	93	3	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	9		
Meiothermus granaticus	Deinococcus-	Thermales	100	3	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	7		
	Thermus										
				RN1	2011						
Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance		
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)		
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	100	58	Cochliobolus kusanoi	Ascomycota	Pleosporales	99	36		
Acidobacteriaceae bacterium K22	Acidobacteria	Acidobacteriales	100	11	Penidiella columbiana	Ascomycota	Capnodiales	100	24		
Acidobacteria bacterium WSF1-34	Acidobacteria	Gp2	96	10	Chironomus plumosus	Arthropoda	Diptera	99	11		
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	96	3	Cryptococcus albidus	Bastidiomycota	Filobasidiales	100	5		
Hydrogenobaculum sp. Y04ANC1	Aquificae	Aquificales	97	2	Surculiseries rugispora	Ascomycota	Xylariales	99	4		

RN1-2012

Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	100	79	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	23
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	96	4	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	16
Rudea sp. YC6842	Proteobacteria	Xanthomonadales	93	3	Penidiella columbiana	Ascomycota	Capnodiales	100	15
Sulfurihydrogenibium sp. Y03AOP1	Aquificae	Aquificales	100	2	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	9
Hydrogenobaculum sp. Y04ANC1	Aquificae	Aquificales	97	2	Cochliobolus kusanoi	Ascomycota	Pleosporales	99	5

RN2

Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	100	31	Pichia kudriavzevii strain IPE100	Ascomycota	Saccharomycetales	100	31
Acidobacteria bacterium IGE-016	Acidobacteria	unclassified	94	16	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	22
Hydrogenobaculum sp. Y04ANC1	Aquificae	Aquificales	97	7	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	18
Thermolithobacter ferrireducens strain KA2	Firmicutes	Thermolithobacterales	89	7	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	9
Sulfurihydrogenibium sp. Y03AOP1	Aquificae	Aquificales	100	4	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	5

RN3

Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	100	64	Chlamydomonadaceae sp. RT1n14cul	Chlorophyta	Chlamydomonadales	99	32
Hydrogenobaculum sp. Y04ANC1	Aquificae	Aquificales	97	9	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	11
Hydrogenobaculum sp. SN	Aquificae	Aquificales	100	3	Metopus palaeformis	Ciliophora	Armophorida	96	10
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	96	3	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	7
Melioribacter roseus P3M	Ignavibacteria	Ignavibacteriales	100	2	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	5

RS1

					-				
Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	100	48	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	28
Chloracidobacterium thermophilum	Acidobacteria	Unclassified	99	12	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	15
Synechococcus sp. C9	Cyanobacteria	Chroococcales	100	9	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	9
Acidobacteria bacterium IGE-016	Acidobacteria	unclassified	94	4	Neolecta irregularis strain ZW-Geo79-Clark	Ascomycota	Neolectales	92	6
Sulfurihydrogenibium sp. Y03AOP1	Aquificae	Aquificales	100	4	Parasonderia vestita	Ciliophora	Plagiopylida	87	6

RS2

Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	100	42	Ascoidea hylecoeti strain NRRL Y-17634	Ascomycota	Saccharomycetales	83	22
Bacterium Ellin5258	Acidobacteria	Acidobacteriales	97	24	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	14
Ktedonobacteria bacterium Hsw-67	Chloroflexi	Ktedonobacterales	94	3	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	7
Thiomonas sp. 6C	Proteobacteria	Burkholderiales	99	3	Pseudostichococcus monallantoides	Chlorophyta	Trebouxiophyceae	100	5
Paludibacter propionicigens WB4	Bacteroidetes	Bacteroidales	93	2	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	4

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Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	100	58	Neolecta irregularis strain ZW-Geo79-Clark	Ascomycota	Neolectales	92	24
Sulfurihydrogenibium sp. Y03AOP1	Aquificae	Aquificales	100	13	Uronema sp. CCAP 334/1	Chlorophyta	Chaetophorales	100	20
Meiothermus granaticus	Deinococcus-	Thermales	100	8	Pinus wallichiana	Streptophyta	Coniferales	99	13
	Thermus								
Meiothermus granaticus strain AF-68	Deinococcus-	Thermales	91	5	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	5
	Thermus								
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	96	3	Codosiga minima strain IOW73	Choanoflagellida	Codonosigidae	87	4

RS4

Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	100	60	Neolecta irregularis strain ZW-Geo79-Clark	Ascomycota	Neolectales	92	54
Tepidimonas sp. AA2	Proteobacteria	Burkholderiales	98	4	Chlorella protothecoides var. acidicola	Chlorophyta	Chlorellales	100	12
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	96	3	Chlamydomonadaceae sp. RT1n14cul	Chlorophyta	Chlamydomonadales	99	5
Rudea sp. YC6842	Proteobacteria	Xanthomonadales	93	2	Metopus palaeformis	Ciliophora	Armophorida	96	4
Bacterium Ellin5258	Acidobacteria	Acidobacteriales	97	2	Mesotaenium caldariorum	Streptophyta	Zygnematales	98	3

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Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	100	77	Talaromyces purpurogenus isolate BMC1	Ascomycota	Eurotiales	100	20
Sulfurihydrogenibium sp. Y03AOP1	Aquificae	Aquificales	100	3	Pseudostichococcus monallantoides	Chlorophyta	Trebouxiophyceae	100	8
Chlorogloeopsis sp. Greenland 5	Cyanobacteria	Stigonematales	96	2	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	8
Sparobacteria bacterium NM5	Verrucomicrobia	Spartobactieria	93	2	Penidiella columbiana	Ascomycota	Capnodiales	100	6
Chthonomonas calidirosea	Armatimonadetes	Chthonomonadales	92	2	Cyanidioschyzon merolae strain 10D	Rhodophyta	Cyanidiales	100	5

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Bacteria (16S)			Identity	Abundance	Eukarya (18S)			Identity	Abundance
Best BLASTn Hit	Phylum	Order	(%)	(%)	Best BLASTn Hit	Phylum	Order	(%)	(%)
					Fibraurea tinctoria	Streptophyta	Ranunculales	100	>99
					Fibraurea tinctoria	Streptophyta	Ranunculales	96	<1
No 16S rRNA gene amplicons obtained					Fibraurea tinctoria	Streptophyta	Ranunculales	97	<1
					Fibraurea tinctoria	Streptophyta	Ranunculales	97	<1
					Fibraurea tinctoria	Streptophyta	Ranunculales	96	<1
APPENDIX B: Photographs of sample sites taken on the day of sampling (unless otherwise noted).









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*Photo taken July 2011



RS3





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