

DRAFT: Development of Layered Photosynthetic Mats *in situ* - Community Dynamics and Microbial Dispersal

Running Title: Photosynthetic mat formation *in situ*

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ABSTRACT

We modeled multi-layered photosynthetic mat formation at Fairy Geyser, Yellowstone National Park by suspending sterile glass rods in the thermal run-off. Population dynamics were assessed by comparing biofilm populations from replicates at 1, 2.5, 2.5 and 13 months using microscopy, pigment assessment, and bacterial 16S rRNA gene analysis (both general bacterial and Chloroflexi-targeted). To address potential dispersal mechanisms for mat and biofilm formation, we also assessed geothermal vent water from Fairy Geyser. Initial biofilm colonization appeared to be by Thermus-like bacteria and Synechococcus-like Cyanobacteria, although green Chloroflexi were detected using a targeted strategy. For the next two months, Cyanobacteria were the dominant member of the biofilm, with notable increases in chemotroph diversity. At 13 months, mats with outer green layers and inner red layers had fully developed. The green layer was composed of at least 2 different Cyanobacteria, Synechococcus and Fisherella. The red layer was more diverse in terms of both phototrophs and chemotrophs, with Planctomycetes, Acidobacteria, and red Chloroflexi sequences representing the highest portions of the retrieved 16S bacterial population. Fairy Geyser vent source water contained predominantly Deinococcus/Thermus, as well as Chloroflexi using a targeted approach. However, we were unable to detect Cyanobacteria or intermediate biofilm chemotrophs, leaving open the possibility that something other than water (e.g. air, soil) is seeding mat communities. Although our initial aim and hypothesis for this study regarded the specific development of red Chloroflexi layers, we gained insight into several comparable portioning events, including early transitions from chemotrophs to Cyanobacteria-dominated biofilms, and green layer transitions from mostly Synechococcus to Synechococcus/Fischerella. Taken together, these community changes reflect new niches being established, likely defined by different temperature, light, and nutrient conditions.

INTRODUCTION

In 1967, Thomas Brock immersed slides into several near-boiling pools in Yellowstone National Park's Lower Geyser Basin, demonstrating an accumulation of thermophilic microorganisms, including Cyanobacteria, pink and yellow filaments, and many colorless rods (REF: **Bott and Brock, 1969; Brock, 1967**). Contemporary biofilm studies in Yellowstone continue to support Brock's historic findings, with most aimed at understanding how microbes contribute to biogeochemical processes and geomorphology. For example, Fouke et al., working at carbonate spring, Angel Terrace in Mammoth Basin, reported a rich diversity of 16S rRNA sequences in geothermal vent source water and downstream travertine deposits, with each particular community selected by varying geochemical and temperature gradients. These studies also demonstrated that erupting geothermal groundwater contains an abundance of microorganisms, suggesting a mechanism for microbial dispersal (REF: **Fouke et al., 2002, 2003 and 2006 submitted**).

Given these studies, we examined dispersal mechanisms and mat formation in the context of our laboratory's focus, red Chloroflexi that occupy underlayers within some communities (35-60°C and pH 7.5-8.5), beneath Cyanobacteria and green Chloroflexi (REF: **Boomer 2000, 2002; Ward Review**). 16S rRNA sequences retrieved from red layer communities (designated Red Layer Microbial Observatory, RLMO, sites) are most similar to Roseiflexus, a Chloroflexi that contains only bacteriochlorophyll (Bchl) a and was isolated from red surface mats in Japan (REF: **Hanada 2002**). Unlike Roseiflexus, RLMO Chloroflexi show distinct Bchl a absorbance profiles, have not been observed to colonize the surface of these mat systems, and have not been amenable to pure culture methods using published approaches by Hanada et al. (REF: **Boomer 2000, 2002; Terry's Thesis**).

In this multi-year study, we modeled photosynthetic mat formation in the run-off channel of Fairy Geyser (Figure 1, Plate A), a previously described RLMO site (REF: **Boomer 2002**). Prior to designing these experiments, we had observed that natural debris which fell into thermal channels (70-90°C) fed by RLMO spring water became covered with multi-layered photosynthetic mats in the cooler splash zones (35-50°C) above the run-off water (REF: **unpublished - Boomer, Lodge**). Consequently, we hypothesized that red Chloroflexi were present in geothermal groundwater, capable of seeding downstream niches if provided a substrate with an appropriate temperature zone. Because RLMO Chloroflexi are non-sulfur photoheterotrophs that metabolize under low intensity light and low oxygen conditions (REF: **Boomer, 2000**), we further hypothesized that red layer formation would require the presence of a shielding green layer and would thus form after initial colonization by green phototrophs. Indirectly supporting these hypotheses, Ward et al. demonstrated that photosynthetic mats at Octopus Spring (55-62°C) readily re-grow following the scraping away of the

top 3 mm, primarily Synechococcus (REF: **Ward, 1997**). Although Octopus Spring mats contains both red and green Chloroflexi, it does not contain a distinct red underlayer.

To test our hypotheses, we suspended sterile glass rods in the thermal run-off at Fairy Geyser and collected replicates at defined intervals. We assessed biofilm accumulation using microscopy, pigment analysis, and 16S rRNA studies, both general bacterial and Chloroflexi-targeted. This report represents our second year-long study at Fairy Geyser (2004-5), following a successful preliminary study with fewer collections, replicates, and analyses in 2003-4. Although other researchers have grown monolayers on artificial substrates or performed disturbance and recovery studies on existing mat systems in Yellowstone (REF: **Bott and Brock, 1969; Brock, 1967, Ward 1997**), this study likely represents the first report of multi-layered photosynthetic mats being grown and studied in this manner.

MATERIALS AND METHODS

Study site: Our study site was at Fairy Geyser (Figure 1, Plate A), Yellowstone National Park, Wyoming (N44.32.53, W110.51.68). Previously, only the splash mats (48-53°C, pH 8.0) around the vent source have been collected and described (REF: **Boomer 2002**). Fairy Geyser's run-off channel maintains a temperature of 65-80°C for approximately 200 m. Along this drainway, several splash zones with ephemeral, multi-layered mat communities grow.

Mat formation study: On May 30, 2004, sixteen sterile glass rods (20 cm long, 1 cm diameter) were placed approximately 100 m downstream (70.5°C, pH 9.0) from the vent source in accordance with low-impact requirements defined by the Yellowstone Center for Resources (REF: **personal communication, Christie L. Hendrix, Research Permit Facilitator**). In the field, each rod was held in place using a sterilized clothespin that was secured onto a native stick placed across the run-off channel (Figure 1, Plate B). Of each rod, 6-7 cm was physically in the run-off water, 6-7 cm was between the water surface and the point of attachment, and 6-7 cm was at or above the point of attachment. Four rod replicates were collected at each of the following timepoints: 1 month (June 30, 2004), 2.5 months (August 17, 2004), 3.5 months (September 14, 2004), and 13 months (July 8, 2005). After unclipping, the top attachment region of each 2004 rod was aseptically removed, with each sample end placed in a sterile conical 15 ml tube. 2005 rod samples contained so much growth that each had to be dissected on site into outer green and inner red layer sub-samples using sterile forceps and scalpels. All specimens were frozen at -20°C within 2 hours of collection, transported to Oregon on dry ice, and stored at -80°C.

Rod biofilm sample preparation: Given that our goal was to assess and compare similar amounts of biofilm growth from each timepoint, efforts were made to measure, resuspend, and aliquot defined amounts of each sample. For 1, 2.5, and 3.5 month samples, 10 ml of GTE was added to each tube. A combination of 5-10 minutes of vortex agitation and light stripping using a sterile scalpel removed and homogenized biofilm material from each rod. Biofilm resuspensions were subdivided into 10 pre-weighed tubes, centrifuged at 10,000 rpm for 4 minutes at room temperature to pellet cells, and weighed following removal of the buffer supernatant. Although we did not perform a true dry weight measurement, we obtained consistent sample weights based on averaging 2-3 rod samples for each replicate set.

Water collection, filtration, and analysis: Geothermal groundwater was collected at the Fairy Geysers vent source on June 9, 2006 (pH 8.0, 93°C) using 6 L in acid-cleaned and sterilized 2 L Nalgene Polypropylene Boston Round bottles. Within 2 hours of collection, 1.0 L water was filter-concentrated onto sterile Millipore Isopore Polycarbonate Membrane filters (37 mm diameter, 0.4 micron pores) using a 250 ml filter chimney tower (FisherBrand, Pittsburgh, PA) and a vacuum pump (Gast, Benton Harbor, MI). Each filter was placed in a 50 mm diameter individual tight-lid petri dish (FisherBrand, Pittsburgh, PA), frozen at -20°C, transported to Oregon on dry ice, and stored at -80°C until further analysis.

DNA extraction, PCR amplification, and cloning: To isolate genomic DNA, each sample (0.2 g each biofilm timepoint or water filters) was combined with GTE, 1% SDS, phenol-chloroform (50% total volume), and Zirconia/Silica beads (Biospec Products, Inc., Bartlesville, OK). Visible lysis was achieved using a Mini-Beadbeater (Biospec Products, Inc., Bartlesville, OK) at a setting of 6 for 60 seconds, repeated 3 times. Following 2 additional phenol-chloroform extractions, aqueous layers were subjected to ethanol precipitation at -80°C for 1 hour. In all cases, two independent PCR amplifications were applied to each sample: one using universal bacterial 16S rRNA primers (8FPL, 1492 RPL) that amplified a nearly full-length product (**REF: Reysenbach 1994**), and another using Chloroflexi-specific 16S rRNA primers (CCR-344-F, CCR-1338-R) that amplified a shorter, internal product (**REF: Ward 2001**). All PCR amplifications were performed using a suite of twelve buffers as previously described, with products from 4 different buffers combined for each clone library (**REF: Boomer 2002**).

Sequence analyses: For each sample, twenty universal bacterial clones and ten Chloroflexi clones were assessed. Nucleotide sequence analysis was performed as described previously, with one end-read performed per clone (REF: **Boomer 2002**). All datasets were analyzed using Bellerophone (REF: **Huber, G. 2004**), with predicted chimeric sequences culled. Non-chimeric sequences were compared with the GenBank database using the basic local alignment search tool (BLAST) network service (REF: **ALTSCHUL ET A. 1990**) to establish division-level assessment (and, in the case of Chloroflexi, to green, red, or chemotrophic subdivision). For each sequence, the sequence length (base pairs/bp), percent similarity (%ID), and best match organism (highest-ranking BLAST retrieved) were recorded.

Microscopy: All microscopy was performed using an Olympus BX41 microscopy with an Olympus Reflected Fluorescence System/BX-URA2 Model and a DP11 digital camera system (Olympus America, Inc. Melville NY). To consistently examine defined volumes for each rod sample, we used CELL-VU disposable hemacytometers (**Millennium Sciences - GET MORE?**), assessing and averaging six random counting squares (each 0.25 mm X 0.25 mm X 0.02 mm) using manufacturer instructions. After troubleshooting, we determined that the appropriate dilution of sample material for counting biofilm suspensions was 0.0052 g in 10 ul buffer. Given hemocytometer limitations, counts were biased toward large, pigmented cell types. While this approach worked for 1, 2.5, and 2.5 month samples, whole mat sections prepared from the 13-month timepoint would not homogenize and thus we were unable to count these samples. All samples were also examined using UV to assess for red-fluorescing chlorophyll (Chl) and green- or non-fluorescing Bchl.

To microscopically assess water filtrate samples, we removed intact cells by combining each filter with 6 ml GTE buffer in a 15-ml conical tube and gently rocking overnight at 4°C. Buffer containing removed cells were subdivided into 4 microfuge tubes and pelleted at 10,000 RPM for 10 minutes at room temperature. Following supernatant removal, visible pellets were combined with 50 ul GTE prior to microscopic assessment using the same counting methods described above.

Pigment analysis: Total pigment was methanol-extracted from all rod biofilm samples as previously described (REF: **Boomer 2002**). In each case, 0.02 g biofilm resuspension was analyzed. Absorbance measurements for pigment peaks at 436-440 nm (carotene), 664-669 nm (Chl a or Bchl c), and 768-771 nm (Bchl a) were recorded.

Accession numbers: Accession numbers for all June/1-month rod biofilm samples are as follows: universal bacterial clones are DQ984378-DQ984397; Chloroflexi-targeted clones are DQ984398-DQ984406. Accession numbers for all August/2.5 month rod biofilm samples are as follows: universal bacterial clones are DQ984288-DQ984307; Chloroflexi-targeted clones are DQ984308-DQ984317. Accession numbers for all September/3.5 month rod biofilm samples are as follows: universal bacterial clones are DQ984407-DQ984426; Chloroflexi-targeted clones are DQ984278-DQ984287. Accession numbers for all July/13 month rod biofilm green layer samples are as follows: universal bacterial clones are DQ984318-DQ984337; Chloroflexi-targeted clones are DQ984338-DQ984347. Accession numbers for all July/13 month rod biofilm red layer samples are as follows: universal bacterial clones are DQ984348-DQ9843647; Chloroflexi-targeted clones are DQ984368-DQ984377. Accession numbers for all source water samples are as follows: universal bacterial clones are DQ984497-DQ9843516; Chloroflexi-targeted clones are DQ984427-DQ984436.

Website and database information: All data in this publication and related materials can be found at the Red Layer Microbial Observatory (RLMO) database (<http://www.wou.edu/rmodb> - **CHECK**).

RESULTS

Biofilm accumulation: In this study, we partially immersed sterile glass rods in the thermal run-off channel of Fairy Geyser, collecting sample replicates at 4 timepoints over 13 months. At all collection timepoints, macroscopic evidence of biofilm accumulation could be observed (Figure 1, Plates D-F and I). Table 1 shows biofilm accumulation as measured by mass and microscopic cell counts, the latter biased toward large unicellular rods (2-3 μm wide, 8-10 μm long) that fluoresced red under UV and thin filaments (1 μm wide, 10-100 μm long) that did not appear to fluoresce under UV. Although more difficult to see, filaments conservatively comprised 5-10% of the population after 2.5 months. Representative biofilm microscopy images are shown in Figure 1, Plates G-H. Although 13-month biofilm samples were not amenable to resuspension, green and red layers were assessed for cell morphology and autofluorescence properties. Figure 1, Plates J-K show the 13-month green layer, dominated by green heterocyst-containing filamentous cells and curved unicellular rods, both of which strongly fluoresced red under UV. Figure 2, Plates G-H show the 13-month red layer, dominated by red-orange thin filaments that weakly fluoresced light green under UV. Figure 2 shows relative amounts of methanol-extracted pigments, including carotenes, green Chl a, Bchl c, Bchl a, in 0.02 g of each biofilm sample.

Biofilm 16S library analysis: To characterize and compare general bacterial populations present in biofilm samples over time, 16S rRNA gene libraries were retrieved from each timepoint and 20 clones were assessed via nucleotide sequence analysis and similarity analysis against GenBank. These data are summarized in Table 2 and Figure 3, Panel A. Most retrieved sequences were 90% or more similar to other bacterial sequences in GenBank, with 51/100 most similar to isolates from other Yellowstone thermal features and an additional 20/100 most similar to isolates from thermal features elsewhere in the world. Notably low similarity scores were observed for a 3.5 month Candidate WS6-like sequence (81%), a 3.5 month Planctomycetes-like sequence (83%), and a 13 month red layer Proteobacteria-like sequence (64%).

Cyanobacteria- and Thermus-like sequences represented the majority of sequences at 1 month (40 and 45%, respectively). Cyanobacteria-like sequences continued to dominate at 2.5 (60%) and 3.5 (60%) months. During the first 3.5 months, Cyanobacteria-like sequences were most similar to either Synechococcus or uncultured hot spring sequences. At 13 months, sequences resembling Fisherella represented 55% of the retrieved population. Red Chloroflexi-like sequences did not appear at any timepoint except the 13-month red layer sample where they represented 15% of the retrieved population; Planctomycetes-like sequences represented the majority (45%) of the retrieved population in the 13-month red layer sample.

Given our specific interest in Chloroflexi, we also made targeted 16S rRNA libraries using Chloroflexi-specific primers (**REF: Ward 2001**), assessing 10 clones from each timepoint. These data are summarized in Table 3 and Figure 4. Most retrieved sequences were 90% or more similar to other bacterial sequences in GenBank, with 49/50 most similar to isolates from other Yellowstone thermal features and the final sequence most similar to isolates from thermal features elsewhere in the world. For the first 3.5 months, green Chloroflexi-like sequences dominated all libraries. At 13 months, the green layer sample contained 80% green Chloroflexi-like sequences and 20% red Chloroflexi-like sequences. The 13 month red layer sample contained 90% red Chloroflexi-like sequences and 10% green Chloroflexi.

Geothermal water assessment: In order to determine whether geothermal groundwater from the source contained bacteria that could seed downstream community formation, we collected and filtered water samples from Fairy Geyser in June 2006. For three years prior, we had performed preliminary water analyses at Fairy Geyser, observing varying filament counts ranging from 10^{3-5} filaments/L in June/July to 10^{1-2} filaments/L in August/September (**REF: Williams/Lodge**

unpublished). The June 2006 water filter sample, also used for companion 16S rRNA studies, contained approximately 0.003 g of pellet mass, with an estimated 1.6×10^3 filaments/L. A representative light micrograph used for counting is shown in Figure 1, Plate C. In contrast with biofilm samples, little to no fluorescence was observed for any filtrate sample, despite many smaller rods (1-2 μm wide, 5-7 μm long; conservative estimate = 2.0×10^5 rods/L).

As with biofilm samples, both general bacterial and Chloroflexi-targeted 16S rRNA libraries were retrieved from water filtrates. General bacterial library data are summarized in Table 2 and Figure 3, Panel A. Retrieved source water sequences were 96% or more similar to other sequences, with 18/20 most similar to bacterial sequences from other Yellowstone thermal features. The majority of retrieved sequences (75%) were Deinococcus-Thermus-like, with 15/17 most similar to uncultured Thermus from hot springs in Yellowstone National Park. Notably, no phototroph-like sequences were retrieved using general bacterial primers. Chloroflexi-targeted library data are summarized in Table 3 and Figure 4. All retrieved sequences were 93% or more similar to other sequences, with 10/10 most similar to isolates from other Yellowstone thermal features. The distribution of Chloroflexi in source water was 60% Green, 20% Red, and 20% chemotrophic, specifically Thermomicrobium-like.

DISCUSSION

In this study, we demonstrated that multi-layered photosynthetic mats could be grown *in situ* at Fairy Geyser, a previously-described RLMO site in Yellowstone National Park (REF: **Boomer 2002**). Mat formation was accomplished by suspending sterile glass rods in the thermal run-off, with sample replicates collected through 13 months. Population dynamics were assessed by comparing biofilm populations from sample replicates using microscopy, pigment assessment, and 16S rRNA gene analysis.

After the first month of mat formation, biofilm accumulation appeared macroscopically white to light yellow (Figure 1, Plate D), with microscopic analysis demonstrating an abundance of red-fluorescing unicellular rods (represented in Figure 1, Plate G). These data suggested Synechococcus-like Cyanobacteria but did not rule out the presence of chemotrophic microbes too small to detect using our microscopy assessment techniques. Pigment analysis (Figure 2) also supported the presence of Cyanobacteria, showing absorbance peaks for carotenes and Chl-like green pigment. The majority (55%) of retrieved bacterial 16S sequences most resembled thermophilic chemotrophs, including Deinococcus-Thermus and Aquificae, both of which could account for the white to light yellow biofilm coloration (Figure 3, Panel A). Further supporting microscopy and pigment data, Cyanobacteria-like

sequences (40%) were most related to either uncultured hot spring sequences or Synechococcus-like sequences, in both cases from Yellowstone habitats such as Octopus Spring and Obsidian Pool. When we applied a Chloroflexi-targeted retrieval strategy to this sample, we observed all green Chloroflexi-like sequences (Figure 4). Although no filaments were observed during microscopic cell counting at this timepoint, it is possible we missed some of these smaller cells. Given 16S data, it is likely that a portion of the green pigment absorption data was Chloroflexi-associated Bchl c.

Over the next two months, there were continued increases in biofilm mass, with all samples appearing green (Figure 1, Plates E-F). Microscopic analysis continued to demonstrate an abundance of strongly fluorescence unicellular Cyanobacteria-like rods, although non-fluorescing filaments comprised 5-10% of each sample (represented in Figure 1, Plates G-H). Pigment analysis (Figure 2) partially supported the cell count data, demonstrating an increase in green phototrophs through mid-August followed by a modest decrease through mid-September. General bacterial 16S rRNA analysis (Figure 3, Panel A) suggested that Cyanobacteria-like sequences increased to 60% of the population through mid-August and maintained this proportion through mid-September, generally supporting observed pigment data. As with the one-month timepoint data, Cyanobacteria-like sequences were most related to either uncultured hot spring sequences or Synechococcus-like sequences, in both cases from aforementioned Yellowstone habitats. Interestingly, Aquificae-like sequences were not retrieved and Deinococcus-Thermus-like sequences dropped to 10% for both timepoints. Replacing them were a more diverse array of chemotroph-like sequences, including Acidobacteria, Planctomycetes, Proteobacteria, and Candidate Division WS6, none of which would be easily detected microscopically using our methods. Green Chloroflexi-like sequences were also retrieved from the 3.5 month/mid-September sample using this general retrieval approach. We hypothesize that these diversity changes reflect new lower-temperature niches being established on the rod substrate and more diverse energy and/or carbon sources being produced by the growing biofilm matrix. When we applied a Chloroflexi-targeted retrieval strategy to this sample (Figure 4), we observed all green Chloroflexi-like sequences, supporting both microscopic evidence and general bacterial 16S rRNA studies, and suggesting that a portion of the green pigment absorption data represented Chloroflexi-associated Bchl c.

Owing to the difficult logistics associated with winter access at this backcountry thermal feature, we did not perform sample collections until 13 months/July 2005. By this point, multi-layered mats had visibly formed on all rods, each weighing nearly 7 g (Figure 1, Plate I). Consequently, all analyses were performed on dissected green and red layer sub-samples. At 13 months, the outer green layer

appeared to be dominated by Cyanobacteria based on all assessment techniques. Although we were unable to perform cell counts, microscopy demonstrated two distinct Cyanobacterial morphologies, both of which strongly fluoresced red under UV: unicellular rods that resembled Synechococcus from Yellowstone, and heterocyst-containing filaments that resembled Fisherella, the best match organisms for which were Fisherella from freshwater hot springs in Iceland that erupted in tidal zones (REF: **Hobel et al 2005**) (Figure 1, Plate J). A number of non-fluorescing cell types, including smaller rods and filaments, were also visible, suggesting Chloroflexi and any number of chemotrophs. Pigment analysis (Figure 2) indicated the presence of green phototrophs, albeit with reduced overall pigment absorbance as compared with other timepoints. General bacterial 16S rRNA (Figure 3, Panel A) analysis supported microscopy data, showing 100% Cyanobacteria-like sequences (55% Fisherella, 40% uncultured hot spring Cyanobacteria, and 5% Synechococcus). When we applied a Chloroflexi-targeted retrieval strategy to this sample, we observed all green Chloroflexi-like sequences, supporting microscopic evidence of Chloroflexi-like filaments.

These data suggest that the green layer is also undergoing temporal and spatial development, likely in response to new temperature niches established produced by the growing biofilm. Synechococcus generally shows a wider but higher temperature range (45-73°C) (REF: **Miller/Castenholz 2000**), with distinct variants associated with specific temperature zones (REF: **Allewalt/Ward, 2006**). Fisherella (Mastigocladus) in Yellowstone grow at a lower and more narrow temperature range (52-56°C), with distinct variants associated with specific nitrogen zones (REF: **Miller/Curtis, 2006**). Thus, we speculate that Synechococcus colonize the rods first because early biofilms are being directly subjected to higher temperature run-off water (70.5°C, where this study was carried out). As the biofilm thickens and adheres to areas above the water line, Fisherella can establish in lower temperature zones. Similar spatial and temporal variations in natural Cyanobacterial mat populations have also been reported at Octopus Spring, with notable increases in variant diversity particularly during the spring (REF: **Ferris/Ward, 1997**).

After 13 months, the inner red layer appeared, in terms of phototrophs, to be dominated by red Chloroflexi. Additionally, it contained a surprising array of diverse chemotrophs. Although we were unable to perform cell counts, microscopy clearly showed thin filamentous cells that fluoresced light green en masse, a characteristic of Bchl a-containing phototrophs such as red Chloroflexi (Figure 1, Panel K). This was also the only sample timepoint that showed all major pigments (Figure 2), including Bchl a (carotene abs. = 0.583; green abs = 0.175), suggesting the presence of Cyanobacteria, red and green Chloroflexi. While Chloroflexi-targeted 16S library data (Figure 4)

supported microscopic and pigment data, general bacterial 16S rRNA (Figure 3, Panel A) analysis suggested a far more diverse array of chemotroph-like sequences, including Planctomycetes (40%), Acidobacteria (20%), and Proteobacteria (10%). Phototrophs, in contrast, represented only 25% of the population (15% red Chloroflexi and 10% Cyanobacteria). Given that previously published studies of the Fairy Geyser mat community (**REF: Boomer 2002**) had only involved a Chloroflexi-targeted retrieval approach, we analyzed July 2005 upstream mat red layer samples using the same general bacterial strategy for rod and water samples in this report. As with the downstream rod red layer population, we retrieved an even more diverse array of chemotrophs, including Planctomyetes (20%), Acidobacteria (10%), Proteobacteria, (15%), Verrucomicrobia (15%), Candidate WS6 (15%), Firmicutes (10%), and Candidate OP10 (5%). Phototrophs represented only 10% of the population (5% red Chloroflexi and 5% Chlorobi). Taken together, these red layer data significantly alter our view of red layer systems, suggesting a much more important role for chemotrophs in these systems. That red Chloroflexi like Roseiflexus are photoheterotrophs and perform alternative chemotrophy in the dark suggests that related Yellowstone RLMO variants may rely on as-yet-unknown metabolic relationships with or carbon source products from their cohabitants, possibly explaining why this group of phototrophs has been notoriously difficult to isolate in pure culture.

In terms of potential sources for all biofilm-colonizing microbes, we hypothesized that geothermal source water contained seed microorganisms and provided a mechanism for their dispersal. To test this hypothesis, we performed microscopy and 16S gene analysis on geothermal water collected at Fairy Geyser's vent source upstream. Upon sample removal and concentration, all water filtrates produced detectable white to light green pellets that contained an abundance of smaller unicellular rods and long, thin filaments, neither of which fluoresced (Table 1, Figure 1, Plate C). These data suggested the presence of chemotrophic microbes and, possibly, Chloroflexi-like filaments. Retrieved bacterial 16S sequences (Table 2, Figure 3) most resembled thermophilic chemotrophs, including Deinococcus-Thermus and Aquificae, both of which could account for the white pellet and microscopy data. When we applied a Chloroflexi-targeted retrieval strategy, we retrieved green, red, and chemotroph-like Chloroflexi sequences (Table 3, Figure 4), further supporting microscopy data. Although these data could explain the initial colonization data by chemotrophs and the presence of Chloroflexi, they do not account for a large array of other biofilm members, most prominently Cyanobacteria and other chemotrophs after 2.5 months. One explanation is that the number of general bacterial 16S clones we analyzed was too limited. Larger-scale geothermal water analysis by Fouke et al. at Mammoth have readily screened (about 50-200 clones per sample site) and retrieved more diverse bacteria, including Cyanobacteria, Planctomyetes, Proteobacteria, and Firmicutes

(REF: Fouke et al., 2003). Another explanation is that Cyanobacteria and secondary chemotrophs are not originating from geothermal run-off water but from nearby locations like air or soil, both of which deserve more attention in future work.

Combined biofilm and water data generally supported our hypothesis that a distinct red layer would not appear until after the establishment of a shielding green layer. Although 16S rRNA and pigment data showed no evidence of red Chloroflexi-like organisms until 13 months, microscopic assessments suggested the presence of Chloroflexi-like filaments as early as 2.5 months. Precisely when the red layer formed, however, is unknown given the lack of sampling timepoints between fall and spring. To address this question, we have already begun a new study with collaborators who can access and collect at this site every 2 months starting October 2006. Equally interesting but not predicted outright, population dynamics during early colonization, intermediate and red layer chemotroph populations, and within the green layer both reflected the appearance of new niches, likely defined by different temperature, light, and nutrient conditions. Finally, given that red Chloroflexi remain challenging to isolate in pure culture, using this rod-based approach could provide an important, alternative tool for studying and propagating these interesting phototrophs without heavily impacting natural vent habitats.

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FIGURES AND TABLES

Figure 1: Plate A shows Fairy Geyser mat and vent source. Plate B shows rod field set up (about 100 m down the channel from Fairy Geyser) in June, 2004. Plate C shows a representative light micrograph of vent source water filtrate (grid square edge = 0.25 mm, 0.02 mm deep). Plates D-F show rod biofilm samples from 1 month (June 30, 2004), 2.5 months (August 17, 2004), and 3.5

months (September 14, 2004), respectively. Plate G shows micrographs of the 1-month biofilm sample counting grid (square edge = 0.25 mm, 0.02 mm deep) under light (left) and UV (right). Plate H shows higher magnification micrographs of 2.5-month biofilm sample under light (left) and UV (right); the upper right bar in the light micrograph indicates 10 μm and the arrow indicates a filamentous cell that does not fluoresce under UV. Plate I shows a 13-month, multi-layered biofilm sample; adjacent sub-plates show outer green dissected sub-samples (top) and inner red dissected sub-samples (bottom). Plate J shows micrographs of the 13-month green sub-sample under light (left) and UV (right); the upper right bar in the light micrograph indicates 20 μm . Plate K shows micrographs of the 13-month red sub-sample under light (left) and UV (right); the upper right bar in the light micrograph indicates 20 μm .

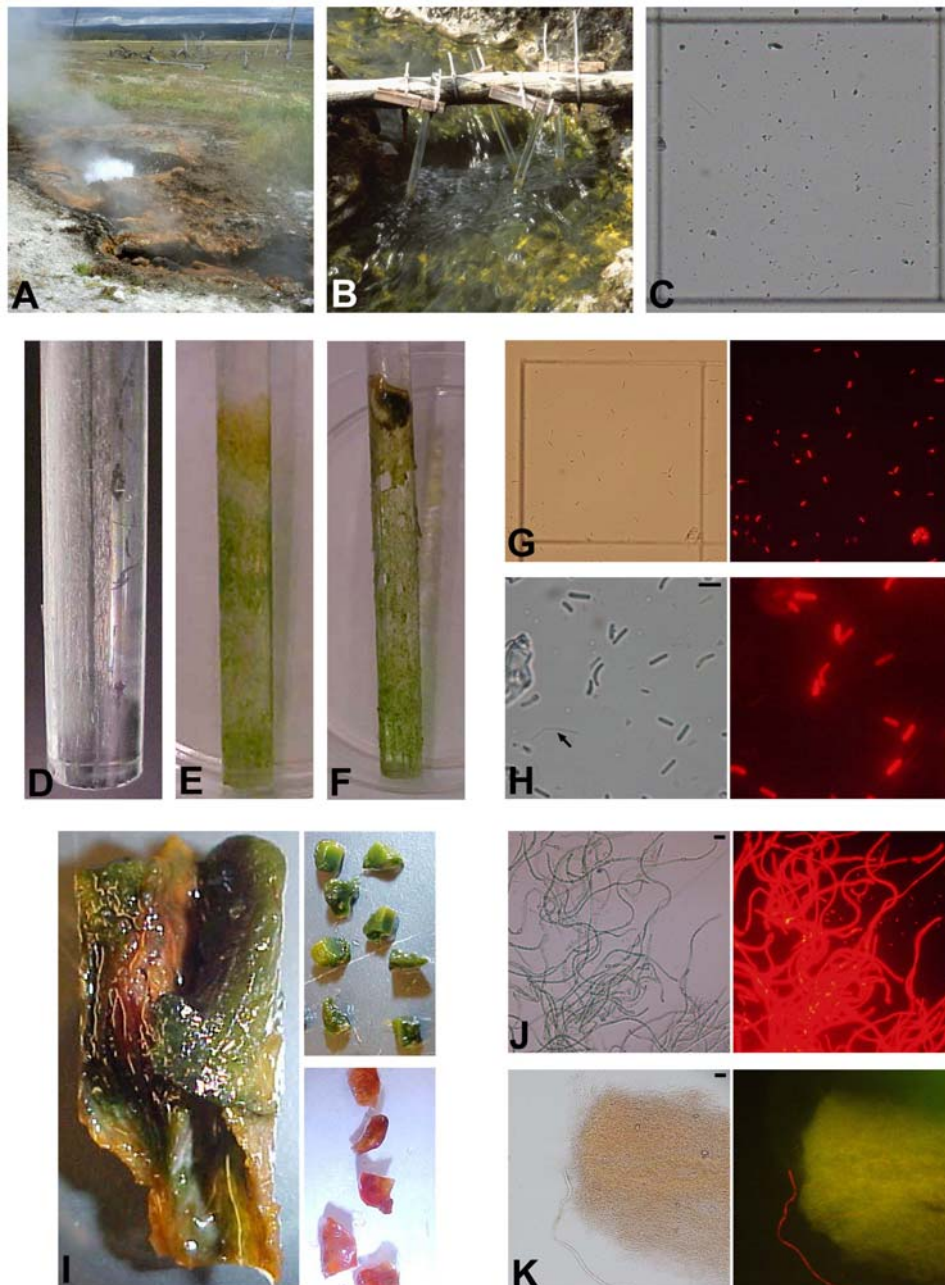


Figure 2: This graph shows comparative pigment absorbance data for each rod sample timepoint. For each, 0.02 g biofilm sample was used for methanol extraction. In all cases, major peaks for each of the following pigment groups were recorded: carotene pigment absorbance at 436-40 nm for each sample is shown in diagonal stripe; green pigment (including Chl a and Bchl c) absorbance at 664-669 nm for each sample is shown in a solid gray; and red pigment (Bchl a) absorbance at 768-771 nm is shown in solid black (but only observed in the 13-month red layer sample).

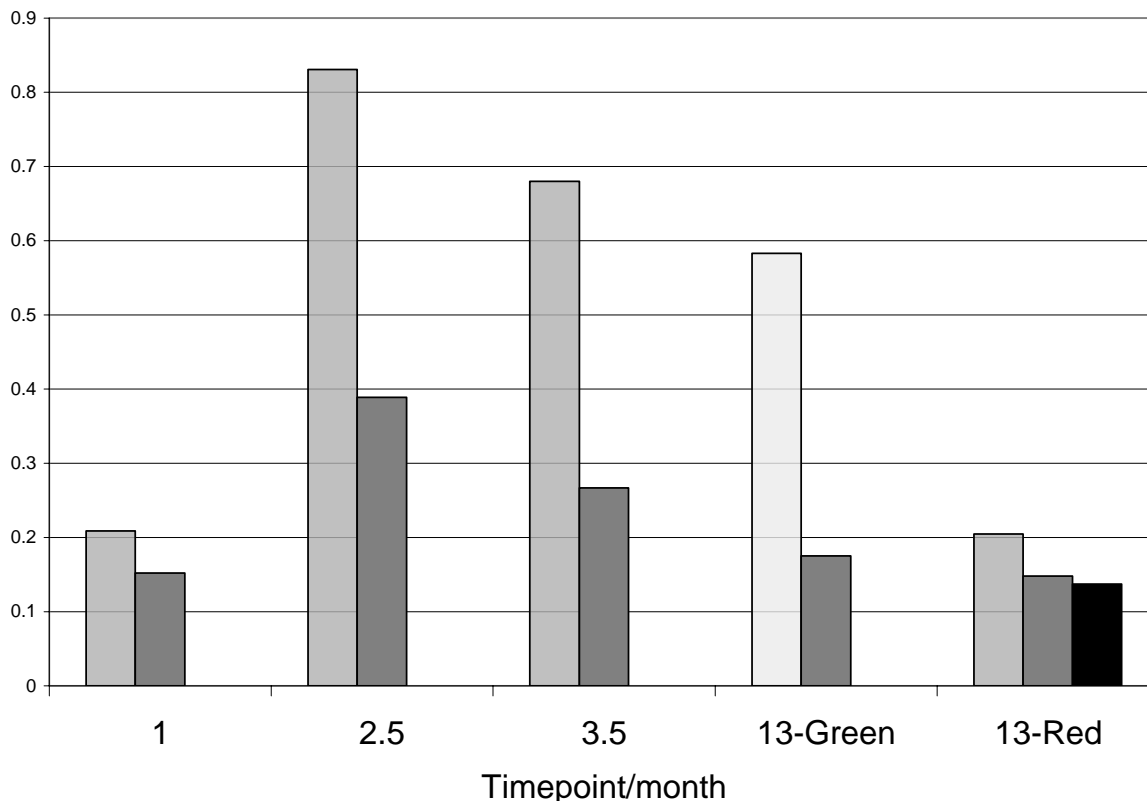


Figure 3: This graph shows comparative general bacterial 16S rRNA library composition for each rod and water sample timepoint. For each sample library, twenty clones were retrieved and assessed via nucleotide sequence analysis and BLAST to establish division-level assignment, with additional information about clones in Table 2. The % abundance of each retrieved division in a given library is indicated by the area of each division-specific color in a given column, where Acidobacteria = yellow; Aquificae = dark blue; Candidate WS6 = light blue; green Chloroflexi = light green; red Chloroflexi = red; Cyanobacteria = dark green; Deinococcus/Thermus = gray; Firmicutes = black; Planctomycetes = purple; and Proteobacteria = brown.

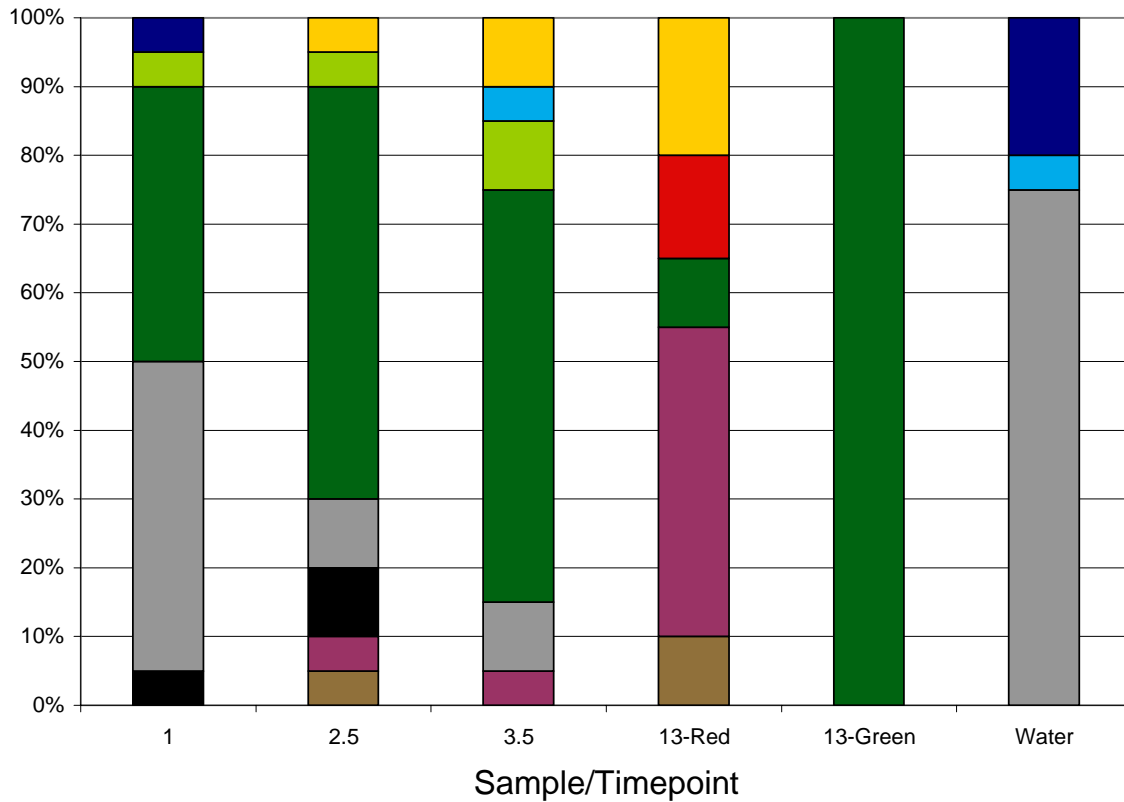


Figure 4: This graph shows comparative Chloroflexi-targeted 16S rRNA library composition for each rod and water sample timepoint. For each sample library, twenty clones were retrieved and assessed via nucleotide sequence analysis and BLAST to establish subdivision-level assignment, with additional information about clones in Table 3. The % abundance of each retrieved subdivision in a given library is indicated by the area of each division-specific color in a given column, where green Chloroflexi = light green; red Chloroflexi = red; and chemotroph-like Chloroflexi = orange-brown.

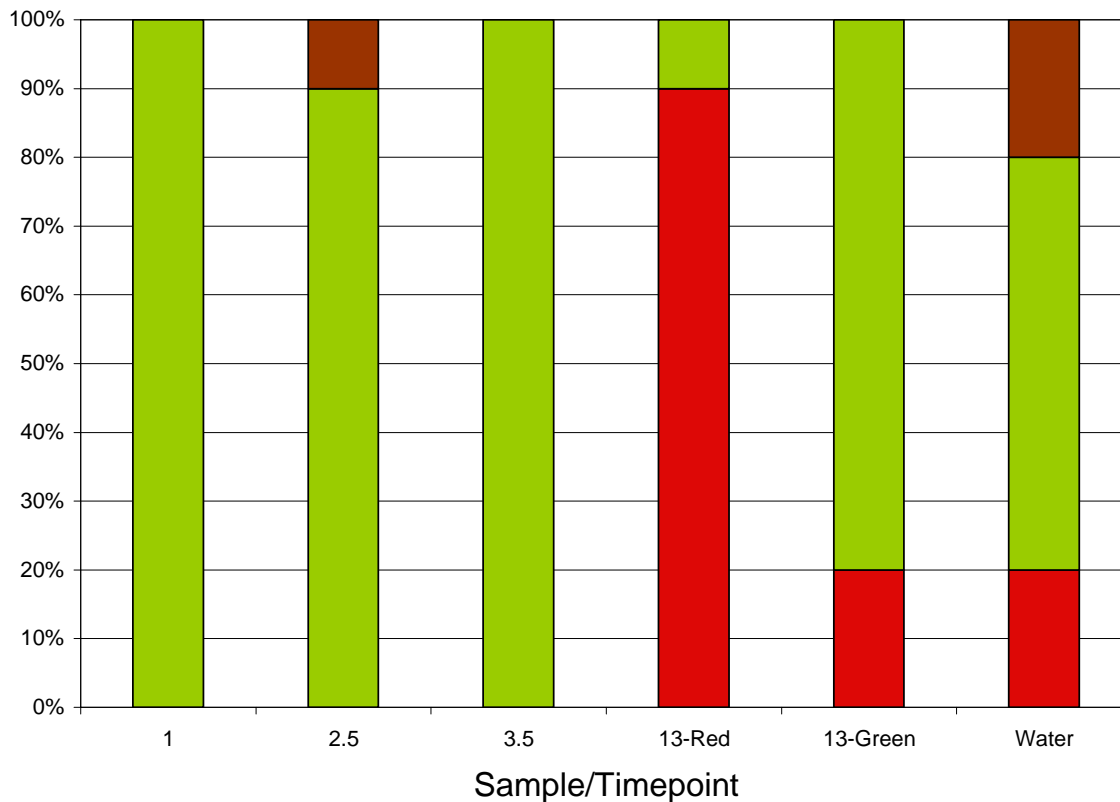


Table One

<u>Sample/Timepoint</u>	<u>Mass (g)</u>	<u>Hemocytometer Count</u>
Biofilm - 1 month	0.0438	2.43 X 10 ⁶ rods per total sample ^a
Biofilm - 2.5 months	0.1450	4.57 X 10 ⁷ rods per total sample ^a 2.28 X 10 ⁶ filaments per total sample ^b
Biofilm - 3.5 months	0.2400	7.24 X 10 ⁷ rods per total sample ^a 7.24 X 10 ⁶ filaments per total sample ^b
Biofilm - 13 months	6.7890	ND
Source Water - June 2006	0.0030	1.20 X 10 ⁵ filaments per L ^b 2.00 X 10 ⁶ rods per L ^c

^anearly all fluoresced red under UV, with most measuring 2-3 um wide, 8-10 um long

^bnone fluoresced under UV, with most measuring 1 um wide, 10-100 um long

^cconservative estimate; none fluoresced under UV, with most measuring 1-2 um wide, 5-7 um long

Table Two

<u>Sample</u>	<u>Division (%)</u>	<u>Length (bp)</u>	<u>ID%</u>	<u>Best Match Organism</u>
Biofilm - 1 mo.	Aquificae (5%)	631	97	Thermocrinis*
	Chloroflexi-Green (5%)	575	95	UC, HS*
	Cyanobacteria (40%)	433-798	84-99	75% UC, HS*; 25% Synechococcus*
	Deinococcus-Thermus (45%)	476-716	88-99	55% UC, marine; 45% Thermus*
	Firmicutes (5%)	468	96	UC, HS*
Biofilm - 2.5 mo.	Acidobacteria (5%)	519	90	UC, soil
	Chloroflexi-Green (5%)	574	98	UC, HS*
	Cyanobacteria (60%)	402-752	94-99	75% UC, HS*; 25% Synechococcus*

	Deinococcus-Thermus (10%)	569-700	97	50% UC, marine; 50% Thermus*
	Firmicutes (10%)	445-712	96-98	UC, HS*
	Planctomycetes (5%)	645	96	UC, soil
	Proteobacteria (5%)	416	97	UC, HS*
Biofilm - 3.5 mo.	Acidobacteria (10%)	526-793	89-90	UC, marine
	Candidate WS6 (5%)	691	81	UC, soil
	Chloroflexi-Green (10%)	441-451	87-96	UC, HS*
	Cyanobacteria (60%)	459-692	87-99	92% UC, HS*; 5% Synechococcus*
	Deinococcus-Thermus (10%)	577-652	96-97	50% UC, marine; 50% Thermus*
	Planctomycetes (5%)	644	83	UC, soil
Biofilm/R - 13 mo.	Acidobacteria (20%)	465-705	95-96	UC, HS*
	Chloroflexi-Red (15%)	494-773	95-98	UC, HS*
	Cyanobacteria (10%)	588-780	91-97	UC, HS
	Planctomycetes (45%)	509-790	86-96	33% Gemmata, freshwater; 67% UC, soil
	Proteobacteria (10%)	472-547	64	UC, freshwater
Biofilm/G - 13 Mo.	Cyanobacteria (100%)	474-775	97-99	55% Fisherella; 40% UC, HS; 5% Synchococcus*
Source Water	Aquificae (20%)	589-717	97-98	UC, HS*
	Candidate WS6 (5%)	660	97	UC, HS*
	Deinococcus-Thermus (75%)	500-685	96-98	87% UC, HS*; 13% UC, marine

R = red layer; G = green layer; UC = uncultured; HS = HS; * = Yellowstone

Table Three

Sample	Division Subgroup (%)	Length (bp)	%ID	Best Match Organism
Biofilm - 1 mo.	Chloroflexi-Green (100%)	314-520	88-99	UC, HS*
Biofilm - 2.5 mo.	Chloroflexi-Green (90%)	303-607	85-97	80% UC, HS*; 20% Chloroflexus sp. 396-1*
	Chloroflexi-Chemotroph (10%)	735	95	Thermomicrobium
Biofilm - 3.5 mo.	Chloroflexi-Green (100%)	226-586	89-97	88% UC, HS*; 22% Chloroflexus sp. 396-1*
Biofilm/R - 13 mo.	Chloroflexi-Green (10%)	510	96	UC, HS*
	Chloroflexi-Red (90%)	421-680	93-98	UC, HS*
Biofilm/G - 13 mo.	Chloroflexi-Green (80%)	480-642	86-98	UC, HS*
	Chloroflexi-Red (20%)	510-607	94-96	UC, HS*
Source Water	Chloroflexi-Green (60%)	561-632	93-98	83% UC, HS*; 17% Chloroflexus sp. 396-1*
	Chloroflexi-Red (20%)	575-601	96-99	UC, HS*
	Chloroflexi-Chemotroph (20%)	596-593	98	UC, HS*

R = red layer; G = green layer; UC = uncultured; HS = HS; * = Yellowstone