

DRAFT (July 2006) - The Growth Patterns of Colorful Bacterial Mats in Hot Springs Water at Yellowstone National Park

By Jennifer Esparza

Introduction

Microbial mats are “structurally coherent macroscopic accumulations of microorganisms” that vary in size and thickness and are found in many diverse locations all over the planet from the arctic’s to the equator (Pierson 247). What makes the microbial mats so interesting is their evolutionary and biological significance, first observed by Ferdinand Cohn regarding the extreme limits of a species existence (Brock 1012).

Microorganisms accumulate and form complex communities all around us and in Yellowstone National park there are colorful microbial mats that surround the mouth and run off areas of the hot springs, with the water ranging in temperature from 80-90°C (176-194°F) and the mats themselves ranging from 40-70°C (104-158°F). The study of a species growth in these extreme environments intrigued Brock and in 1967 he was the first one to grow a biofilm on an artificial substrate in the hot spring itself by placing a glass slide or a cotton string in the water of the hot spring for several days and observing the community of microorganisms that had grown there (1014). Since that time the microbial mats have been studied to improve our knowledge of the diversity of different phylogenetic groups, to gain a better understanding of the evolution of photosynthesis (Boomer 346), to better interpret stromatolites (Pierson 247), and to study disease-causing bacteria like legionella (Sheehan 507).

Although much progress has been made we are still only able to cultivate less than one percent of the microorganisms observed by microscopy and alternate isolation procedures must be found (Nubel 422). Brock commented that it was impossible to replicate all of the variables found in nature to be able to cultivate and study a microorganism in the lab (Brock 882). The question then is how researchers can solve this problem with cultivation and continue to make new discoveries and solve unanswered questions. The solution can be found at the original source. The microbial community can be cultivated on an artificial substrate in the natural environment that would allow researchers to study the mats as they occur naturally without disturbing the original mat system.

The microbial mat system at Fairy hot spring in Yellowstone National Park can be imagined as a hamburger with the different layers of microorganisms representing the layers of bun, lettuce, tomato, and hamburger. The first layer of the mat system you see when you approach the spring is a bright orange color all around the mouth and run-off. This layer represents the burnt *Cyanobacteria* which are constantly exposed to intense light and oxygen (Boomer 153). The second layer, just below the orange surface is green in color and composed of *Cyanobacteria* that utilize light and Chlorophyll (Chl) a pigments to produce oxygen just like plants (Boomer 155). The third layer is olive green and is composed of the bacteria called *Chloroflexus*. The fourth layer is red in color and is most similar to *Roseiflexus*. *Chloroflexus* and *Roseiflexus* both perform photosynthesis, however, it is different in both the process and the pigments plants use.

The characterization of the mat is very distinct, and a basic knowledge of the characteristics of each layer in terms of pigments, microscopy and DNA fingerprinting is needed in order to be able to study the growth patterns of the mat system and be able to distinguish the differing organism’s establishment in that system at Fairy. The mat is translucent and allows for an attenuation of the visible and near-IR solar spectrum to penetrate through the layers of bacteria; therefore, the deeper into the mat the less visible light will be available to sustain the *Cyanobacterial* species, which is why they are found closest to the surface. The near IR radiation penetrates deeper into the layers of the mat and this is how the *Chloroflexus* and *Roseiflexus* are able to use light to perform photosynthesis with Bacteriochlorophyll (Bchl) c and Bchl a pigments, respectively (Boomer 155). Experiments have shown that both the *Chloroflexus* and *Roseiflexus* perform best with low levels of oxygen and light intensity (Boomer 157). The *Cyanobacteria* found at Fairy use Chl a pigments to absorb red light at 450/660nm. The size and shape is large filamentous or unicellular rods and it fluoresces red when hit with ultraviolet light (Boomer 155). The *Chloroflexus* uses Bchl c to absorb light at 450/660nm. The

size and shape is small, thin filaments and it does not fluoresce. The *Roseiflexus* uses Bchl a to absorb light at 770nm. The size and shape is intermediate filaments which also do not fluoresce.

An interesting question is about the formation of the mat—in terms of the chronological sequence in which microorganisms in the community establish themselves. I.e., which organism starts the mat and which organisms establish themselves later to help make the thick mat system that we observe upon arrival at the spring. Based on the physiological necessities of each bacterial layer it is hypothesized that:

- The first bacteria to establish itself on the rods (see experimental set up) will be *Cyanobacteria* due to its high tolerance for light and oxygen.
- The second bacteria to establish itself on the rod will be the *Chloroflexus* due to its medium tolerance of light and oxygen.
- Finally, the third bacteria to establish itself on the rod will be the *Roseiflexus* due to its very low tolerance of light and oxygen.

Therefore, at one month, based on pigment analysis, microscopy, and DNA analysis, only *Cyanobacteria* will have established itself on the mat; at three and four months some *Chloroflexus* species will be present due to the minimal growth in depth of the mat and the subsequent protection from oxygen and light intensity; and finally at one year *Roseiflexus* will be present due to the substantial growth in depth or thickness of the mat for greater protection.

Materials and Methods

Experimental Set-up

Four sets of four glass rods were set up in the run off of Fairy Hot Spring in Yellowstone National Park. The hot spring has a water temperature of 92.7° C with a pH of 7.5 and the mats have a temperature of 46.0°C with a pH of 8.5-9.0. The temperature was measured with a Traceable universal digital thermometer (Friendswood, TX). The pH was measured using ColorpHast pH 5-10 pH indicator strips.



Figure 1 (left): Fairy Hot Spring at Yellowstone National Park. The bright orange surrounding the water is the mat system. Figure 2 (right): This is a picture of the experimental set up with the glass rods. The rods were placed so that they were in the water but not touching the floor of the run-off and had enough area above the water for splashing and mat formation to occur.

Clothes pins and zip ties were utilized to attach the rods to a branch so that they could catch the water splashing from the run off (Figure 2). The rods were set up in May of 2004 and collected at one month in June, three months in August, four months in September, and 13 months in July of 2005. The temperature at this run-off site of Fairy was 70.5°C and the pH was 9.

Collection took place with a glass scorer to cut the rods, which were then placed in sterile 15mL conical tubes and frozen with dry ice for transport back to the lab. The July 2005 mats were too large to fit the mat and rod in the tubes, therefore, large-scale dissection in the field took place using sterile scalpels to remove the mat from the rod. The mat was then cut into large quarters with a diameter that barely fit into the conical tube, approximately (centimeters). Each conical tube contained two quarter pieces, therefore, a total of two conical

tubes were used for each dissected mat and one final conical tube was used for transportation of the rod, of which mat samples remained. All samples were frozen with dry ice for transport.

Mat Processing

The early samples were simple biofilms (one, three, and four months) that were physically removed from the rod via a scalpel and sterile gloves. The biofilm and the rod were then placed in a sterile 15ml conical tube with 3ml of GTE buffer and vortexed to remove any bacteria that remained on the rod. The rod was then removed and the conical tube was then filled with 7ml more, to equal a total of 10ml (Figure 3).



Figure 3 (left image): This is a comparison of the amount of mat yielded from August (three months) on the left with September (four months) on the right. The diluted mat samples are a result of the dissection off the rod and the vortexing. Figure 4 (right 3 images): the mat sample that was dissected into two halves in the field so that it could be frozen and transported back to the lab. Three visible layers can be seen, closest to the rod there is red, then green and the outermost layer is the burnt orange. The right 2 pictures represent the green and red layers dissected apart so that pigment, microscopy and DNA analysis could take place.

The sample was then divided 10 times into 1.5ml tubes; therefore, each tube contained 1000µl of diluted biofilm sample. The dilutions were made to quantitatively equalize the samples so that further analysis could be performed on the same amounts. Each sample was then spun using a mini centrifuge and the excess GTE was removed to yield a small sample of the mat, which was then weighed and frozen at -80° C. The June 2005 mat sample first went through a dissection into quarters in the field as described previously in the experimental set up and then underwent extensive dissection in the lab because the mat was so large and visible layers could be seen. Therefore, the mat sample was dissected into two layers, a red layer and a green layer.

Growth/Weight Analysis

The growth of the microbial community could be seen visually from the early stage biofilms to the late stage, complete mat formation (Figure 3 and Figure 4a, b). The weights of the biofilms were taken following removal from the rod and allocation into the ten 1.5ml tubes. First the tube was weighed (need reference?) and then the tube and the vortexed sample, to remove the buffer, were weighed to obtain the weight of the sample.

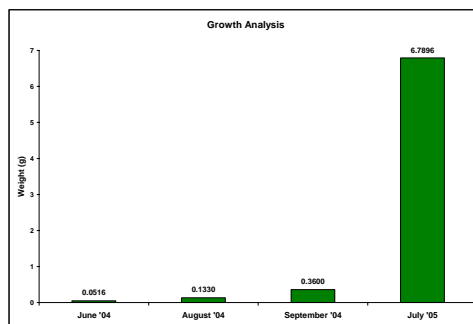


Figure 5: Growth of the microbial community on each rod via the amount of sample that was collected from the rods in the lab.

Pigment Analysis

Methanol extraction (see Table 1 for weight of each sample) was carried out first by resuspending the aliquots of biofilm in 500µl of GTE in order to use the mortar and pestle to break apart the large pieces of biofilm formed upon centrifugation. The samples were then spun to create a pellet so that the GTE could be removed and replaced with 1ml of methanol, vortexed for resuspension and finally wrapped in foil and placed on ice for

30 minutes. In-vivo extraction was carried out with a resuspended biofilm sample in GTE buffer. The cells were physically lysed using 0.1mm zirconium beads and a mini bead-beater (GlenMills), also wrapped in foil and placed on ice until viewed with the spectrophotometer. For the July 2005 mat sample each piece (Figure 4c,d) was homogenized using a mortar and pestle as explained previously for the biofilm samples. Spectra from both of these extracts were determined using a U-2000 UV/Vis spectrophotometer (Hitachi, Inc., Tokyo, Japan).

Pigment Analysis Sample Weights		
Sample	Methanol extraction	In vivo extraction
June 2004	0.00938g/500µl	0.00938g/500µl
August 2004	0.00727g/500µl	0.00727g/500µl
September 2004	0.01091g/500µl	0.01091g/500µl
July Green Layer 2005	0.00918g/500µl	0.00918g/500µl
July Red Layer 2005	0.00846g/500µl	0.00846g/500µl

Table 1: This table shows the weight of the biofilm that was analyzed for pigment analysis. Each is approximately 0.01g.

Microscopy

Light and Fluorescence microscopy were both carried out using an Olympus Reflected Fluorescence System/BX-URA2 Model with a DP11 digital camera system. Population diversity, growth and size were determined visually by analyzing 10µl from a 50µl GTE resuspended biofilm sample (refer to Table 2 for the weight of each sample analyzed). The relative numbers of groups with the sample population was determined using a counter and the pictures taken with the light microscope. For each sample, ten random spots were chosen to photograph at a magnification of 20X and 100X under both light and fluorescence. Upon completion of all the photos for that sample, the pictures were transferred to a computer and saved so they could be viewed and counted at a later point in time.

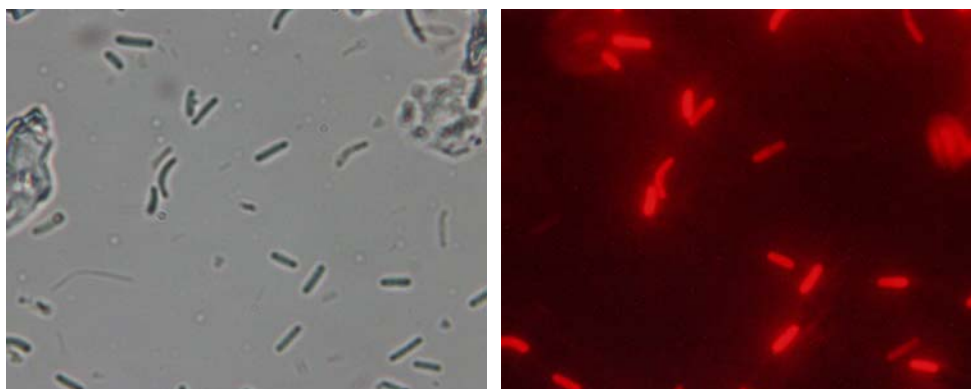


Figure 6: These two pictures represent a light microscopy image from the August sample taken at a magnification of 100X (left) and a fluorescent microscopy image of the same magnification and the same spot (right).

Microscopy Analysis Sample Weights	
Sample	Weight
June 2004	0.00188g/50µl
August 2004	0.00146g/50µl
September 2004	0.00218g/50µl
July Green Layer 2005	0.00184g/50µl
July Red Layer 2005	0.00169g/50µl

Table 2: This table shows the weight of each sample from which 10µl was viewed under the microscope. The September sample went through a 1/10 dilution (new weight not shown).

DNA Analysis

Genomic Isolation and PCR Amplification:

50µl of a previously resuspended mat sample was added to a 1.5ml tube filled approximately halfway with 0.1mm zirconium beads, 800µl of Phenol/Chloroform and 66µl of a 10% SDS detergent. This was then placed in a mini bead-beater and went through a series of beatings and icings in order to break open the cells. The tubes were then spun using a mini centrifuge to pull down all of the heavy cell debris and leave the light DNA on top. The supernatant, containing the DNA was removed and placed in a second 1.5ml tube and another Phenol/Chloroform extraction was performed. Again the supernatant was removed and placed in a new 1.5ml tube following a spin to pull down unwanted extracellular debris. 3M sodium acetate, 95% ethanol, and freezing at -80°C were used to make the DNA precipitate out of the solution. Finally the extracted DNA was resuspended in ultra pure water and placed in a water bath to go into solution. PCR analysis was carried out using the Master Amp PCR Optimization Kit (Epicenter technologies). Each final reaction cocktail contains 21.8µL of water, 1µL of general 16SrRNA forward primer (Reysenbach) or Ward Chloroflexi forward primer (Ward ref.), 1µL of general 16SrRNA reverse primer (Reysenbach) or Ward Chloroflexi reverse primer (Ward ref.), 0.2µL of Taq, 1µL of template, and 25µL of a 2X buffer dNTP mix (providing varying pH and salt conditions). Thermal cycling was carried out with an Eppendorf Mastercycler with 35 cycles as follows: One minute at 94°C for denaturation, one minute at 60°C for annealing, and three minutes at 72°C for extension (boomer). The results of the PCR analysis determine the buffers from the Optimization kit that will be combined for cloning.

Cloning and Nucleotide Sequence Analysis:

Cloning was carried out using the pCR-TOPO TA Cloning Kit (with E. coli) and liquid broth media according to manufacturer specification. Two PCR product buffers were mixed. A mini-prep used in conjunction with EcoR1 restriction enzymes were used to determine which of the clones had good insert. The gels were stained with ethidium bromide and viewed using Foto/Analyst Investigator PCImage software (Fotodyne Inc.). mini-prep is set up for sequencing using a Promega Wizard Plus SV Minipreps DNA Purification System (Fisher Scientific International) and a dNA Analyzer Gene Reader 4200. (Need reaction primer info) Sequencing analysis is carried out with E-Seq V2.0 program which allows us to physically edit any of the nucleotides that the computer could not recognize. The edited sequences are then compared to the National Center for Biotechnology Information (NCBI) by using the Basic Local Alignment Search Tool (BLAST) to determine the possible identity of the sequences. 20 16s and 10 ward sequences were run from each June, August, September, July green layer and July red layer time points.

Results

Pigment Analysis:

Pigment analysis uses a spectrophotometer to analyze what pigments are in a sample based on the wavelength that they absorb. Unique pigments for each of the layers of the mat were described in the introduction.

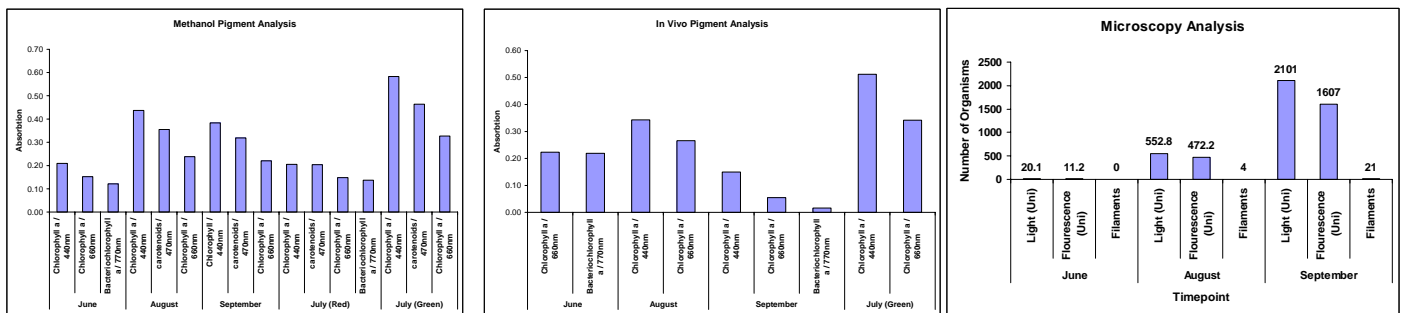
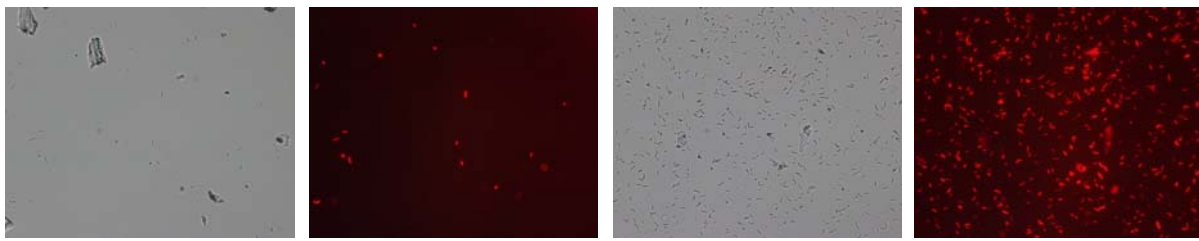


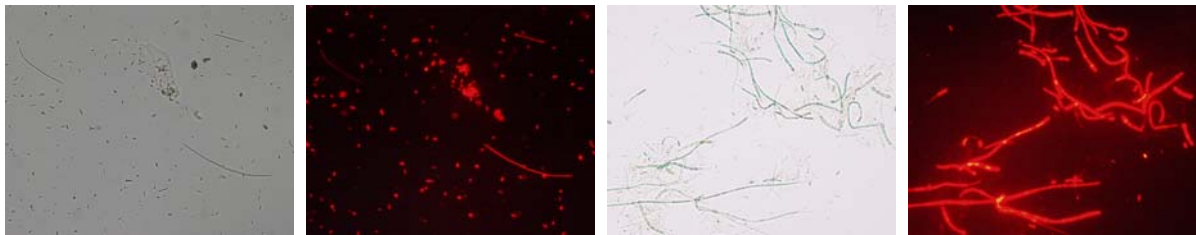
Figure 7: pigment analysis for the methanol extraction; Figure 8: pigment analysis for the in vivo extraction. The July Red layer data is not shown because there were no in vivo peaks present when the analysis was performed. Figure: Average unicellular (labeled as Uni) *Cyanobacterial* rods and filamentous *Chloroflexus* organisms present over the differing timepoints. The September sample was diluted by taking 20µl of the 100µl already diluted and adding 200µl—making a 1/10th dilution. July sample is not shown due to the complexity of the pictures and counting difficulties.

Microscopy

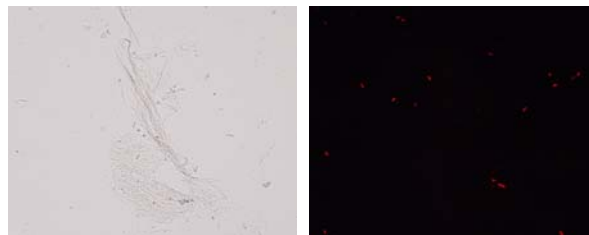
The samples were diluted with 100µl of GTE buffer and 10µl were viewed underneath both the light and the fluorescence. There is a general trend of progression in number of organisms present. As the rods were left for longer periods of time the population size grew tremendously as seen from Figure 9 and Figure 5.



(a). June 2004 Light 20x; June 2004 UV 20x; (b). August 2004 Light 20x; August 2004 UV 20x



(c). September 2004 Light 20x; September 2004 UV 20x; (d). July 2005 (Green) Light 20x; July 2005 (Green) UV 20x

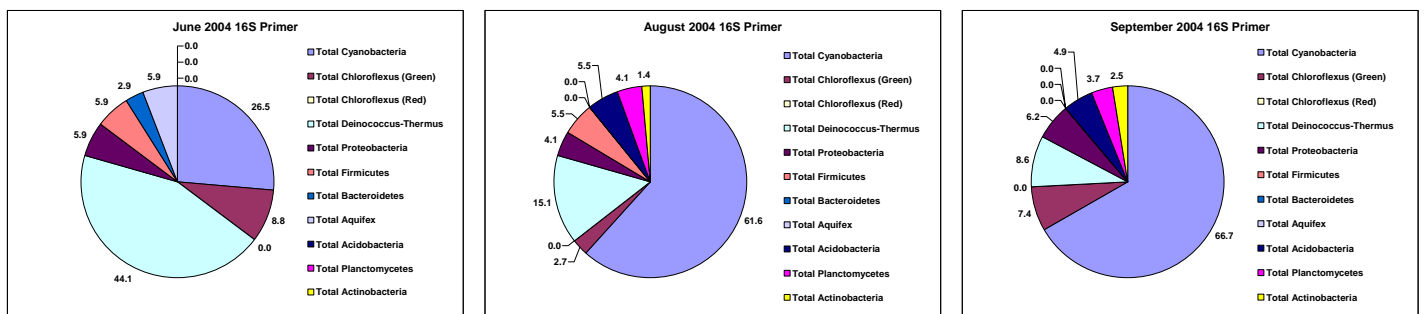


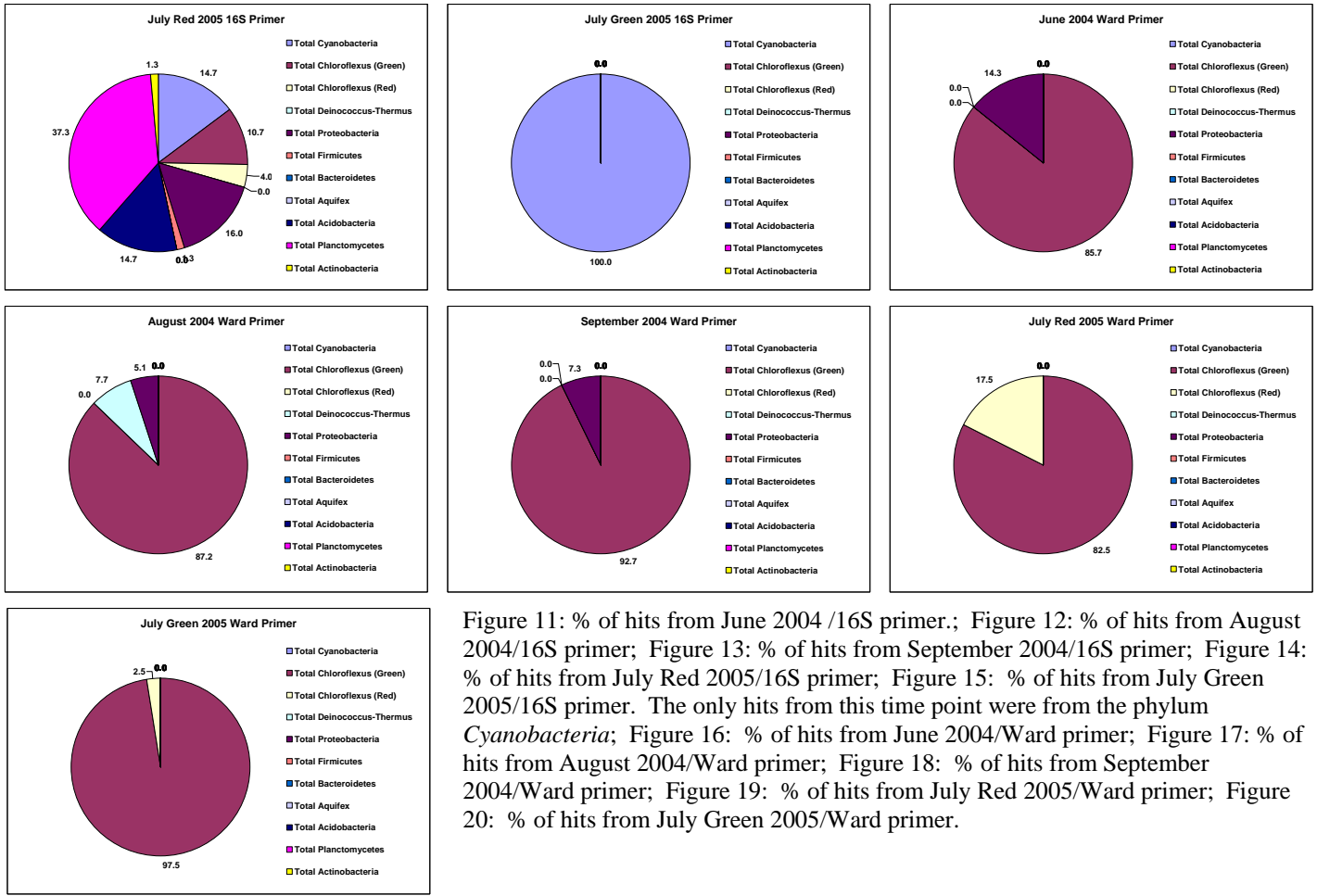
(e). July 2005 (Red Layer) Light 20x; July 2005 (Red Layer) Fluorescence 20x

Figure 10: Both June and August samples in (a) and (b) are composed entirely of unicellular *Cyanobacteria*. In (c) there are filamentous bacteria, however, they must be *Cyanobacteria* because they are fluorescing. In (d) there can be seen both filamentous and unicellular *Cyanobacteria*. Finally in (e) there are filaments in the light photograph that do not fluoresce in the photo to the right, indicating that they are either *Chloroflexus* or *Rosieflexus*, also present are unicellular *Cyanobacteria*, fluorescing in photo to the right.

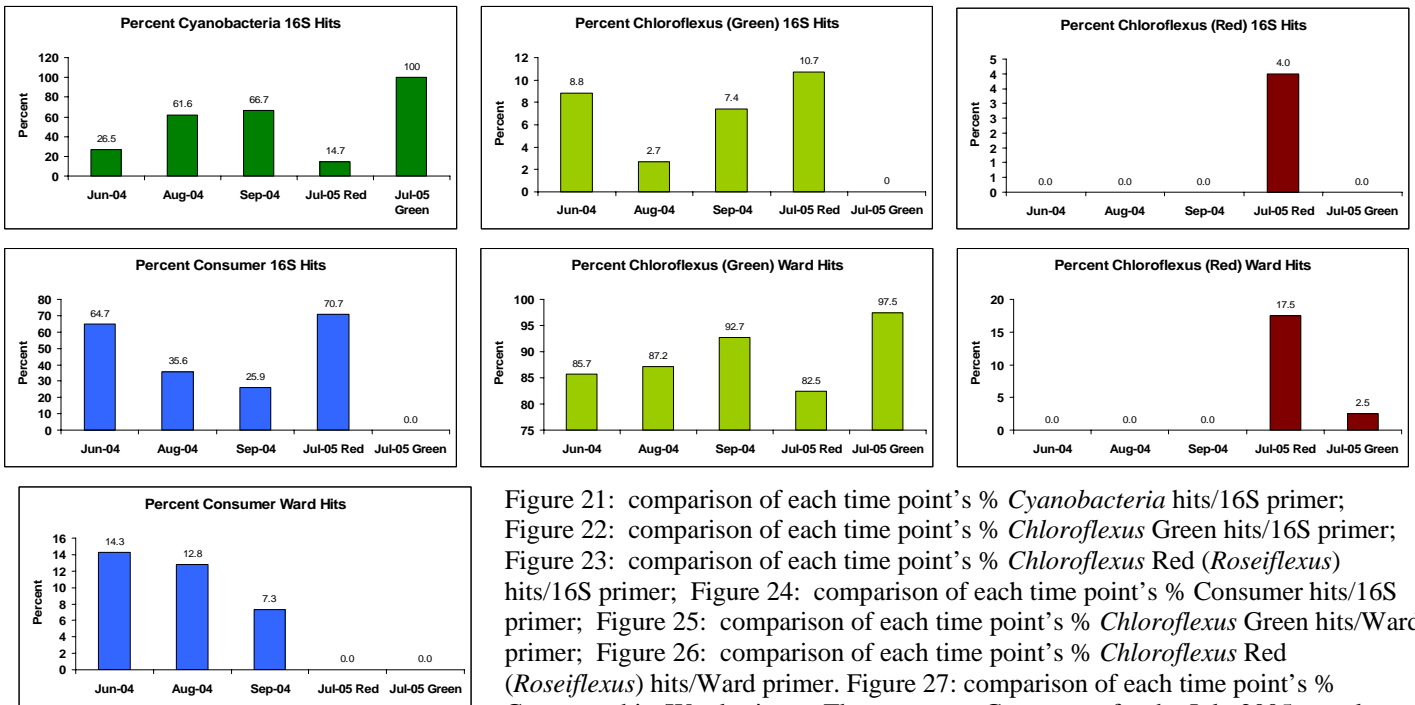
DNA Analysis

The DNA analysis is used to identify individual organisms in the mat community that may have been missed with the microscopy and pigment analysis either due to their size being too small, or the fact that they do not possess pigments for photosynthesis. Analysis of the data collected was done via reviewing the worksheets for a particular time point and recording the number of times a hit for *Cyanobacteria*, *Chloroflexus*, *Proteobacteria*, etcetera came up in the data base. The data reported in the graphs is limited to the phylums from which organisms were found to be present in the community. The *Chloroflexus* (Red) results refer to the *Rosieflexus*.





A compilation of the time points was made so that a side by side comparison of the *Cyanobacteria*, *Chloroflexus* (Green), *Chloroflexus* (Red), and Consumers could be made. A graph for the *Cyanobacteria* percent of Ward primer hits is not shown because there were no hits for cyanobacteria using the Ward primer.



Discussion

Clearly the microbial community grew from a simple biofilm to a full mat system with a visual difference in the color of the layers in just one year (Figure 4). This is very important in that researchers can have a fully grown mat system to study in a very short amount of time and therefore they will be able to make the most of their time performing research on the mats that have been artificially grown in the field.

The pigment analysis aided in identifying key pigments found in specific phototrophic phyla and allowed for a visual representation of the amount of pigment in each organism. This information is useful because it shows that for the methanol extraction the most abundant pigments for the early months were Chl a which is found in *Cyanobacterial* species (Figure 7). There is a very early and small appearance of the Bchl a pigment found in the *Chloroflexus* species but the majority of the appearance of this organism is found in the July (Red) layer (Figure 7). The in-vivo pigment analysis resulted in a more scattered appearance of the Bchl a pigment. There were peaks found in the June and September samples (Figure 8). These results prove that the *Chloroflexus* can easily survive with very little protection from the *Cyanobacterial* layer—at least for a short amount of time while the community establishes itself. It is suspected that, based on the results from the DNA analysis and microscopy analysis, this was the *Chloroflexus* Green species that uses both Bchl a and Bchl c to absorb light, and can tolerate a higher intensity of light and oxygen than the *Roseiflexus*.

The microscopy analysis proved overwhelmingly that the majority of the microbial biofilm (in the early stages) is composed of unicellular *Cyanobacteria* (Figure 10a-c). It was not until the July analysis that any filamentous species were seen that did not fluoresce (Figure 10e). This data supports the conclusions of the hypothesis and the methanol pigment analysis in terms of the *Cyanobacteria* species needing to be the first to establish the community so as to offer protection from the high light and oxygen intensity that the *Chloroflexus* and *Roseiflexus* have a medium and low tolerance for. An interesting feature of the complete mat system analyzed in July was that the *Cyanobacteria* species were now predominantly filamentous (Figure 10d) as opposed to the unicellular dominance of the early June, August, and September time points (Figure 10a-c). The early time points were dominated by the unicellular *Cyanobacterial* species: *Synechococcus*, *Gloebacter*, and *Gloeotheca*; while the July time point was dominated by filamentous *Cyanobacteria* such as *Fischerella* and *Hapalosiphon*. This change is thought to be due to the fact that in order for the mat system to be established and maintained, the layers have to intertwine for stability, therefore filamentous bacteria are more practical. While these analyses are useful because they allow for a visual analysis/confirmation of the organisms present, they cannot provide any information about the consumers in the community that do not contain pigments and cannot be detected through the microscope. This is why the DNA analysis is a useful analysis and distinguishing tool because it relies solely on the DNA of the organism, which is unique enough that a more precise analysis can be established.

The DNA analysis was based on two primers, one that was a general 16S rRNA primer that would detect any microbial organism that contained the 16S gene. This primer showed the hidden diversity of the community in the early time points. 64.7% of the microbial community in the biofilm at the June time point was consumers (Figure 24). This analysis shows that the *Cyanobacteria* is not the only species that can provide protection from the intense light and oxygen; which could also explain how the *Chloroflexus* Green species is able to establish itself so early. *Cyanobacteria* species were found in all of the time points, including the separation of the red and green layer (Figure 21). This may indicate that the dissection was not complete or simply that there is, to a certain extent, and intertwining of the layers of organisms. This would add to the overall protection of the community in terms of aiding its survival. The *Chloroflexus* Red (*Roseiflexus*) species, however; is only found in the July 2005 red layer analysis and this supports the fact that the *Roseiflexus* cannot survive in an aerobic environment with such intense light as is found in the early stages of the mat system where it is only a simple biofilm (Figure 23).

The second primer used was specific for the *Chloroflexus* species, which is why the consumer hits for the Ward primer are much lower than they were for the 16S primer (Figure 24 and Figure 27). In addition to the amount of hits being lower, the diversity of the consumers was dramatically reduced with the Ward primer. The only consumers to be recognized by the primer were *Proteobacterial* in the June, August, and September sample

(Figure 16-18); and the *Deinococcus-Thermus* in the August samples (Figure 17). Surprisingly, there were no consumer hits for the July sample (Figure 19-20). This could be due to the fact that the *Cyanobacterial* layer has grown in depth to the point where the consumers are no longer needed in the community, in terms of protection, and are out competed by the photosynthetic organisms. The consumer organisms could also play a role in the establishment of the mat community in terms of adherence. This would correlate with the fact that the July 2005 sample was characterized with filamentous *Cyanobacteria* as opposed to the unicellular bacteria in the early stages of mat development.

References

- Boomer SM, Pierson BK, Austinhirst R, Castenholz RW (2000) Characterization of novel bacteriochlorophyll-a-containing red filaments from alkaline hot springs in Yellowstone National Park.. Arch Microbiol 174:152-161
- Boomer SM, Lodge DP, Dutton BE, Pierson BK (2002) Molecular characterization of novel red green nonsulfur bacteria from five distinct hot spring communities in Yellowstone National Park. Appl Environ Microbiol 68:346-355.
- Brock TD (1967) Micro-organisms adapted to high temperatures. Nature May 27 p 882
- Brock TD (1967) Life at high temperatures. Science 24:1012-1019
- Nübel U, Garcia-Pichel F, Kühl M, Muyzer G (1999) Quantifying microbial diversity: morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophs in microbial mats. Appl Environ Microbiol 65:422-430
- Peirson BK (1992) "Modern mat-building microbial communities: A key to the interpretation of proterozoic stromatolitic communities" The Proterozoic Biosphere ed. J. William Schopf & Cornelius Klein. pp. 247-248
- Sheehan KB, Henson JM, Ferris MJ (2005) Legionella species diversity in an acidic biofilm community in Yellowstone National Park. Appl Environ Microbiol 71:507-511