Microbial Biogeochemistry from Fly Ranch Geysers

March 2019

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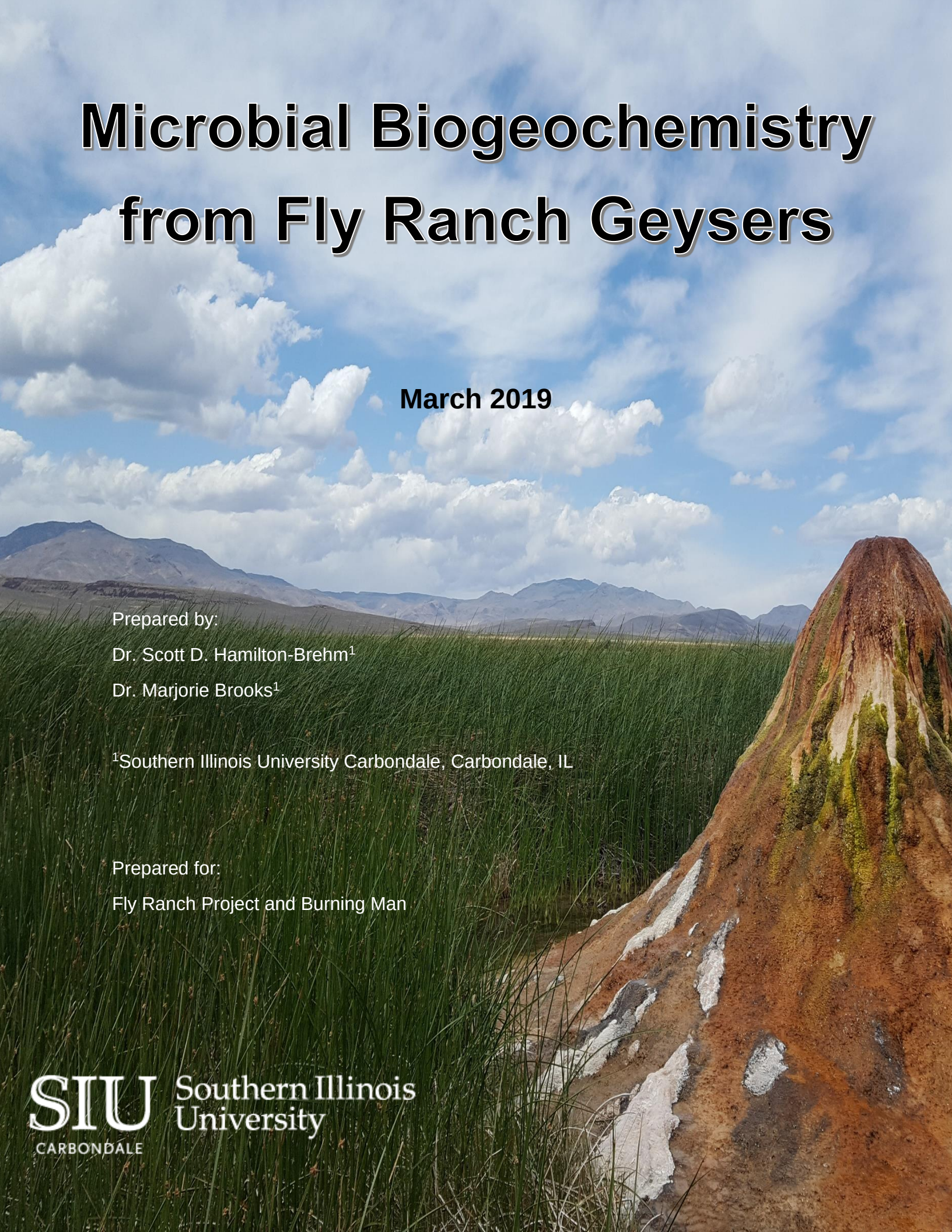
Prepared for:

Fly Ranch Project and Burning Man



SIU Southern Illinois University CARBONDALE

Project title: Microbial Biogeochemistry from Fly Ranch Geysers

Event: Fly Ranch Project Spring Expeditions

Effective Dates: 6-08-2018 to 6-10-2018

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Cover Photo:
Will's Geyser with differentially pigmented cyanobacteria. Currently, 90^{o}C water continuously bubbles down the outside of the geyser. The geysers of Fly Ranch provide a 'window' into the subsurface, allowing researchers to study how microbial colonization occurs across geothermal up-wellings from faults in the earth surface. Knowing which microbes participate in the development of a geyser can help NASA better interpret the origin of life from analogous structures on Mars.

Executive Summary

Key Findings

- The phyla Cyanobacteria, Proteobacteria, Bacteroidetes, Aquificae, Actinobacteria, Candidatus Hydrothermae, and Unclassified Bacteria had at least 12% or greater presence at least once in each dataset of NGS from Fly Geyser and Will's Geyser.

- The Candidate phylum Hydrothermae appears in significant numbers of 14% at Will's Geyser (5948/41956 DNA reads) and 4% at Fly Geyser (5660/143775 DNA reads). There may be a correlation with high temperatures (>80^{o}C), as this microorganism does not appear in significant numbers at any other location except close to the geyser spring source. While its numbers will aid future culturing, the unusual abundance of a previously uncultured organism raises the question why its population is so high at Fly Ranch.

- Candidate Phylum Atribacteria has been detected in both Fly geyser and Will's Geyser. Although it is present in very low numbers, it is interesting that this microorganism has also been detected in several locations around the world from marine and terrestrial subsurface locations. The role of this bacterium in the ecosystem is not clear. The Hamilton-Brehm laboratory is currently characterizing the only isolate ever cultured since the detection of the 16S rRNA gene in 1994 at Yellowstone National Park. It would be interesting to culture the Fly Ranch Atribacteria for comparison against the world population of this enigmatic bacterium.

- The microbes from Fly Geyser Pool have been successfully cultured in the presence of toxic biomass waste (furfurol, furfural, and liquefied leaf litter). While this research is preliminary, the anticipation is that unique microbes from Fly Ranch could be used to transform waste precursor molecules into biofuels for vehicles and jets. This is an exciting development because several previously sampled sites from across the country were not cultured successfully under these conditions.

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1. Background Information

1.1. Project Motivation and Justification

Prior to this exploratory study, almost no microbial research was conducted on the geothermal ecology of Fly Ranch. This study endeavors to raise awareness of the uniqueness of the geothermal features of Fly Ranch and the microorganisms that live within. Through DNA analysis combined with environmental chemistry and physical measurements of conditions, we can provide a preliminary understanding how the microbial producers and consumers control and regulate their extreme environment. This information will help guide scientifically informed decisions by the Burning Man and Fly Ranch projects about the future of the Fly Ranch geothermal site. Historically humans have depended on microbes for the production of cheese, yogurt, beer, and wine. However, microbes adapted to extreme conditions such as the deep subsurface hold the key to fundamental research in medicine, industry, energy, and learning how life evolved from the fiery origins of this planet. Previous investigations into subsurface monitoring boreholes in Southern and Northern Nevada have discovered a diverse array of microbial communities. Up to 30 and 40% of microbes in any community are unknown, sometimes referred to as “microbial dark matter”. We expect to find some known microorganisms but also previously unknown microorganisms with bizarre traits in the geothermal fields at Fly Ranch. Such findings potentially open new fields of research.

1.2. Acknowledgements

Thank you to Zac Cirivello for inviting Dr. Hamilton-Brehm and Dr. Brooks to the first Fly Ranch expeditions to mark the inaugural gatherings meant to plan and discuss approaches for the Fly Ranch Project.

1.3. In Kind Contributions

In the tradition and spirit of Burning Man gift giving, Dr. Scott D. Hamilton-Brehm and Dr. Marjorie Brooks provided the support for time, travel expenses, and reagents necessary for this analysis and report to become a reality.

2. Introduction

Geothermally active sites fascinate microbiologists, geochemists, and ecologists because the extreme conditions at these locations support a unique group of microorganisms known as extremophiles (from the Latin words translating to ‘extreme lovers’). Geothermal sites are found around the world, from magnificent landscapes like Yellowstone National Park, Iceland, and Hawaii, to the submarine ranges of ‘black smokers’ along the Pacific ‘ring of fire’ and tectonic plate boundaries in the Atlantic and Indian oceans. These sites are reminders of the tumultuous beginnings of when our planet formed some 4.5 billion years before present. The latest research suggests that life started very soon after the planet formed at around 3.5 to 4.2 billion years before present. The early earth atmosphere was composed of carbon dioxide (CO_2), carbon monoxide (CO), methane (CH_4), ammonia (NH_3), hydrogen sulfide (H_2S), and was highly reduced, meaning without oxygen (O_2). The surface of the earth was inhospitable compared with modern conditions: temperatures were boiling, meteoric bombardments

common, and the surface was bathed in solar radiation. How early life began in these conditions is not clearly understood.

It was not until the 1960s that Dr. Thomas Brock, an American microbiologist, discovered microorganisms living in the boiling hot geyser waters at Yellowstone National Park. His discovery revolutionized the scientific perception of where microbial life can live. The bacterium he discovered was named *Thermus aquaticus* (translated from Greek and Latin 'hot water microbe'), and it comfortably lives in water temperatures of 70 to 80^{o}C (water boils at 100^{o}C) [1]. *T. aquaticus* represented a novel type of bacterium that the scientific world had never before characterized, which required inventing the new descriptions such as 'thermophile' (50-80^{o}C), and 'hyperthermophile' (80-100^{o}C). These terms translated from Latin, meaning 'heat lover' and 'extreme heat lover' respectively, are based on the microorganism's ideal temperatures for growth. Research on thermophiles and hyperthermophiles resulted in two of the most significant discoveries in human history. The first discovery produced a new molecular tool for replicating DNA resulting in a multibillion-dollar biotechnology industry. The second discovery revealed a completely new domain of life on this planet.

The first discovery examined cell division, during which the genome (the DNA molecule that contains all genes for that organism) must be replicated precisely without error. A specific biochemical reaction is catalyzed by an enzyme that is called a polymerase. During cell division, the polymerase makes a perfect copy of the genomic DNA. The copies are then transferred to the new daughter cells (one each) allowing the continuation of the genetic lineage of that organism. Thermophilic microorganisms also have polymerases for their genomic DNA, but as expected, these enzymes operate at the elevated temperatures where these microbes grow. Non-thermophilic organisms' proteins/enzymes denature at high temperatures (e.g. egg whites on a hot skillet turn from clear to opaque solids indicating denaturation of the proteins). The thermostable polymerases make it possible to artificially copy or 'amplify' any DNA (in conjunction with assisting DNA primers for targeting) just by changing temperatures using a piece of equipment called a thermocycler. This method is known as Polymerase Chain Reaction or more commonly PCR. This discovery created a rapid expansion of biotechnology in the area of molecular biology, cloning, and DNA sequencing. PCR made scientific endeavors previously thought impossible a reality (e.g. the human genome project [2]). This first discovery permitted the second major discovery to occur, which reshaped taxonomic categorization.

After the discovery of thermostable polymerases, it became very easy to copy and sequence any DNA from any organism. In 1977, Dr. Carl Woese and Dr. George E. Fox, from the University of Illinois at Urbana-Champaign, proposed a novel method for the purpose of taxonomy [3, 4]. Their idea was to sequence a common universal gene, found within all living cells, as a marker to categorize living organisms. The gene they selected was a ribosome, a complex molecular machine, which serves as the scaffolding to make proteins by reading transcripts from the genome's gene library. A subset DNA sequence from the ribosome, which is used for Bacteria known as the 16S rRNA gene (the 18S rRNA gene is used for Eukarya). By using this method it was

possible to empirically determine the genetic relatedness of all life, and arrange them in a hierarchical order known as the ‘tree of life’. Initially there was only two domains of life, Eukarya (ancient Greek meaning ‘good kernel or nut’) and Bacteria (ancient Greek meaning ‘rod or stick’). The domain Eukarya describes organisms like plants, protists, fungi, and animals (humans) whose cells have defined organelles that help regulate biochemical processes. The domain Bacteria described all other lifeforms that did not contain organelles like the Eukarya, and typically have the shape of a rod or stick when magnified. While comparing DNA Sequences (amplified by PCR) from the 16S rRNA gene of thermophilic bacteria, Dr. Woese found discrepancies associated with a specific group of microorganisms originally thought to be bacteria that produced methane. His discovery revealed a new domain of life, which he named Archaea (ancient Greek meaning ‘ancient things’) (see **Figure 1**) [4, 5]. It was later discovered that many members of the domain Archaea are thermophiles and hyperthermophiles, and strangely, some of these microorganisms possessed genetic machinery similar to Eukarya (organisms like you and me). Furthermore, the positions of the thermophilic microorganisms on the lower branches of the tree of life may not be coincidental. This is evidence that suggests the origin of life was a geothermal environment (e.g. geysers and black smokers). This connects back to the geological fiery origins of the earth. Some of these same microorganisms can be found in geysers, deep wells, and deep mines today.

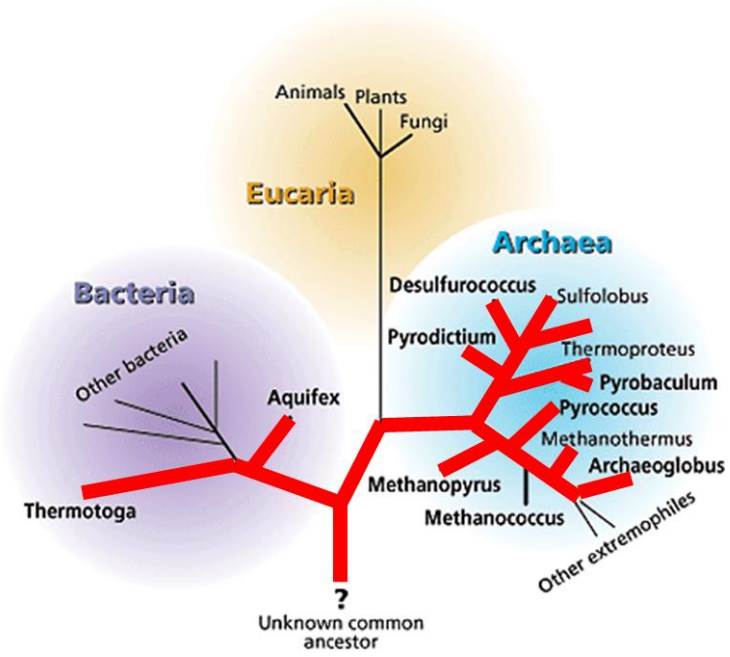

Figure 1. **A phylogenetic tree of the three domains of life on Earth.** This showing the evolutionary relationships between the three domains of life Eukarya (formerly Eucaria), Bacteria, and Archaea. Branch length are based upon similarities and differences in their physical or genetic characteristics. Red lines indicate branches occupied by thermophilic microorganisms.

We owe a debt of gratitude to the thermophiles: they transformed the Earth, allowing biological evolution to produce more complex organisms, and without them, humanity would not have been able to advance in biotechnology the way we have over the last few decades. Molecular DNA-based characterizations of microbial community structure, utilizing the 16S rRNA gene, and variations of this approach are the gold standard for surveys of microbial diversity in environmental samples [6, 7]. This has enabled the identification of >90% of prokaryotes in most natural communities [8, 9]. While we can

sequence almost all microorganisms from any environment, the growth (i.e. culturing) of any of those microbes is only about 1-8% successful, a phenomenon known as the 'Great plate count anomaly' [8, 10]. In the discipline of microbiology we have a lot more to learn how life is supported by geochemistry.

In 2016, the Burning Man Project acquired Fly Ranch, located 25 miles north of Gerlach, NV, USA. The Fly Ranch land covers an area of 3,800 acres encompassing multiple unique ecological environments including sagebrush-grasslands, salt flats (Hualapai Flat), and 640 acres of wetlands fed by geothermal hot springs. There are four original wells and 120 springs depositing $CaCO_3$, either as travertine towers or as terraces of aragonite. The hot springs at Fly Ranch, in the Hualapai Flat area appear to be an isolated part of several localized hydrothermal systems. Research of this area is limited, and the available information is insufficient to fully characterize the hydrothermal processes. What is known is that the thermal areas near Fly Ranch may be related to a series of subparallel faults that cross Hualapai Flat in a northeasterly direction. The temperature profile in the Cordero test hole indicates a reversal in thermal gradient below a depth of 45 m, which suggests lateral flow of thermal water through an aquifer at that depth. The thermal water presumably moves into the aquifer from much greater depth along a concealed conduit, probably of fault origin. Non-thermal surface runoff from the mountainous areas to the west probably migrates toward the basin and mixes with thermal waters migrating up the permeable fault zone. The hottest springs occur on or very near the Fault bounding the area to the east, thus giving some evidence for the migration of thermal waters up the fault zone [11-13].

From unknown origins of life, the great plate count anomaly, and microbial dark matter, all of this this underscores that we struggle to even understand a fraction about the microbial 'engineers' of our planet. Unique and geothermal sites like the geysers of Fly Ranch may provide scientists the opportunity to understand the origins of life, observe how life manages biochemical processes to live in extreme environments, screen for new biochemical processes that will help define our understanding or revolutionize biotechnology, and to look to the stars since extreme environments are analogues to the planets and moons in our solar system and beyond.

3. Materials and Methods

3.1. Sampling Field Site

The Fly Ranch land covers an area of 3,800 acres encompassing multiple unique ecological environments including sagebrush-grasslands, salt flats (Hualapai Flat), and 640 acres of wetlands fed by geothermal hot springs. Large volumes of water discharge at consistent rates characterize major springs in this location. Samples taken from Will's and Fly Geyser were selected for water chemistry analysis (see **§3.3.**), culturing enrichments (see **§3.2.**), and DNA extraction for microbial diversity analysis (see **§3.2.**). Samples for geochemical analysis were also taken from the surrounding areas of these two main geysers.

3.2. Sample Collection and Processing

Sample collection occurred by water filtration and direct water volume removal. All borosilicate glass containers (1 L Fisher bottles), sampling tubes (50 mL Falcon tubes), and platinum-cured silicone tubing (Masterflex LS-14) used in sampling were sterilized by autoclaving or ordered from supplier pre-sterilized. For filter sampling and water chemistry a peristaltic pump with silicone tubing connected to a 'Y' split to two 0.2 µm filters (Sterivex housings, EMD Millipore, Bedford MA) was assembled on site (see **Figure 2**). From four discrete locations, water was pumped at a rate of 0.3 Liters per minute for 1 hour. The resulting filtered water was collected in 3 x 50 mL Falcon tubes and stored on ice for physical and geochemical analysis (see **§3.3.**). Once pumping was complete after 1 hour, filters were voided of remaining water, aseptically stored in sterile Falcon tubes, and placed on ice to be sent to Southern Illinois University Carbondale (SIUC) for extraction of total environmental DNA (see **§3.4.**).

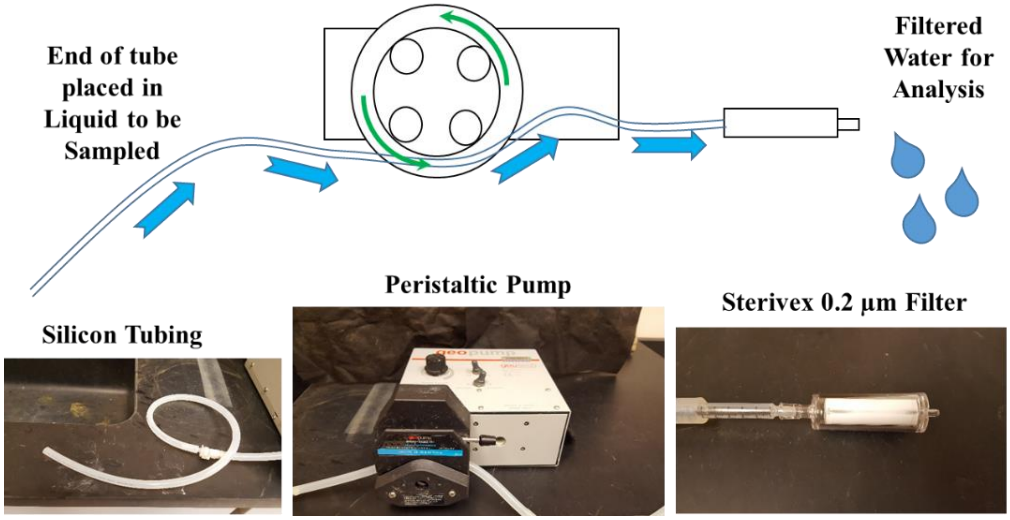

Figure 2. Planktonic microorganism sampling from liquid water medium. This **s**chematic illustrates the method for acquiring a sample of planktonic microorganisms within a water column. The 0.2 µm filter is the accepted size to capture the majority of all microorganisms and therefore sterilizing the water that flows through. Water is pumped by peristalsis through sterilized silicon tubing to the filter. The filter is kept and later removed from plastic casing and DNA extracted to be analyzed by next generation sequencing.

A sterile 1 L bottle was fixed at the end of a 1.5 meter telescoping stainless steel rod for direct water sampling. For each target location with concurrent filter analysis, one bottle is submerged near the inlet of the pumping peristaltic line and allowed to fill to full capacity with water. While under water, the bottle is angled to scoop minerals from the bottom of the sampling site. Upon removal from the water, the glass container is immediately sealed without any air bubbles (using a 23 gauge needle as a vent) with a butyl stopper and cap. The 1 L water samples were also stored at 4^{o}C and sent to SIUC for culturing enrichment studies (see **§3.6.**).

3.3. Physical and Geochemical Analysis.

In field assessments of water quality included dissolved oxygen (D.O.), temperature (ºC), and conductivity (µS) collected using an HQ40d multi-parameter meter (Hach) fitted with the appropriate electrodes. Electrodes have two-decimal point resolution. At locations where temperature exceeded the meter's measurement capacity, ethanol based thermometers were used in triplicate. Alkalinity and acidity were determined by pH paper strip sticks, and sulfide was determined using Lead Acetate strips.

Off field assays of water geochemistry were conducted using standard kits of determining water quality as per manufacturer's protocols [14]. Initial measurements of trace nutrients were conducted for ammonia (NH_3), nitrate (NO_3) and phosphorus (P) using a Surface Water Test Kit (Hach). Hardness, alkalinity, chloride (Cl), sulfide (S), and pH were assessed using Aquacheck 7 dipsticks (Hach).

3.4. Extraction of Environmental DNA

Sterivex filters were stored at -80ºC at SIUC until DNA extraction. Using aseptic technique, 95% ethanol-wiped-flamed utensils (sterilized tweezers/forceps/scissors) inside a scrupulously cleaned UV-treated Labconco Class II Biosafety Cabinet (Kansas City, MO), each filter were removed from their plastic casing. Using disposable sterile surfaces, filters were processed by cutting into small (5mm x 5mm) pieces.Total DNA was extracted from the filters using a commercially available kit (UltraClean Soil DNA Isolation Kit, MoBio, Solano Beach, CA) as per the manufacturer's protocols. The procedure was amended by including three freeze/thaw cycles (-80ºC/75ºC; 20 minutes each [15]) at the very beginning to facilitate cell lysis. DNA concentration was determined using a ThermoScientific NanoDrop® ND-1000 UV-Vis Spectrophotometer (Waltham, MA) with wavelength settings of 260 nm and 280 nm.

3.5 Next Generation Sequencing and Mothur processing

Extracted DNA from the filters was sent to The Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory (Lemont, IL) to be prepared for next generation sequencing (NGS) by an Illumina MiSeq platform. Universal Bacterial primers targeting the V4 region of the 16S rRNA gene [16] were for this diversity analysis. Illumina sequence data were processed in Mothur v1.39.5 [17] using a modification of the pipeline presented by Kozich *et al*. [16] at the "classify.seqs" command. At this step, we used the Silva database for improved taxonomic assignment of sequences. Operational taxonomic units (OTUs) were defined at a genetic distance of 0.03, and any OTUs that could not be taxonomically classified were curated manually. Singleton OTUs, chloroplast DNA, and unknown domains were removed from downstream analyses [18]. All Mothur commands were run in batch mode on an AMD 16 core 'Thread Ripper' CPU computer built using only solid-state technology.

3.6. Culturing Enrichments

Water removed in 1 L bottles was used as inoculum to initiate preliminary growth studies for culturing experiments. The medium used in these studies was designed for subsurface Nevada desert (SND) ecosystems. SND is composed of 3.6 g/L 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 284 mg/L Na_2SO_4, 174.2 mg/L K_2PO_4, 0.3/L resazurin, 400 mg/L $MgCl_2$•$6H_2O$, 500 mg/L KCl, 268 mg/L NH_4Cl, 250 mg/L $NaHCO_3$, 1 mL/L ATCC Minimal Vitamins (ATCC, VA), and 1 mL/L ATCC Minimal Minerals. Culturing medium was prepared anaerobically using a modified Hungate technique using a lower concentration of Na_2S•$9H_20$ (600 mg/L) as a reducing agent [19]. SND Anaerobic media volumes varied from 25 or 50 mL in 160-mL serum bottles. Several carbon and energy sources were used separately in enrichment studies: 10 mM glucose, 10 mM xylitol, 10 mM methylamine, 10 mM furfural, 10 mM furfurol, 0.1% w/v lignin, 80%/20% (v/v) H_2/CO_2, 100% (v/v) CH_4 and ¼ dilution of Oxidative Hydrothermal Dissolution (OHD) treated leaf litter. Resulting cultures were monitored using a phase contrast Axioskop2 Plus microscope (Zeiss, Thornwood, NY, USA) microscopy with a Petroff-Hausser counting chamber (Hausser Scientific Co., Horsham, PA).

4. Results

4.1. Sampling Logistics

The geographic locations for sampling was determined upon arrival at the site. As a preliminary study with limited capacity, it was necessary to identify two locations that could provide the largest and most in-depth view of microbial diversity from the geothermal system. A total of 12 x filtered water, 4 x filters, 4 x culture bottles, and 3 x sediment were collected from two major locations, site #1 (S1) labeled as ‘Fly Geyser’, and site #2 (S2) labeled as ‘Will’s Geyser’ (see **Figure 3**).

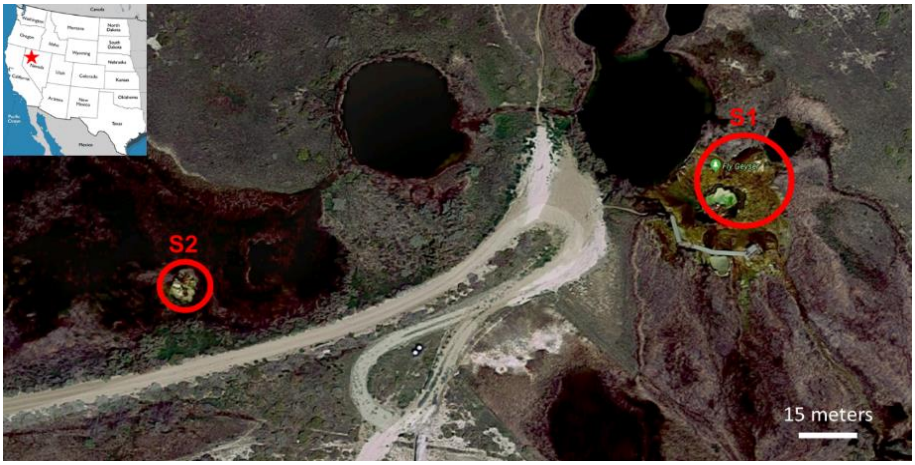

Figure 3. Geographic locations of Samples taken at Fly Geyser. Samples for geochemical and DNA analysis were taken from positions: Fly Geyser (S1) 40º 51’ 33.6276”N/119º 19’ 54.8148”W, and Will’s Geyser (S2) 40º 51’ 32.778”N/119º 20’ 1.2768”W. Inset, red star indicates location of Fly Ranch in the state of Nevada.

Sampling of Fly Geyser focused on acquiring water samples from the travertine/ aragonite terraces. Individual samples taken where labelled ‘Fly Geyser upper tier’ (FGUT) and ‘Fly Geyser lower tier’ (FGLT) (see **Figure 4A**) and the western facing connecting pool, was given the name Fly Geyser Pool (FGP) (see **Figure 4B**). Sterile

tubing and 1L glass bottle were positioned in the middle of each terrace pool during sample collection. Water from the geyser was pumped through a 0.2 µL Sterivex filter by peristaltic pump. The water volumes passing through the filters varies from site to site based on when they became plugged (<5 L per hour). A total of 25 liters was filtered for the FGUT DNA sample, 5 liters for FGLT DNA sample, and 5 liters for the FGP DNA sample. The filters underwent a color change from white to an amber color during filtration.

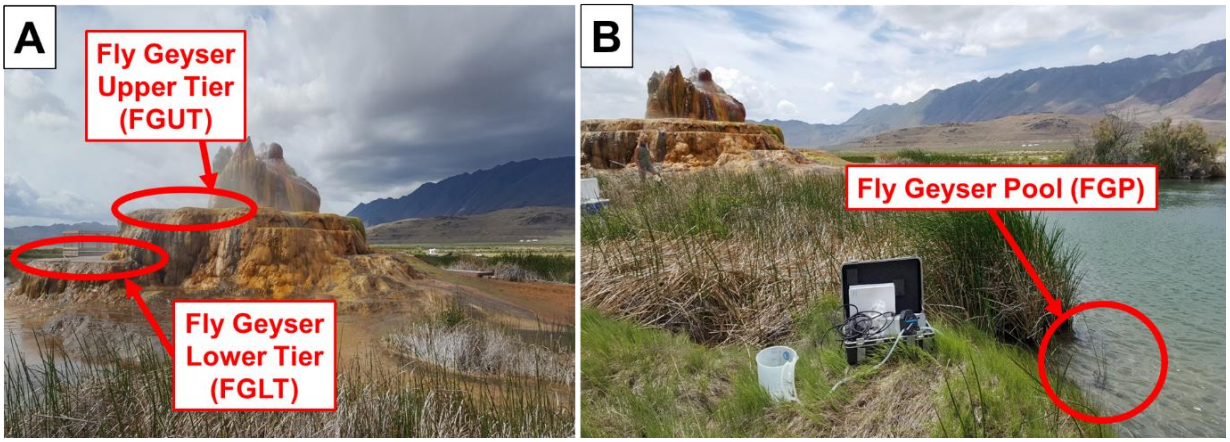

Figure 4. **Sampling locations at Fly Geyser for DNA analysis.** As indicated by red circles and arrows; A) Water samples were taken for culturing studies and DNA analysis from what is referred to as 'upper tier' and 'lower tier'. B) Water and sediment samples were taken from the larger of the pools that Fly Geyser feeds into for culturing and DNA analysis.

Water collection and filtration of Will's Geyser focused on acquiring samples from the core outlet with sediment samples from the side and lower base (see **Figure 5A**). Although the height proved to be a challenge, a telescoping pole was used to direct the tubing into the mouth of Will's Geyser. The Sterivex 0.2 µm filter flow rate reduced to under 5 Liter per hour after 20 liters of was had passed through it. As with Fly geyser, the filter underwent a color change from white to dark amber. Digital devices could not read temperatures of the water coming out of Will's Geyser, indicating the water inside the interior of the geyser was higher than 60^{o}C, which is the rated maximum temperature for the Hach probes. Ethanol thermometers rated for 0.1 ^{o}C accuracy were attached to the end of the telescoping pole and suspended inside the geyser's mouth for 5 minutes. The thermometers were then removed and quickly read, this was done three times in order to achieve an accurate reading. Mineral and sediment samples were chiseled and scooped from the sides of Will's Geyser as noted by the circles (see **Figure 5A**). Samples taken weighed no more than approximately 5 grams. Microscopy analysis of chiseled samples revealed that the green patches were photosynthetic microorganisms, likely cyanobacteria (see **Figure 5B**).

Figure 5. **Sampling location on Will's Geyser for DNA analysis.** As indicated by red circles and arrows, these locations are where samples were acquired and microscopy images of the microorganisms found at this location; A) Three sample sites were selected for DNA analysis and B) Phase contrast microscopy (400X) analysis of microorganisms from side of Will's Geyser.

4.2. Water Chemistry and Physical Properties

The overall chemical and physical characteristics measured from the waters from the Fly Ranch geysers during this preliminary study are shown in **Tables 1** and **2**. Dissolved oxygen (D.O.) ranged from 4.95 mg/L at Will's Geyser outflow pool to 10.57 mg/L at Fly Reservoir. The hardness and alkalinity were consistent over all sampling sites at 120 mg $CaCO_3$/L and 240 mg of $CaCO_3$/L respectively. Conductivity remained constant at an average of 1950 µS/cm with the exception of Fly Reservoir with 4220 µS/cm. The pH of each sampled location, while different, remained within pH values of 7.8 to 8.4. No chloride, sulfide, or nitrate was detected at any of the locations sampled. The same was true for the detection of sulfide by lead acetate paper. Phosphorous ranged from 0.5 to 1.0 mg/L. Ammonia was consistently detected at 0.5 mg/L for the sites that were tested. Colormetric titrations and dipsticks provide insight into the general range of solutes, typically in ~20% increments. We did not test for trace nutrient metals such as copper, iron, or zinc.

Direct microscopy assessments indicate cell densities to average 10^6 cells/mL. Observed morphologies ranged from bacilli (rod), cocci (sphere), and spirochete (rod with zig-zag). Cellular organization included colonial to single free floating cells. Cell densities of a soil sample taken from base camp were 10^7 to 10^9 cells/mL, a factor of 10 to 1000 times higher than the geyser samples.

Table 1. Geochemical data of Fly and Will's Geyser waters.

Location	Time	Lat	Long	Date Collected	Depth (m)	DO (mg/L)	Conductivity (µS/cm)	Temp (°C)	Cell Counts (Cells/mL)
North East Upper Tier	10:30	40°53.34'N	119°19.55'W	20180609	≤ 0.20	no read	1995	60.2	~10^6
North East Brown Midway Tier	10:30	40°53.34'N	119°19.55'W	20180609	≤ 0.20	5.48	1955	44.2	-
North East Moat	10:30	40°53.34'N	119°19.55'W	20180609	≤ 0.20	5.51	1953	32.8	~10^6
North Outflow Pool	10:30	40°53.34'N	119°19.55'W	20180609	≤ 1	5.55	1963	32.8	~10^6
West Upper Tier	12:25	40°53.34'N	119°19.55'W	20180609	≤ 0.20	no read	1988	68.9	-
West Brown Midway Tier	12:25	40°53.34'N	119°19.55'W	20180609	≤ 0.20	6.52	1906	35.8	-
West Moat	12:25	40°53.34'N	119°19.55'W	20180609	≤ 0.20	6.1	1939	41.5	-
South Moat	17:30	40°53.34'N	119°19.55'W	20180609	≤ 0.20	6.86	1927	39.2	-
South Outflow Pool	17:30	40°53.34'N	119°19.55'W	20180609	≤ 0.20	9.25	1922	28.7	-
Will's Geyser, Throat	14:15	40°51.534'N	119°20.018'W	20180609	na	no read	1910	~90.0	~10^6
Will's Geyser, Outfow Pool	17:00	40°51.534'N	119°20.018'W	20180609	≤ 0.20	4.95	1945	26.2	-
North Outflow Lake	12:25	40°53.34'N	119°19.55'W	20180609	0.25	6.13	1984	32.6	-
North Outflow Stream	12:35	40°53.34'N	119°19.55'W	20180609	0.25	6.21	1985	35.0	-
Swimming Hole	15:30			20180609	3.3	6.55	2003	35.1	-
Fly Reservoir	15:00			20180609	≤ 1	10.57	4220	21.3	-
South Ditch	17:50			20180609	≤ 1	6.15	2149	19.7	-

All samples on filtered water except probe readings in pools, reservoir, and swimming hole.

Table 2. Field measures of mineral content, pH, and nutrients in Fly and Will's Geyser waters.

Location	Hardness (as mg $CaCO_3$/L)	Alkalinity (as mg $CaCO_3$/L)	Chloride (mg/L)	Ammonia (mg/L)	Phosphorus (mg/L)	pH	Sulfide (mg/L)	Nitrate (mg/L)
North East Upper Tier	120	240	0	0.5	1	8.4	0	0
North East Brown Midway Tier	120	240	0	0.5	0.5	8.4	0	0
North East Moat	120	240	0	0.5	0.625	8.4	0	0
North Outflow Pool	120	240	0	0.5	1	7.8	0	0
West Upper Tier	120	240	0	0.5	0.75	8.4	0	0
West Brown Midway Tier	120	240	0	0.5	0.5	8.4	0	0
West Moat	120	240	0	0.5	0.85	8.4	0	0
South Moat	120	240	nd	nd	nd	8.4	nd	nd
South Outflow Pool	120	240	nd	nd	nd	8.4	nd	nd
Will's Geyser, Throat	120	240	0	0.5	0.75	7.8	0	0

n.d. = not determined

4.3. Temperature Profile of Will's and Fly Geyser

Temperature varied greatly depending on sampling source. Of the sites that were sampled the temperatures were found to be from 19ºC at the South Ditch to approximately 90ºC at the outlet of Will's Geyser. Water temperature was mapped using a FLIR Thermal imaging camera. Temperature ranges of Fly Geyser were 32.8ºC at the base to 74.2ºC at the outflow across the body of the geyser's mineral depositions (see **Figure 6**). Temperature of Will's Geyser ranged from approximately 40ºC at the base to 80.0ºC at the outflow across the body of the geyser's mineral depositions (see **Figure 7B**).

Figure 6. Fly Geyser thermal profile. Temperature readings from FLIR of Fly Geyser, profiles a significantly drop from 74.2ºC at the origin of spring, cooling to 32.8ºC.

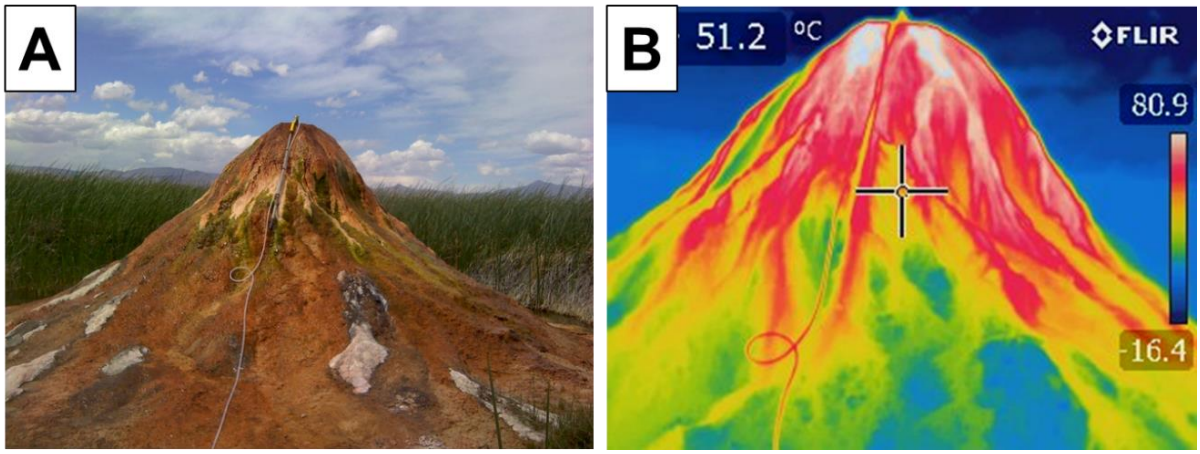

Figure 7. **Will's Geyser thermal profile.** While sampling the waters within, temperature gradient of the exterior was recorded; A) Will's Geyser during water sampling from mouth of geyser, B) Thermal image readings from FLIR profile Will's Geyser during water sampling with temperatures as high as 80.9ºC.

4.4. Microbial Diversity

Next Generation Sequencing (NGS) is a high throughput method that can sequence 16S rRNA genes from all microorganisms within an environment from one sample. Seven samples were collected, four by water filtration and three by sediment sampling. One of the soil samples was a control taken from the parking lot area outside of the Fly Geyser location. Total DNA of each sample was extracted using a kit and the DNA sent to Argon National Laboratories for NGS that generated over 1 million quality-tested sequences. The NGS used only universal bacterial primers for the sequencing, although some archaeal sequences are always generated. The archaeal sequences are typically removed due to primer bias, but for this analysis, they have been kept in the dataset. The distribution of bacterial (and some Archaeal) sequences are shown at the taxonomic level of phylum (see **Figure 8**). From all samples analyzed, a total of 1,004,531 DNA fragments were sequenced, which can be placed into 2,816 distinct genera (species is not possible with short 250 bp DNA sequences) that fall under sixteen phyla.

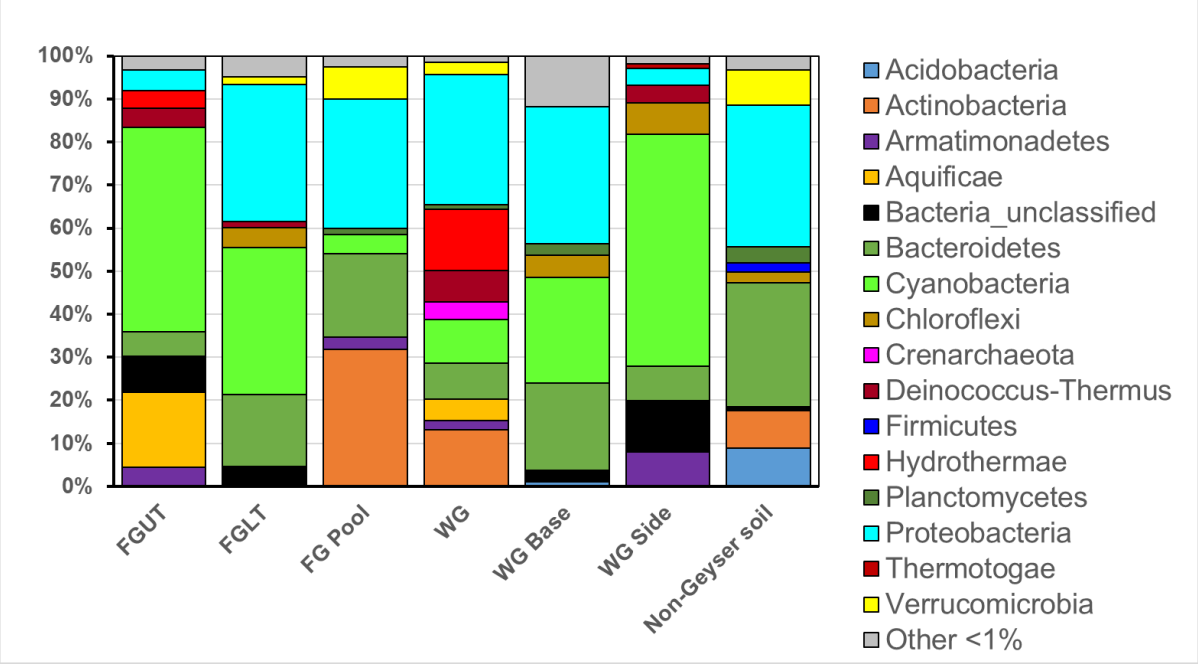

Figure 8. Next generation sequencing results summary from seven sampled locations at Fly Ranch. Samples came from Fly geyser Upper Tier (FGUT), Fly Geyser Lower Tier (FGLT), Fly Geyser Pool (FGP), Will's Geyser (WG), Will's Geyser Base (WGB), Will's Geyser Side (WGS), and a non-geyser soil control sample (NGCS). This dataset represents 1,004,531 sequenced reads of DNA, profiling 2,816 species of bacteria and archaea combined.

The phyla identified are: Acidobacteria, Actinobacteria, Armatimonadetes, Aquificae, Unclassified bacteria, Bacteroidetes, Cyanobacteria, Chloroflexi, Crenarchaeota, Dienococcus-thermus, Firmicutes, Candidatus Hydrothermae, Planctomycetes, Proteobacteria, Thermotogae, and Verrucomicrobia contributed to equal to or greater

than 1% of the overall microbial community. Other phylum sequences that did not amount to more than 1% of the total reads were collapsed into a single category ‘Other <1%’.

4.5. Culturing Enrichments

Water samples collected from Fly geyser lower tier and pool were used as inoculum to grow enrichments for exploratory biofuel or value added molecule assessments. The carbon source tested was 10 mM glucose with three different electron acceptors (fufural, lignin, and fufurol) separately prepared as anaerobic media (see **Figure 9A**). Under these conditions, microorganisms from Fly Geyser Lower Tier and Pool generated sustainable and transferable cultures. While end-products were not analyzed, it is interesting that cultures were generated in the presence of the electron acceptors that typically are very cytotoxic. This exploratory culturing experiment reveals potential for mining microorganisms in the pursuit of value-added molecules and biofuel production.

Figure 9. **Exploratory value added molecule production from culture enrichments of Fly Geyser Pool sediment**. Cultures were successfully grown after three transfers in the presence of A) furfurol, lignin, and furfural. B) A culture was also enriched after three transfers on leaf litter that was subjected to Oxidative Hydrothermal Dissolution (OHD). All of these enrichment experiments indicate that Fly Geyser microbiome may have biofuel or value added transformation properties worthy of further research.

Another intriguing experiment was to use Fly Geyser Pool inoculum with a medium composed of a one fourth dilution of Oxidative Hydrothermal Dissolution (OHD) processed leaf litter (see **Figure 9B**). The OHD process can liquefy almost any non-soluble material into smaller products that are soluble in water. This process can turn coal or cellulose (grass clippings, leaf litter, etc.) into a carbon rich solution. Similarly as with the alternate electron acceptor experiment, a culture was enriched, and microorganisms from Fly Geyser Pool were sustained with this typically toxic media composition.

4.6. Transects

The geochemistry in Fly Geyser and other geothermal features differs significantly from the surface water in Fly Reservoir. The geysers and hot pools have lower oxygen levels due to their warmer temperatures. In contrast, the Reservoir has twice the mineral content of any other water body, meaning that has a different water source, likely mainly surface runoff without input of artesian springs. These findings are very preliminary. Additional analyses will investigate the geochemical signature left by microbial communities, even after a geyser dies.

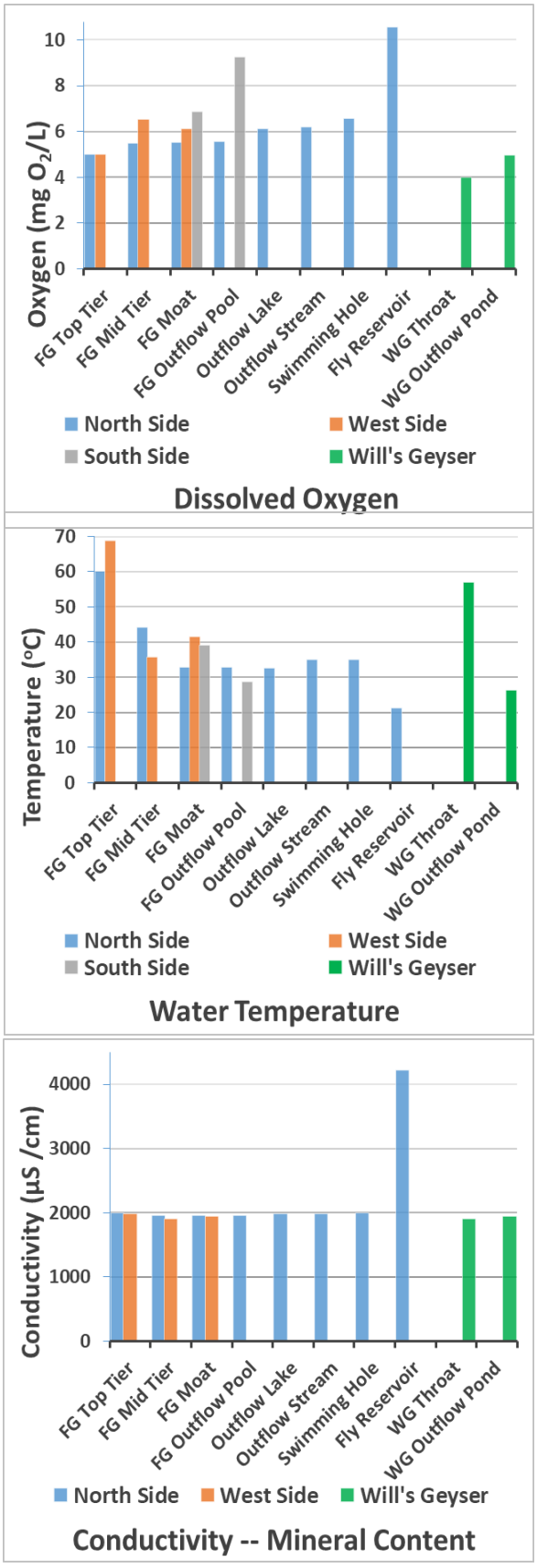

Figure 10. Transects of water geochemistry and temperatures in pools radiating southward and westward from Fly Geyser. The blue bars indicate the transect extending from Fly Geyser across water bodies and northward to Fly Reservoir. Will's Geyser and its westward outflow pool are shown at far right in each graph.

5. Discussion

This preliminary assessment of microbial communities indicates that Fly Ranch contains a significant reservoir of microbial diversity. The importance of this site is underscored by the high percentages of microbial 'Dark Matter' and Candidate Phyla. Why these particular microbial communities are present is not clearly understood. Preliminary culturing enrichments with alternative toxic electron acceptors indicate that some of the microbial communities present in the Fly Geyser Pool may be useful for exploring renewable energy research by producing value-added molecules such as biofuels. Observations during sampling noted that the spring pools and outlet channels are coated with microbial and microalga communities that may represent the bulk of system biomass and likely food resources for fish and other fauna. Further adding complexity to the ecosystem of the Fly Ranch geyser field. A detailed characterization of the benthic microflora of these springs probably represents the next logical step in characterizations of the lower trophic organisms.

5.1. Next Generation Sequencing Analysis

Sequencing environments has become a very easy to do and provides and overload of data and information. It becomes a challenge to have people properly trained in the script line programing languages to process the data and ask the questions from which the data can properly answer.

Following are some brief descriptions of each of the sixteen identified phyla found from all sequenced sites:

- **Acidobacteria**: The Acidobacteria are a relatively recently recognized phylum of environmental bacteria; which, although physiologically diverse and ubiquitous, contain very few cultivated representatives. Of the most notable environments that these bacteria can be found are soil, hot springs, oceans, caves, and metal-contaminated soils. Many Acidobacteria are acidophilic, meaning 'acid lovers'.

- **Actinobacteria**: These bacteria are Gram-positive bacteria, meaning they have only one lipid layer and a thick cell wall made of peptidoglycan. These microorganisms can be found in either terrestrial or aquatic environments. These bacteria decompose organic material and often form mycelia-like structures. These metabolic and morphological characteristics, combined with identified symbiotic relationships with plants, often miscategorizes Actinomycetales (the actinomycetes) as Fungi rather than Bacteria. Within this phylum, members of Mycobacterium are important pathogens.

- **Armatimonadetes**: initially the 16S rRNA gene from environmental samples was the only identification of the phylum Armatimonadetes. A placeholder for this phylum was used known as candidate phylum OP10. However, in 2011 a bacterial strain belonging to the phylum was isolated from an aquatic plant in Japan. The species was named *Armatimonas rosea* and became the first species of bacteria representing this phylum.

- **Aquificae**: These bacteria are Gram-negative, meaning these cells have two bi-lipid membranes and a very thin cell wall. They are non-spore-forming and typically have rod morphology. This phylum contains a diverse collection of bacteria that live in ‘extreme’ environmental settings. The name 'Aquificae' was given to this phylum based on an early isolated genus Aquifex (“water maker”), which is able to produce water by oxidizing hydrogen. These bacteria have been found in springs, pools, and oceans, metabolically they are autotrophs, fixing carbon dioxide from their environments.

- **Unclassified bacteria**: Bacteria that have not been cultured or taxonomically classified. These are considered the part of the microbial ‘Dark matter’.

- **Bacteroidetes**: These bacteria are Gram-negative (two membranes) and are a diverse group of nonsporeforming, anaerobes. They can be found in many environments such as soil, benthic sediments, sea water, on skin, and in the intestines of animals. This particular phylum of bacteria may hold answers to how the human microbiome interacts with the body and maintain health.

- **Cyanobacteria**: The Cyanobacteria, formerly known as “blue green algae” are often a dominant group in aquatic ecosystems. A combination of cyanobacteria and algae serve as primary producers in the above-aground portions and surfaces of the geysers. Cyanobacteria are important because some species can turn atmospheric nitrogen gas into organic nitrogen. They are also responsible for toxic blooms in lakes because of uncontrolled nutrient influxes.

- **Chloroflexi**: These filamentous bacteria that mostly stain Gram negative, although this group of bacteria have only one bi-lipid membrane. Isolates within this phylum have a wide diversity of phenotypes including anaerobes (without oxygen), aerobes (with oxygen), thermophiles (‘hot temperature lover’), mesophile (‘moderate temperature lover’), photosynthesis (green non-sulfur bacteria), and halorespirers (the use of halogenated organics, such as the toxic chlorinated ethenes and polychlorinated biphenyls as electron acceptors).

- **Crenarchaeota**: This group of microorganisms are not bacteria, but rather members of the domain Archaea (‘the ancient ones’). This phylum under archaea known as Crenarchaeota (Greek for "spring old quality", as the first isolates came from geothermally heated sulfuric springs in Italy). While Archaeal membrane physiology is different from Bacteria, these microorganisms do stain as Gram negative. Morphologically the Crenarchaeota can be rod, cocci, filamentous and some very oddly shaped cells (e.g. stars). Most of these microorganisms are sulfur-dependent hyperthermophiles (‘extreme hot temperature lover’), some of which have the ability to grow at up to 113°C (this temperature is above boiling water). Recent studies of environmental 16S rRNA gene indicate they these archaea have a previously unrecorded major presence in marine environments.

- **Dienococcus-thermus**: This group of bacteria are cocci (sphere) shaped cells that have two bi-lipid layer membranes and a thick peptidoglycan cell wall, and stain Gram positive. This phylum of bacteria are highly resistant to environmental hazards, from temperature, pH, high salt concentrations, and radiation. Thus they have earned the description of extremophiles ('love of extreme conditions'). Because of these characteristics, this clade is sometimes referred to as the Hadobacteria (from Hades, the Greek underworld, meaning 'bacteria from hell'). The famous bacterium *Thermus aquaticus* comes from this phylum, whose discovery and applied use of its thermal stable polymerase created the multibillion dollar industry based on the technique called polymerase chain reaction (PCR).

- **Firmicutes:** This group of Gram positive bacteria are known to have a thick cell wall made of peptidoglycan. They are also able to form what is called an endospore, a hardened cell state in which metabolic activity is slowed or halted, no growth occurs, in this state the cell can withstand many unfavorable conditions (with the exception of autoclave and some caustic chemicals). In the endospore state cells have been revived from thousands to millions of year old samples. The Firmicutes are a large and complex group of importance in a range of habitats ranging from soil and the human gut to the deep terrestrial and marine biosphere.

- **Candidatus Hydrothermae**: There are no cultured representatives of this group of microbes. This is a place holder for future isolated microorganisms.

- **Planctomycetes**: This group of aquatic bacteria and are found in brackish, marine and fresh water ecosystems. They reproduce by budding, which is similar to yeast (a Eukaryotic cell, like cells that make up trees, fungi, and humans). The morphological structure of this group of microorganisms is ovoid in shape with a thin cylindrical extension from the cell body called a stalk. The life cycle of many planctomycetes involves alternation between sessile cells and flagellated swarmer cells.

- **Proteobacteria**: This major group of Gram-negative bacteria have a widespread environmental impact of every corner of the Earth. The name of this phylum comes from the word Proteus, the name of a Greek subject to the God Poseidon. Proteus was a sage who knew many things but did not want to divulge the information to seekers of knowledge, in order to evade captors he was able to shapeshift into any form. This mythology fits this phylum of bacteria very well. Some of the bacteria include a wide variety of pathogens, such as Escherichia, Salmonella, Vibrio, Helicobacter, Yersinia, Legionellales and many other notable genera. While others are free-living (non-parasitic) and include many of the bacteria responsible for nitrogen fixation. Class level of taxonomy from Proteobacteria include many well-known names such as Alphaproteobacteria, Betaproteobacteria, Hydrogenophilalia, Gammaproteobacteria, Acidithiobacillia, Deltaproteobacteria, Myxococcales, Epsilonproteobacteria, and Oligoflexia.

- **Thermotogae**: While this group of microorganisms exhibit one bi-lipid layer, they stain as Gram-negative. While mostly anaerobic, thermophilic and hyperthermophilic

bacteria, recently some moderate temperature preferring microorganisms have been identified. The name is derived from the existence of many of the high temperatures loving cells that have a characteristic sheath structure, or "toga", surrounding the cells. Many Thermotogae species are considered attractive targets for use in industrial processes, because of their ability to withstand harsh conditions and ease of genetic manipulation. The metabolic ability of Thermotogae to utilize different complex-carbohydrates for production of hydrogen gas led to these species being cited as a possible biotechnological source for production of energy alternative to fossil fuels.

- **Verrucomicrobia**: This grouping of bacteria contains only a few characterized species (*Verrucomicrobium spinosum*, is the type strain that defined the phylum's name). Several species have been isolated from fresh water, soil environments, and human feces. Culture independent 16S rRNA genes have been identified in association with eukaryotic hosts including extrusive explosive ectosymbionts of protists and endosymbionts of nematodes residing in their gametes. While verrucae is another name for the warts often found on hands and feet, this phylum is so called not because it is a causative agent thereof, but because of its wart-like morphology.

5.2. Geochemistry

The physical and aqueous chemistry are critical to understanding how an environment's conditions shape the microbial communities present and helps understand what the driving forces for the ecosystem are. Regarding the geochemistry of Fly Geyser and the surrounding waters, we offer preliminary evaluations that will benefit greatly from high-resolution analyses from future research analysis. Nonetheless, we have a preliminary outline of the site that has already generated some interesting questions.

Within the geothermally active plain, much of the water chemistry does not vary greatly. This seems to include the water in South Ditch, which is important for suppressing dust at BRC. If the water in South Ditch is continually fed by geothermal inputs, it will likely be more resistant to future droughts. Fly Reservoir is quite different in its total amount of minerals, oxygen levels, and temperature (see **Figure 10**). Based on the waterfowl present, it is an important feeding ground for wildlife. The difference suggests less influence from geothermal sources and far greater influence by surface runoff.

It is also important to think about nutrient concentrations because they can limit plant growth as well as microbial growth in the ecosystem. Nitrogen-nutrient levels, (using these preliminary assessment methods) are very low. On the other hand, phosphorus levels are exceptionally high. Algal blooms in lakes or streams are associated with phosphorus of 0.2 mg/L or greater (i.e. the cut-off between moderate and excessive nutrients). The data listed in **Table 2** shows that phosphorus levels range from about 2-times to 5 times the levels that cause algal blooms. High phosphorus without other nutrients suggests not only that the ecosystem is nitrogen-limited, but also that it might lack important trace metal nutrients like iron, copper, and zinc. Although this likely explains why the cyanobacteria we identified haven't grown explosively, since this type

of microorganism is capable of acquiring nitrogen from the atmosphere, something else is holding back their growth.

6. Conclusion, Recommendations and the Future

This preliminary study provides an outline of future research possibilities and applied experimentation that strongly supports the Fly Ranch Project initiative to “Prioritize Environmental Stewardship and Initiate Research Partnerships”. This work will also assist with the development of ideas to “Begin Crafting a Long-Term Vision for the Project”. The hydrothermal ecology found on Fly Ranch is a rare and delicate resource. The only other location in the United States that has the same quality is Yellow Stone National Park, but with one chemical difference: there is no detectable sulfide (the rotten egg smell) coming from the geysers. This is very unusual and piques curiosity about how the microorganisms living in the geothermal water conduct life supporting chemistry.

The knowledge of the uniqueness of this site should initiate a priority to preserve the microbial diversity found at this location, begin discussions to support future research plans, and begin organizing a public outreach program. All of these endeavors are achievable and can be accomplished with sustainable outcomes for the Fly Ranch Project. For example, a zero carbon footprint, solar or geothermal powered visitor’s center can be constructed near the entrance of Fly Geyser to provide protection and services to the geothermal area. This will also allow for the Fly Ranch Project to test and showcase sustainable ideas. More importantly, the Fly Geyser location is known and revered by social media. This knowledge causes several instances of trespassing throughout the year and potential dangerous encounters with the geothermal springs (e.g. Visitor deaths at Yellowstone National Park). Planning for visitation can protect this rare site, provide a venue for science communication and positive public relations, and generate a small revenue flow to support innovation, research, and preservation of the geothermal site.

In closing, Dr. Brooks and Dr. Hamilton-Brehm would like to submit a research proposal to the National Aeronautics Space Administration (NASA) Exobiology program to acquire funding to continue fundamental research at Fly Ranch. The NASA Exobiology solicitation for the pre-proposal letter of intent is due April 16, and upon acceptance, the full proposal will be due on May 24, 2019. The full proposal will require letters stating the support of Fly Ranch Project to the proposed research. It is not guaranteed we will win the grant, but we feel that our expertise, clear access to the unique site, and the presence of interesting extreme microorganisms fall within the interests of the NASA mandate to detect life on other planets and moons.

If the proposal is successful, both Burning Man and the Fly Ranch Project can report they are working with researchers funded by NASA. Funded research would also enable discovery and characterization of new microorganisms from the geothermal springs, opening an opportunity to name them. The names given to microorganisms, upon acceptance by the scientific community, would be immortalized in human time.

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