Leah TRUTSCHEL Project Report Final report rcvd. 9.22.21
Lewis and Clark Fund Grant in Astrobiology
Project title: **EXPLORING MICROBIAL HABITABILITY AND DIVERSITY IN A MARINE-LIKE TERRESTRIAL SERPENTINIZING SPRING**-*NEY'S SPRING*

Background and Location of Field Work

Field work for this project was carried out at Ney Springs, which is located at 41°16'14.0"N 122°19'27.3''W in Siskiyou County, California. The spring was discovered in 1887 by John Ney and had infrastructure built around it so that it may be purported for its "medicinal" waters (**SF1**). The water was initially characterized in a 1915 U.S. Geological Survey and was noted for its exceptionally high pH, salinity, sulfide, ammonia, and silica content (Waring, 1915). Later the unique geochemistry of the spring was found to be caused by serpentinization, a process in which water hydrates iron and magnesium rich minerals in the Earth's crust resulting in the production of hydrogen, methane, and high pH fluids. These environments serve as excellent analogs for early Earth, as well as other celestial bodies where serpentinization is suspected to be occurring or has occurred. While the microbial communities and geochemistry of serpentinizing systems were popularized by deep sea hydrothermal vents, easier to access terrestrial systems have been increasingly studied. Ney Springs is thus far the most marine-like terrestrial serpentinizing system described to date, but it also contains several geochemical elements not observed in any other system, such as ammonia and silica, as well as other elements that are exceptionally higher than in other systems, such as sulfide, methane, and dissolved organic carbon (DOC). The novel geochemistry of this system also influences its microbial community; it shares taxa found in both terrestrial and marine systems, as well as some members that are unique.

Research Conducted

Field work for this trip was carried out May 22nd-28th 2021 and consisted of both geochemical and microbial sampling. Geochemical analyses conducted on site included the quantification of ammonia, sulfide, nitrate, nitrite, and iron. A multimeter was used to measure pH, total dissolved solids, temperature, and conductivity of the spring fluids. Additional fluid samples were collected for ion chromatography and DOC analysis upon return. For the microbial community analysis, fluid, sediment, and biofilm samples were collected. For fluid samples, 4+ liters of water were passed through a 0.1µM filter using a peristaltic pump in order to collect biomass. Sediment and biofilm samples (**SF2**) were collected in sterile 15 mL conical tubes. These samples were preserved on dry ice and later at -80℃ until DNA extraction could be performed on them using a Qiagen DNAeasy soil kit. This DNA would then later be amplified using 16S rRNA universal primers, which allows us to observe a taxonomic snapshot of the microbial community. For enrichment and isolation of microorganisms, agar petri plates that included 20 mM sulfur, 5 mM glucose, or 10 mM sulfate/10 mM acetate were streaked with water from the spring. These plates were incubated aerobically (sulfur plates) or anaerobically (sulfate/acetate and glucose plates) until colonies grew that could be re-streaked for isolation. Additional enrichments included serum vials (small glass bottles) containing 20 mM ammonia, 10 mM methanol, or 10 mM sulfate/10 mM acetate. These were inoculated with sediment or a 0.1µ filter after 2L of spring water had been passed through it. These serum vials were kept at room temperature for several weeks and were monitored for changes that may be caused by microbial activity, e.g. sulfate being converted into sulfide and acetate disappearing.

Results of research

Geochemical analysis of the spring revealed several viable energy sources for microorganisms such as sulfide (470 mg/L), acetate (1.4 g/L), and ammonia (101 mg/L). DOC was high at 22 mg/L when compared to other terrestrial serpentinizing systems such as the tablelands, which contained at most 2.04 mg/L (Szponar *et al*, 2013). While oxygen was very limited in the spring (87 µg/L), other compounds that could be used for anaerobic respiration such as nitrate (35 mg/L) and sulfate (265 mg/L) were plentiful. The sulfur species thiosulfate (851 mg/L) and tetrathionate (324 mg/L) were quantified as well and may serve as energy sources/sinks for microbes.

16S rRNA analysis for taxonomic composition

Comparing samples from June 2021 to ones from May 2019 revealed that the microbial community was much the same and still primarily dominated by a few species belonging to the *Clostridiales* and I*zemoplasmatales* (**SF3).** However, samples collected from the biofilms as seen in (**SF2**) were more enriched in sulfur-oxidizing taxa than seen in the primary cistern. Of the top ten most abundant families collected from these outflow sources, seven of them contained known sulfur-oxidizing members. Especially exciting was the high abundance of *Thiomicrospira* detected in these outflows. *Thiomicrospira* are typically autotrophic sulfur-oxidizers and are also one of the most abundant taxa detected in the Lost City Hydrothermal field (Brazelton *et al*, 2006). Other potential sulfur-cycling bacteria detected in high abundance in these outflows were *Desulfurivibrio* and *Dethiobacter* spp.. These genera are less characterized, but were originally isolated from alkaline soda lakes as sulfur-reducers (Sorokin *et al*, 2008). Metagenome assembled genomes for these bacteria from a previous sampling trip suggest that the strains from Ney Springs are capable of sulfate reduction as well.

Isolate identification & enrichment cultures

Additionally, several isolates were obtained from Ney's Spring on polysulfide, glucose, and sulfate/acetate containing petri plates. These microorganisms have proven amenable to growth under laboratory conditions and have now been identified via full length 16S rRNA sequencing. Two of the strains isolated are *Thiomicrospira sp.,* one of the most abundant taxa from the 16S surveys and a putative autotrophic sulfur oxidizer. Other isolates obtained from these plates that may utilize sulfur include *Halomonas* and *Salinarinonas* sp, though they are more likely to do this in conjunction with heterotrophic growth rather than as a primary energy source (Sorokin, 2003; Liu *et al* 2010). In addition to the plates, serum vials of sulfate/acetate containing media inoculated with sediment from the spring have also shown promising activity indicative of sulfate-reducing microorganisms. These enrichments have shown sulfate and acetate levels decreasing and sulfide increasing over the span of several months, which is a good indicator of activity for sulfate-reducing microorganisms. These serum vial enrichments will eventually be used for specific sequencing of the dissimilatory sulfite reductase (*dsr*) gene, which is a known metabolic marker for sulfate-reduction and is often used for taxonomic comparisons between sulfate-reducing bacteria.

Significance of research

Because the fluids of Ney's Spring are so rich and can often interfere with colorimetric based methods or even destroy the analytical instruments, a significant part of the geochemical portion of this research project has been devoted to developing and comparing methods for measuring nitrogen and sulfur species, as well as dissolved organic carbon. Detailed methodology for these amended assays will later be published and hopefully provide beneficial insight into what corrections and steps are necessary to obtain accurate measurements for these components in even the most extreme high pH environments. Additionally, adaptation of the cyanolysis assay (Kelly *et al*, 1969) for a portable field spectrophotometer allowed us to measure thiosulfate and tetrathionate for the first time in a serpentinizing system. These sulfur species are often important intermediates in sulfur-oxidizing and reducing metabolisms, but the extent of their usage in poorly studied in high pH systems (Sorokin, 2003). By quantifying sulfur species directly in this serpentinizing system, as well as in our microbial enrichments, we will gain much-needed insight into how and which sulfur species may be utilized as energy sources in serpentinizing systems.

Microbial community analysis revealed that the community was very similar to samples collected almost exactly two years prior. While this suggests the community is likely fairly stable, we hope to gain even better resolution on this in the future. Currently we are planning to sample the spring every two months, which will allow us to see how the geochemistry may change seasonally and how that will in turn affect the microbial community. Additionally, several outflow spots **(S2**) tested during this trip revealed a very different microbial community composition compared with the cistern. These outflow areas contained sulfur-oxidizing taxa also found in the cistern, but in much greater abundance. This discovery may make isolation of these organisms easier in the future since they make up the majority of these outflow microbial communities. Future work will also involve looking at the geochemistry of the fluids leeching out of these areas to see how much their composition differs from the main cistern in terms of dissolved oxygen, DOC, and sulfur species. While these areas may simply be where the main cistern infrastructure has started to give way and is leaking, they could also potentially be deeper fluids reaching the surface and may give us better insight into the subsurface community. Either way, by studying the geochemistry of these outflow areas we will be able to better determine what conditions are more favorable for these sulfur-cycling taxa compared to the more abundant fermentative species located in the cistern.

Finally, perhaps the most significant portion of this research has been the isolation of several putative sulfur-oxidizing microorganisms from the spring. Very few isolates exist from serpentinizing systems, with only one thus far having optimal growing conditions above pH 11 (Suzuki *et al*, 2017). Taxa associated with sulfur-oxidizing and reducing microorganisms as well as several genes necessary for sulfur metabolisms have been detected in serpentinizing systems, but these may be the first isolates to shown to engage in *in vitro* sulfur oxidation. The *Thiomicrospira* strains isolated in particular appear capable of autotrophic growth, which will make them an excellent resource for studying carbon fixation in high pH environments with limited inorganic carbon sources.