SUPPLEMENTAL FILES for:

Investigations of microbial metabolisms in an extremely high pH marine-like terrestrial serpentinizing system: Ney Springs

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Supplemental Methods 1: Complete liquid/gas sampling procedures
Description of sampling activities:

Initial Ney Sampling in 2016

The Ney Spring is located 3m up the northern bank of the Ney Creek and housed in an open, hexagonal cistern approximately 1.7m across and composed of man-made cement dating back more than 100 years (Sisson Museum, Shasta CA, personal communication). The bottom of the cistern is lined with cobbles and decaying leaves and plant debris. An L-shaped, metal pipe can be seen at the bottom of the cistern, though its extent and function is unclear. The water depth of the cistern varies from ~85-105 cm, and bubbles emanate from the spring at four primary locations along two sides and the middle of the hexagon.

Preliminary water and gas samples were collected from the Ney Spring in September of 2016 during a three-person scouting trip to the site. Collection vessels for water and gas were provided by Thermochem (Thermochem.com; Santa Rosa, CA) and returned to their analysis facility, in person, within twenty-for hours of sampling for subsequent analysis.

At the time of sampling, the pH of the spring was measured at 12.2, determined using a Fisherbrand pH pen meter. The spring temperature was 18.6°C, and ambient (air) temperature varied between 19-21°C.

Water Collection

Water samples from the Ney cistern were collected using a sterile, disposable 250 ml syringe submerged ~10 cm below the surface to extract water, which was then transferred to containers.

Samples for water chemistry analysis were collected into two 1L Nalgene narrow mouth LDPE containers pre-loaded with 400 ml Milli-Q deionized water. Unfiltered water from the Ney cistern was transferred directly into one of the containers. Water for the second container was passed through a 0.45 micron membrane using a National Scientific Target2™ PES (polyethersulfone) filter attached directly to the syringe.

Unfiltered samples for dissolved gas analysis were collected in sterilized 250 ml Nalgene narrow-mouth glass serum (“Wheaton”) bottles, filled completely with water to avoid air in the sample and sealed using a hand-held crimping device and aluminum crimp top caps with PTFE silicone septa. Bottles, septa, and caps were rinsed three times with Ney water prior to sampling.

Collection of Non-condensable Gases

Gas bubbles emanating from the Ney Spring were collected into two single stopcock valve gas (“Giggenbach”) bottles, each configured with a screw top TFE fluorocarbon plug and a single sampling inlet nipple, prepared following internal Thermochem standard protocols (Thermochem, 2013 unpublished) the day before sample collection. One bottle was dry evacuated. The second bottle was dry evacuated after addition of 25 ml of a preservative prepared as 3:1 ratio of 4M

NaOH and 1M $CdCl_2$ solutions. This preservative, a white slurry, was added to remove CO_2 (through reaction with NaOH to form sodium bicarbonate) and H_2S (through reaction with $CdCl_2$ to form CdS) from the gas phase to allow for more accurate measurement of minor volatile constituents. Sampling followed methods pioneered by Giggenbach and Goguel (1989) and described in ASTM # E 1675-04 (2004).

A gas bubble collection device was created using an HDPE Nalgene funnel (24.8 cm mouth, stem OD 2.3 cm, stem length 6.7 cm) and Nalgene 180 clear plastic ¼-inch ID PVC tubing. The angled tip of the funnel was removed using a razor blade and connected to a straight, polypropylene connector (VWR). The tubing was attached to the other end of the connector and locked in position using a stainless-steel hose clamp.

The entire assembly (funnel + tubing) was submerged in the Ney cistern to remove air from the line. Next, the mouth of the funnel was positioned over a spot of bubble activity; bubbles could be seen rising from the bottom of the cistern, entering the funnel, and moving through the tubing before they exited the open end of the line. The open end of the tubing (still filled with water) was placed just below the top of the water level of the cistern to ensure that no air would enter the line, and then the flow of bubbles into the funnel and through the line was observed. After observing this process for 5-7 minutes, the open end of the tubing was finger-pinched to allow gas to accumulate in the funnel and line. Gradually, the collection assembly was only partially submerged and gas had displaced the water in the funnel to within a few cm of the mouth of the funnel. At this point it was difficult to keep the funnel mouth horizontal to avoid loss of gas.

While one person held the funnel, a second person raised the pinched end of the tubing out of the water and pushed it onto the nipple of the Giggenbach bottle. Once the tubing was attached, the stopcock was opened slowly to allow Ney gas to enter the bottle. Because the bottle was initially under vacuum, it pulled in the gas within the funnel and tubing assembly (the rate will depend on the degree to which the stopcock is opened). Once the pressure of the gas in the sample bottle equalized with that of the funnel/tubing assembly, the stopcock was closed and the tubing was removed from the nipple. This sample collection process was repeated for the second bottle, which contained preservative.

Analysis

Water Chemistry

A comprehensive chemical analysis to determine the cation and anion composition of the Ney Spring samples was performed by Thermochem, Inc., following industry standards and protocols. Trace metal abundances were determined for the filtered Ney water sample diluted with DI water using a PinAAcle 900F Atomic Absorption Spectrophotometer and a Perkin Elmer Optima 7000DV ICP Optical Emission Spectrophotometer. Anion analysis was completed for the DI-diluted, raw water sample using a Thermo Scientific 5000+ Dual-Channel HP Ion Chromatograph with Eluent Generation and a Thermo Scientific 5000+ Dual Channel HPIC with Gradient System. Total alkalinity is reported as the measured value of bicarbonate (HCO_3^-), and the total dissolved

solids was calculated as the sum of the laboratory-reported concentrations of the dissolved constituents.

Gas Chemistry

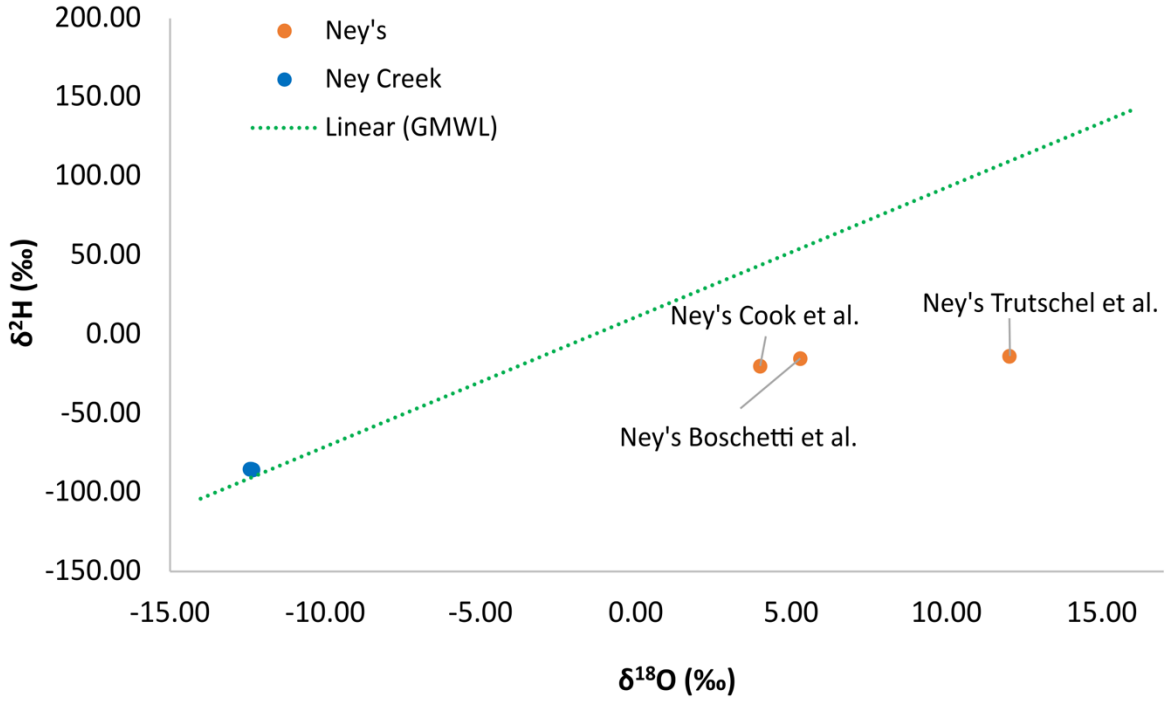
The concentration of dissolved gases in Ney Spring water was determined using the gas-stripping method of partitioning liquid and gas phases after sample collection. To accomplish this, a head space volume was generated through the introduction of an inert carrier gas through the glass sample bottle septum, and the glass bottle was shaken vigorously for five minutes (cf., Capasso & Inguaggiato, 1998; helium was used in lieu of Ar gas, as described by these authors, to facilitate GC analysis methodology). Analytical measurements were made using Agilent GCMS 7890/5975 gas chromatographs with packed, capillary, and mega-bore columns and cryogenic LN_2 cooling, along with thermal conductivity detectors, ECD Flame Ionization Detectors, a purge and trap concentrator, CO_2 and NH_3 Flow Injection Analyzers, and a Thermo Scientific Trace 1310 GC with ECD and TCD detectors.

The two Ney gas samples were processed using Thermochem protocols and analyzed using the same instrument suite listed above. Both samples contained some air contamination. An air correction was made using the assumption that all oxygen gas is atmospheric and air contains 78.09% N_2, 20.95% O_2, 0.93% Ar, and 0.04% CO_2 by volume.

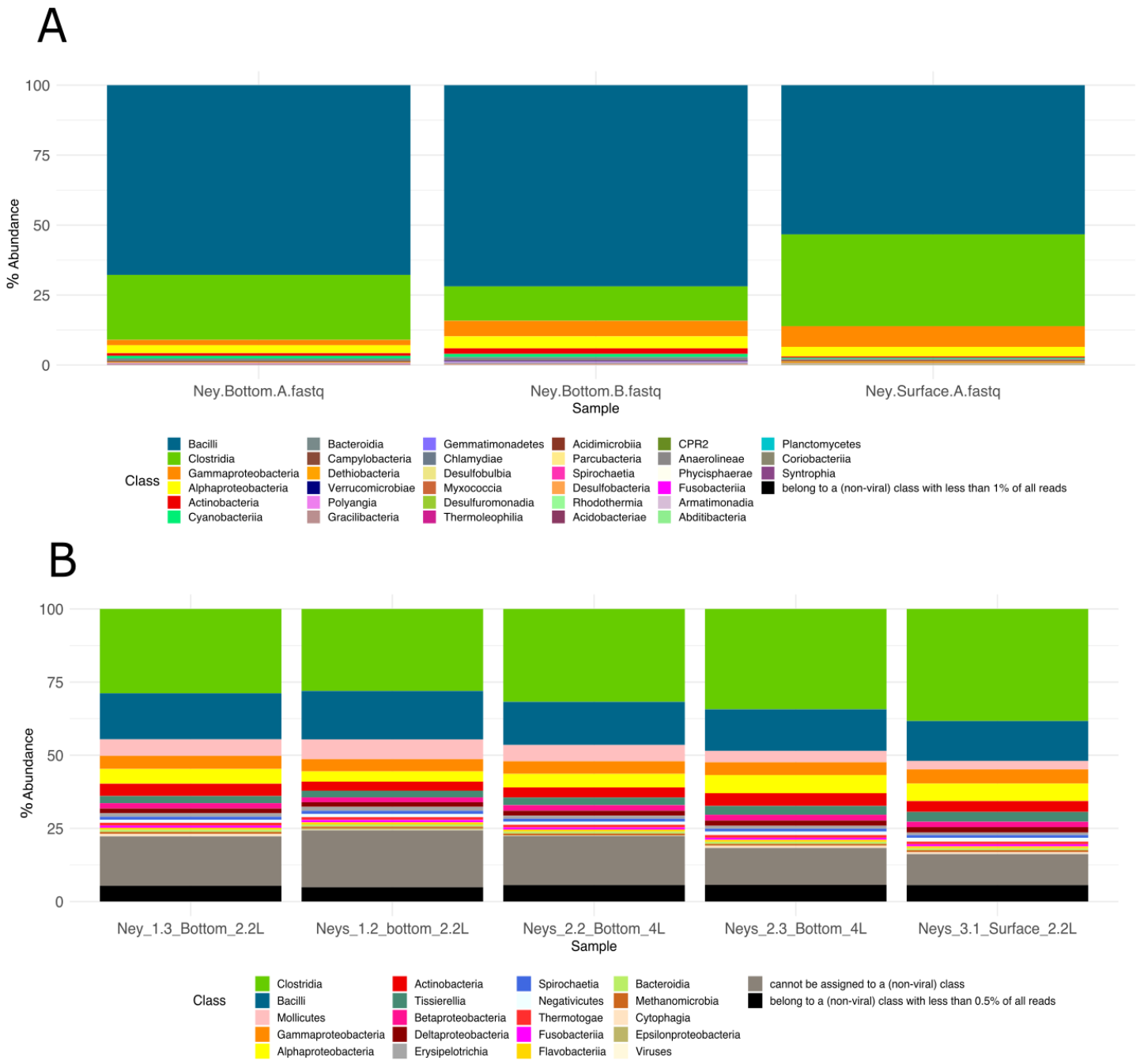
Supplemental Table 1: Table of geochemical data collected and used for Thermodynamics calculations in Geochemist's Workbench

[1]The Nitrate value used for these calculations was obtained via a colorimetric cadmium-reduction method and may be an underestimate, as Ney is filled with highly reducing substances known to inhibit this reaction which we are unable to adjust for.

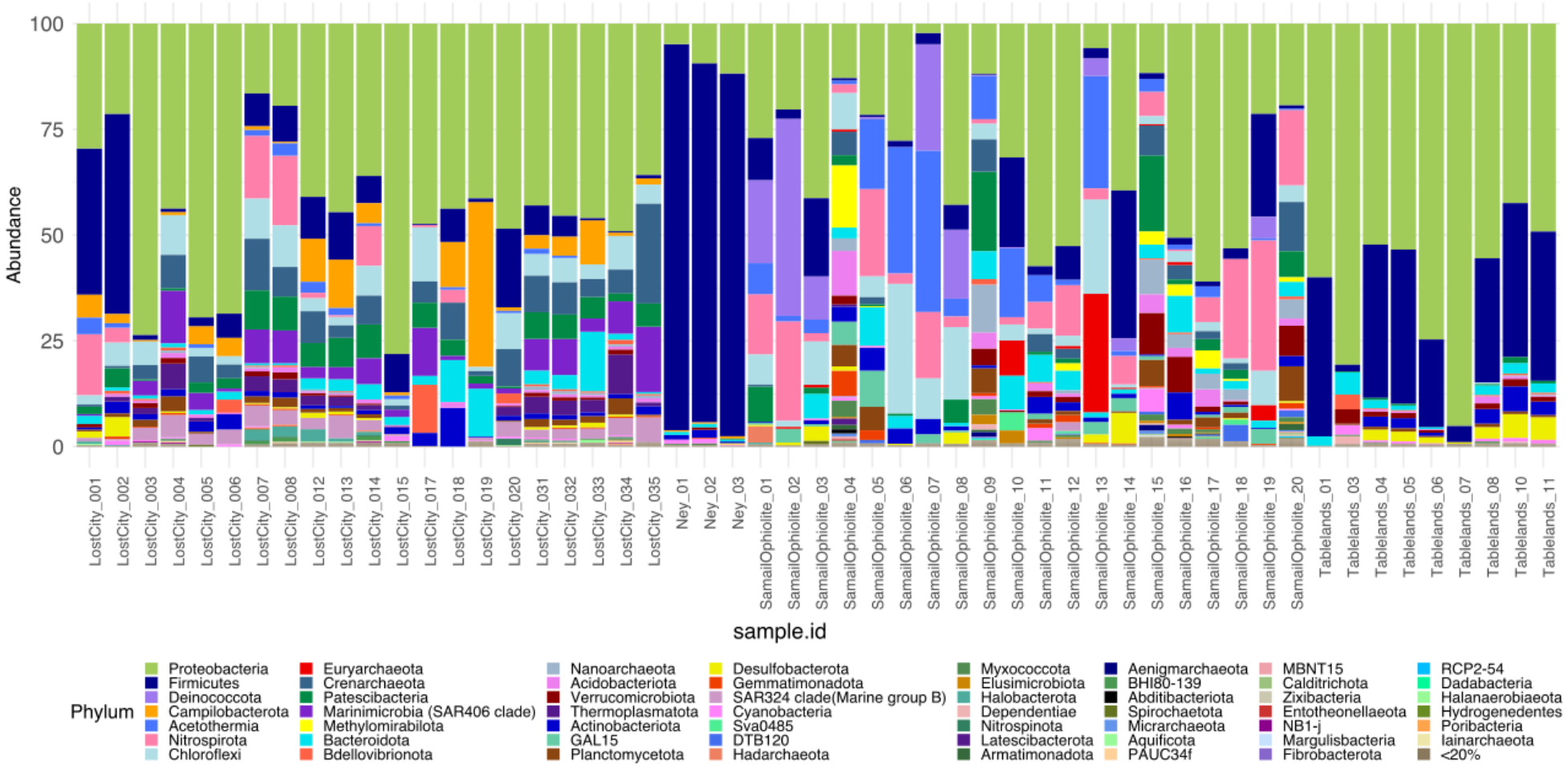
Species	Value	units
H2O	1	free
Sodium (Na+)	10700	mg/kg
Potassium (K+)	139	mg/kg
Calcium (Ca++)	0.161	mg/kg
Magnesium	0.070	mg/kg
Lithium	4.14	mg/kg
Strontium	0.088	mg/kg
Barium	0.315	mg/kg
Iron (Fe++)	0.025	mg/kg
Boron	243	mg/kg
Silica (SiO2)	4150	mg/kg
Aluminum	0.040	mg/kg
Antimony	0.253	mg/kg
Arsenic	0.107	mg/kg
Cesium	<0.4	mg/kg
Manganese	<0.02	mg/kg
Rubidium	<0.4	mg/kg
Chloride (Cl-)	7450	mg/kg
Fluoride(F-)	1.65	mg/kg
Sulfate (SO4--)	373	mg/kg
Total Alkalinity (as HCO3-)	16900	mg/kg
Ammonia (NH3)	35.6	mg/kg
Hydrogen Sulfide (S--)	516	mg/kg
Acetate	500	uM
DO (O2 (aq))	33	ug/L
[1]Nitrate (NO3-)	0.4	mg/L
Carbon Dioxide (CO2)	3200.00	ppm
Argon (Ar aq)	0.63	ppm
Nitrogen (N2 aq)	36.10	ppm
Methane (CH4 aq)	17.50	ppm
Hydrogen (H2 aq)	<1.46E-02	ppm
Ethane (C2H6 aq)	0.00	ppm
Lab pH (units) (H+)	12.0	pH
temperature	25	C
TDS (Calculated)	39900	mg/kg



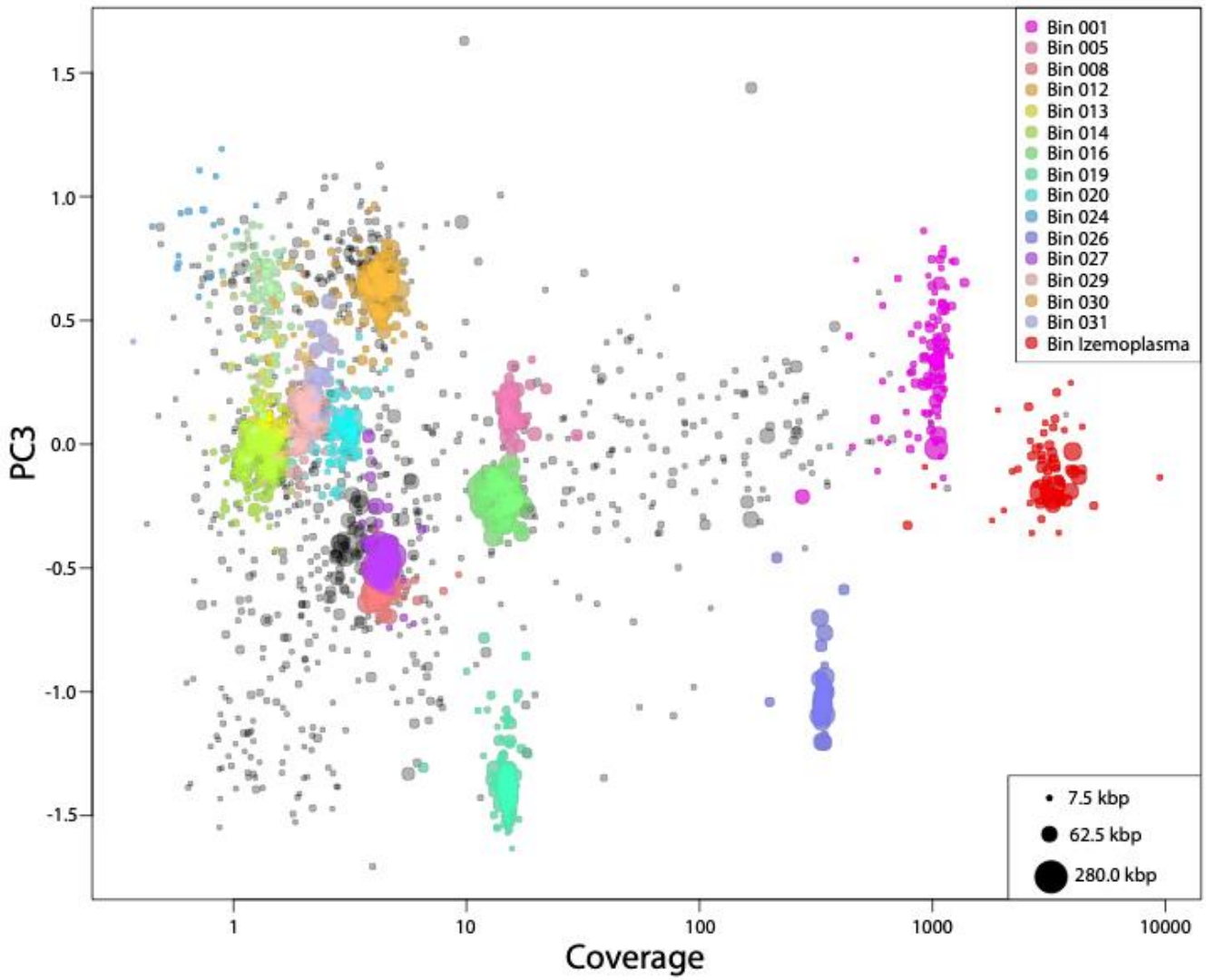
Supplementary Figure 1: Water isotopes of Ney Springs vs. Global Meteoric Water Line



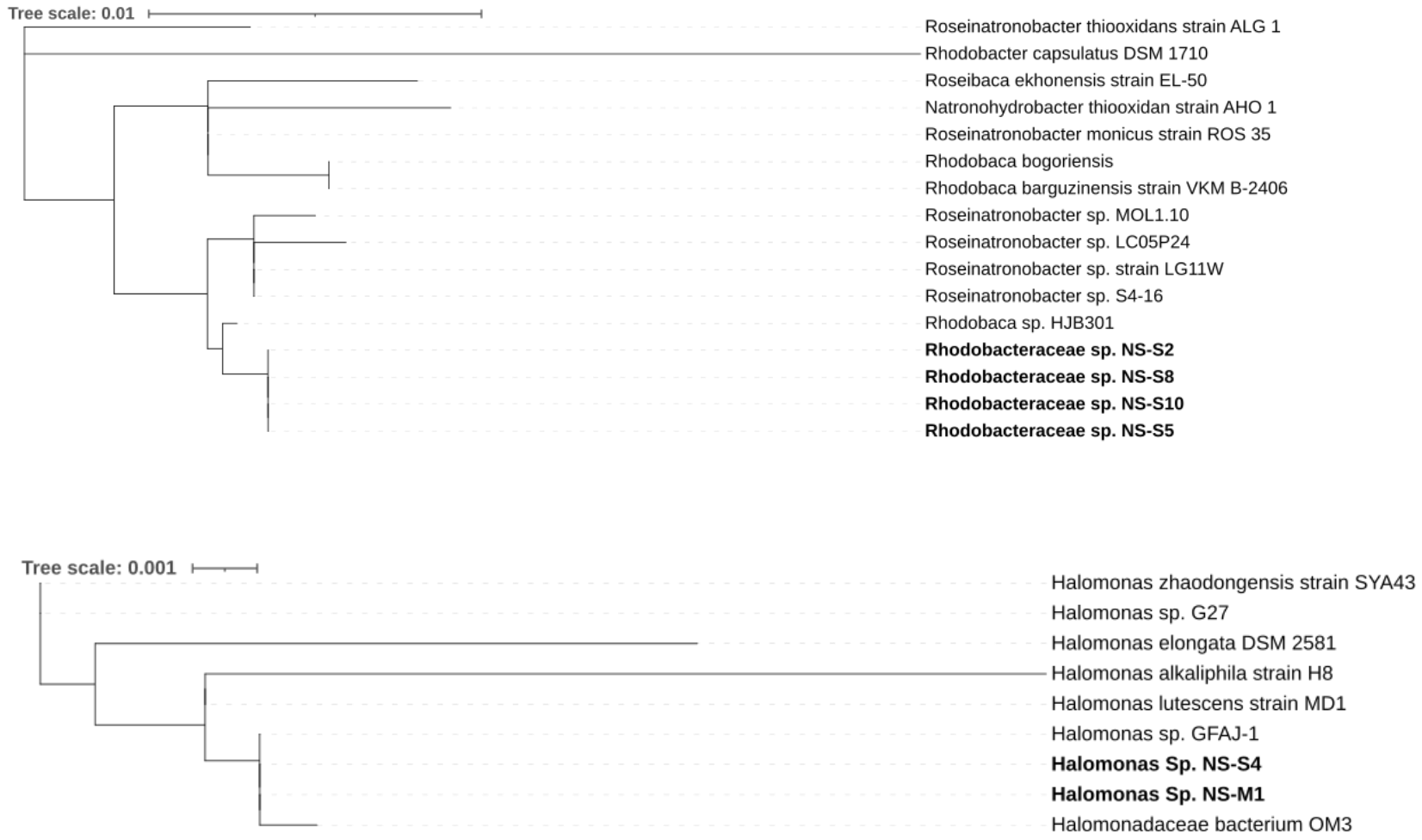
Supplemental Figure 2: Taxonomic bar chart of Ney Springs microbial community as classified at the Class level. (A) 16S rRNA amplicon sequences from three samples as classified by SILVA naive Bayesian classifier (B) Metagenome contigs as classified by Kaiju. Counts were transformed to out of a percentage per sample to normalize data.



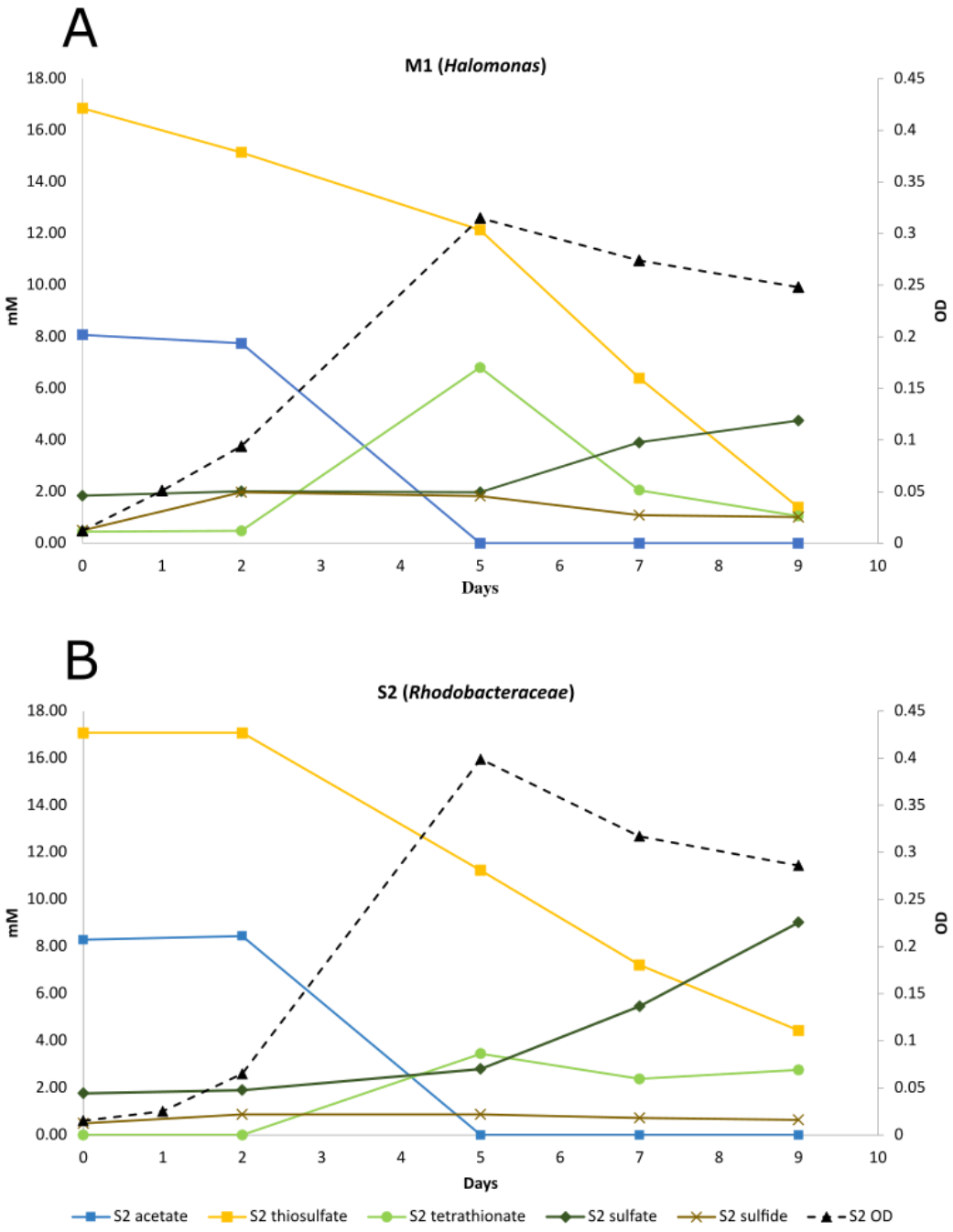
Supplemental Figure 3: Taxonomic bar chart of Phylum level distributions among all serpentinizing system samples used in the community comparison analysis. Samples are listed numerically here for clarity, but a complete list of original sample terminology from their respective system can be found in **Supplemental data 3.**



Supplemental Figure 4: kmer content vs. abundance. The y-axis is the third principle component of the tetranucleotide frequency with the x axis corresponding to read coverage. The size of the points corresponds to the length of the contig, with only contigs greater that 7.5kbp plotted.



Supplemental Figure 5: Trees of *Halomonas* and *Rhodobacteraceae* isolates. Isolates in bold compared to most closely related sequences and type strains for family (*Rhodobacter capsulatus* and *Halomonas elongata*).



Supplementary Figure 6: Usage of thiosulfate and growth curve of M1 (*Halomonas sp.*) and S2 (*Rhodobacteracea sp.*) Initial concentrations in media were as follows: 15 mM Thiosulfate, 10 mM acetate, and 2 mM sulfate. *n*=1 for each data point.

Supplemental Data 1: List of AA bait sequences used in Metagenome gene search (Found as separate file)

Supplemental Data 2: Excel sheet of all 16S sequences used in pivot table form and with a complete sequence list. Includes illumina 16s data and collective serpentinizing system sequences. (Found as separate file)

Supplemental Data 3: Excel sheet on Shannon and Simpson's diversity index values for each individual sample as well as metadata for what the original sample location was deemed in their respective papers. (Found as separate file)