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

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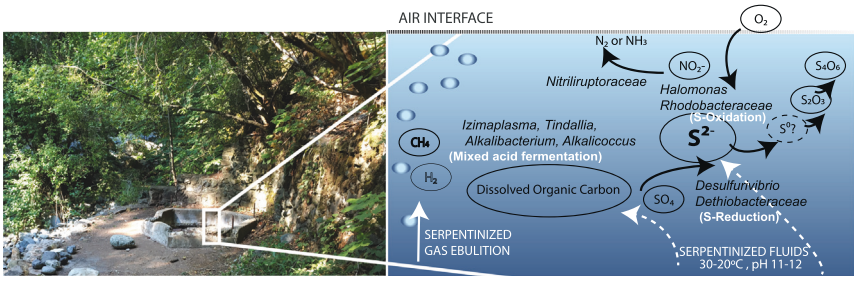
HIGHLIGHTS

- The specific chemistry of serpentinized fluids can vary based on water chemistry and local geology.
- Differences in the geochemistry of these serpentinizing system fluids shapes the endemic microbial communities.
- Ney Springs is a geochemically distinct serpentinizing system due to high amounts of sulfide, methane, and ammonia.
- The Ney Springs microbiome is dominated by poorly characterized firmicutes undetected in other serpentinized fluids.
- Metagenomic and isolate metabolic activity evidence highlight the role of sulfur within this system.

GRAPHICAL ABSTRACT

Proposed microbial metabolic interactions at Ney springs; a terrestrial serpentinizing system.



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ABSTRACT

Ney Springs, a continental serpentinizing spring in northern California, has an exceptionally high reported pH (12.4) for a naturally occurring water source. With high conductivity fluids, it is geochemically more akin to marine serpentinizing systems than other terrestrial locations. Our geochemical analyses also revealed high sulfide concentrations (544 mg/L) and methane emissions (83% volume gas content) relative to other serpentinizing systems. Thermodynamic calculations were used to investigate the potential for substrates resulting from serpentinization to fuel microbial life, and were found to support the energetic feasibility of sulfate reduction, anaerobic methane oxidation, denitrification, and anaerobic sulfide oxidation within this system. Assessment of the microbial community via 16S rRNA taxonomic gene surveys and metagenome sequencing revealed a community composition dominated by poorly characterized members of the *Izemoplasmatales* and *Clostridiales*. The genomes of these dominant taxa point to a fermentative lifestyle, though other highly complete (>90%) metagenome assembled genomes support the potential for organisms to perform sulfate reduction, sulfur disproportionation and/or sulfur oxidation (aerobic and anaerobic). Two chemolithoheterotrophs identified in the metagenome, a *Halomonas* sp. and a *Rhodobacteraceae* sp., were isolated and shown to oxidize thiosulfate and were capable of growth in conditions up to pH 12.4. Despite being characteristic products of serpentinization reactions, little evidence was seen for hydrogen and methane utilization in the Ney

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Springs microbial community. Hydrogen is not highly abundant and could be consumed prior to reaching the spring community. Other metabolic strategies may be outcompeted by more energetically favorable heterotrophic or fermentation reactions, or even inhibited by other compounds in the spring such as ammonia. The unique geochemistry of Ney Springs provides an opportunity to study how local geology interacts with serpentinized fluids, while its microbial community can better inform us of the metabolic strategies employed in hyperalkaline environments.

1. Introduction

Though energy captured from sunlight through photosynthesis is the predominant driver of life on our planet, the potential for geologically driven reactions to fuel microbial metabolism has important implications for pre-photosynthetic life and life elsewhere in our solar system. Serpentinization, which is the hydration and subsequent oxidation of iron and magnesium-rich minerals such as olivine and pyroxene that are often the dominant constituents of ultramafic rocks, is a pertinent example of a geologic process that has been shown to fuel microbial life. Serpentinization has likely occurred throughout the history of liquid water on Earth (Arndt and Nisbet, 2012) and has either occurred or is occurring on other celestial bodies in our solar system (Martin et al., 2008; Taubner et al., 2020). Serpentinizing systems are viable candidates for the origin of life on Earth as many byproducts of serpentinization have the potential to serve as energy sources when coupled with a terminal electron acceptor; serpentinized fluids mixing with other fluid sources, such as meteoric water, can also form pH gradients that could act as a proto-membrane gradient (Russell et al., 2010). This series of water-rock reactions results in high pH fluids that are rich in hydrogen (H_2) and/or methane (CH_4) (Mccollom and Seewald, 2013), and has been shown to generate globally-relevant contributions of hydrogen in both marine and terrestrial systems (Lollar et al., 2014).

Microbial communities, especially autotrophic ones, within these hyperalkaline (pH 10–12) environments must adapt to physiological challenges, such as maintaining ion homeostasis and low bioavailability of inorganic carbon (e.g. CO_2/bicarbonate) (Schrenk et al., 2013). Though predominantly a subsurface process, migration of the resulting serpentinized fluids to the surface can also result in terrestrial springs. While most serpentinizing fluids often contain ample electron donors for autotrophic metabolisms, terrestrial serpentinizing systems in particular often contend with a lack of terminal electron acceptors (Suzuki et al., 2013). Despite these conditions, distinct and persistent microbial communities have been observed in serpentinizing fluids. The metabolic strategies and physiological adaptations of microorganisms in these environments, however, are still poorly understood.

Previous work on serpentinizing microbial communities has included both marine and terrestrial systems. The study of deep-sea serpentinizing systems, such as the Lost City hydrothermal field along the Mid-Atlantic Ridge, has provided important insights into the geochemical drivers (hydrogen, methane, and sulfate/sulfide) and metabolisms of marine microbial communities (Kelley et al., 2001; Lang and Brazelton, 2020). Notable microbial community members in marine serpentinizing systems include methane producing and/or consuming members of the *Methanosarcinales*, which dominate the archaeal component of the Lost City microbial community and the shallow Prony Bay hydrothermal field (Brazelton et al., 2006; Postec et al., 2015). However, the scarcity of methanogenic substrates at high pH, such as carbon dioxide, poses energetic and kinetic challenges for methanogens living in these environments. While evidence supportive of methanogenesis has been detected in several alkaline environments (Brazelton et al., 2017; Kraus et al., 2021; Sorokin et al., 2015), no cultured taxa have been isolated with the ability to make methane above a pH of 10.2 (Hoehler et al., 2018). Sulfur also likely plays an important metabolic role in the Lost City, as sulfur-oxidizing *Thiomicrospira* and sulfate-reducing *Desulfotomaculum* are dominant taxa observed in the microbial community (Brazelton et al., 2006).

Significant differences between the geochemistry of terrestrial and marine systems reflect the interaction of serpentinized fluids with local geology and source waters (Morrill et al., 2013; Ortiz et al., 2018; Rempfert et al., 2017). For example, terrestrial systems often derive fluids from meteoric water and are typically limited for anaerobic terminal electron acceptors (nitrate, iron, sulfate, etc.) as well as sodium, which can be utilized by alkaliphiles to generate an electron motive force (Schrenk et al., 2013; Suzuki et al., 2013). These chemical differences are reflected in the distinct microbial communities observed at terrestrial sites (Brazelton et al., 2013; Canovas et al., 2017; Suzuki et al., 2013). Aerobic hydrogen oxidizers, such as the eponymous *Serpentinomonas* (a distinct group closely related to the *Hydrogenophaga*), are typically the dominant inhabitants of freshwater serpentinized springs and pools such as The Cedars and Tablelands (Suzuki et al., 2017; Woycheese et al., 2015). Due to the mixing of serpentinized fluids with varying lithologies (i.e. peridotite vs. gabbro), other serpentinizing systems such as the Samail ophiolite, have localized fluid chemistries that are more enriched in nitrate and sulfate (Rempfert et al., 2017). This also impacts the microbial community composition throughout the system, with hyperalkaline peridotite fluids containing more of the heterotrophic *Meiothermus* and alkaline gabbro and peridotite fluids containing large amounts of the nitrifier *Nitrospira* (Rempfert et al., 2017) Similarly, marine deposits have been shown to affect the geochemistry and microbial community composition in terrestrial serpentinizing systems within the Franciscan Subduction Complex, such as wells within the Coastal Range Ophiolite Microbial Observatory (CROMO) and the spring GPS1 at the Cedars in northern California (Morrill et al., 2013; Ortiz et al., 2018). Fluids from both systems contain enhanced ion concentrations and the presence of putative sulfur-reducing taxa such as *Dethiobacter* (Ortiz et al., 2018; Sabuda et al., 2020; Suzuki et al., 2013). While these terrestrial systems have some indicators of marine influence such as elevated sulfate and sodium levels, they still have concentrations that are lower than marine systems by orders of magnitude and this is reflected in differences between the overall microbial community composition of marine and terrestrial settings.

Ney Springs is a serpentinizing spring in Northern California located near the Franciscan Subduction Complex previously studied for its unique geochemistry (Boschetti et al., 2017; Feth et al., 1962; Waring, 1915), though its microbiology has yet to be described. Compared to other terrestrial systems, even others within the Franciscan Subduction Complex, Ney springs has more marine-like characteristics making for a distinct geochemical composition. The spring is located at 41°16′14.0″N 122°19′27.3″W in Siskiyou County, California and was discovered in 1887 by John Ney, who arranged for the spring waters to be collected into a cistern for use in a bath house (Fig. 1). In 1915, the U.S. Geological Survey described the spring as saline, having caustic alkalinity, and smelling strongly of ammonia and sulfur (Waring, 1915). In 1961, the unique geochemistry of the spring was proposed to result from mineralization rather than volcanism associated with Mt. Shasta (Feth et al., 1961). However, the discovery of serpentinization along California's coastline further shed light on the source of Ney Springs fluids (Barnes et al., 1967). Ney Springs has consistently been measured at pH > 11 (Feth et al., 1962; Mariner et al., 2003) and is among the highest pH values measured in serpentinizing systems (Table I). Here we report on the distinct geochemistry and microbial community present at Ney Springs, as assessed through 16S rRNA gene amplicon and metagenomic surveys. We propose metabolic strategies utilized by these organisms based on geochemistry, thermodynamics, and metagenomic evidence. We also compare the geochemistry and microbial community of Ney Springs to other well-characterized terrestrial and marine serpentinizing systems to highlight the unique nature of this system. Finally, we assess the physiology and pH tolerance of two isolates from the spring, a *Rhodobacteraceae* sp. and *Halomonas* sp., that are capable of oxidizing sulfur species in hyperalkaline conditions.

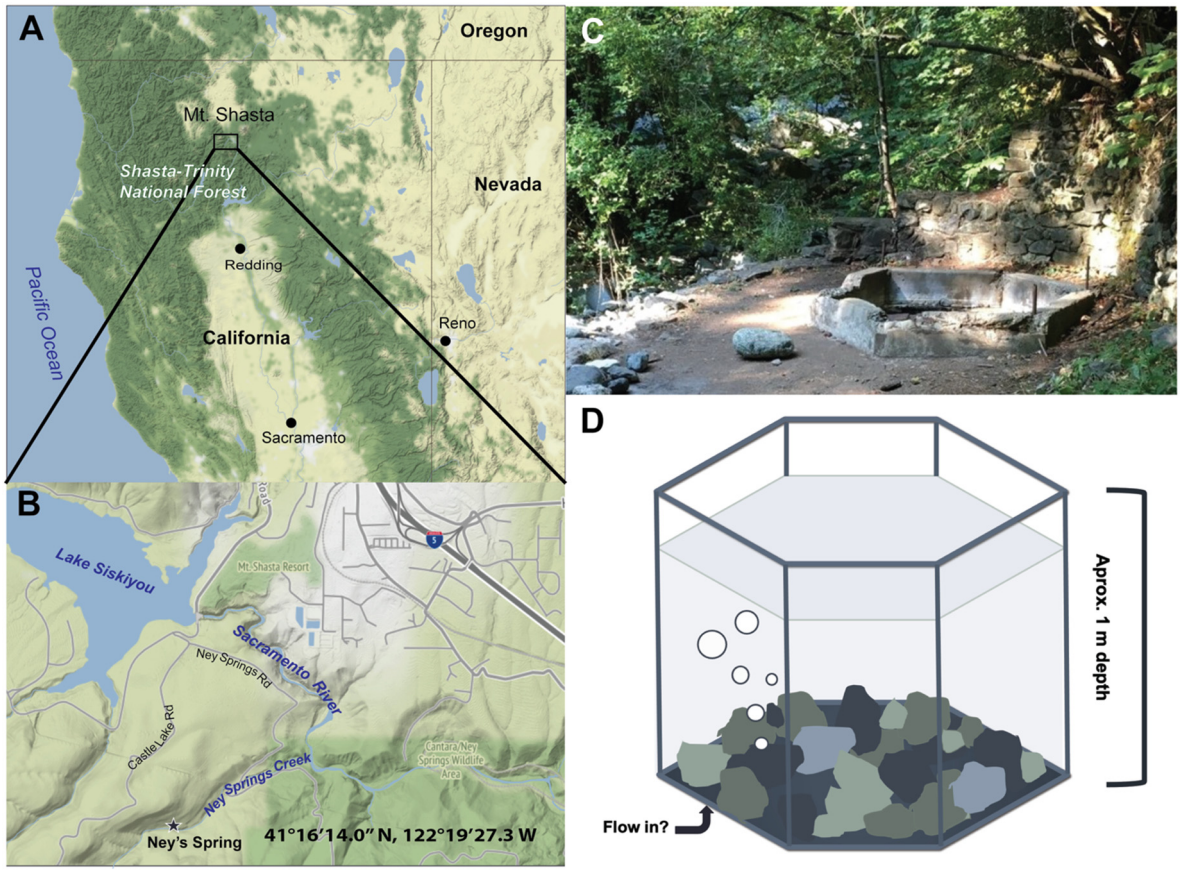


Fig. 1. Geographic location and diagram of Ney Springs (A) Location of Ney Springs in Northern California, USA at 41°16′14.0″N 122°19′27.3″W (B) Location of Ney Springs within Mt. Shasta-Trinity Forest near the resort town of Mt. Shasta in Siskiyou County (C) Photograph of the Ney Springs cistern, which sits approximately 3 m up the bank of the Ney Springs Creek in the remains of a historic site (D) Schematic of the cistern.

2. Materials & methods

2.1. Field sampling and geochemical analyses

Dissolved oxygen (DO) and major ions such as S_2^-, NO_3^- and Fe^{2+} were quantified in the field with a HACH (Loveland, CO, USA) portable spectrometer using standard methods downloaded from HACH.com (methods 8316, 8311, 8171, and 8146 respectively). A Mettler-Toledo (Columbus OH, USA) multi-probe was used to measure pH, conductivity, total dissolved solids (TDS), and oxidation-reduction potential (ORP) in the Ney cistern and nearby Ney Springs Creek. Fluid samples were collected from within 20 cm of the air-water interface (surface) or within 20 cm of the sediment (bottom) portion of the cistern via a modified miniature van Dorn sampler (Wildco, Yulee FL, USA). Using an autoclaved 500 mL Nalgene vacuum filter unit, approximately two liters of water was filtered through a 0.2 μm polycarbonate membrane filter (47 mm diameter, Millipore, Tullagreen, Carrigtwohill Co. Cork, IRL) to collect biomass on three filters (two bottom and one surface) for 16S rRNA analysis. These were frozen on dry ice and stored at −80 °C until extraction. Samples for metagenomics were collected by pumping approximately 2–4 L of fluid using a Geopump™ peristaltic pump (GeoTech, Denver, CO, USA) and autoclaved MasterFlex® PharMed® BPT tubing (Cole-Palmer, Vernon Hills, IL, USA) through 0.2 μm Sterivex™ filters. Three filters were obtained from this: one from 2.2 L of bottom water, one from 4 L of bottom water, and one from 2 L of surface water. Filters were frozen on dry ice after filtration was complete and stored in a −80 °C freezer until extraction. Filtered fluids were collected and used for inorganic anion (F^-, Cl^-, NO_2^-, Br^-, NO_3^-, PO_4^{3-}, SO_4^{2-}) and acetate quantification via a Dionex aquion Ion Chromatograph (IC). Water for dissolved organic carbon (DOC) analysis was collected into acid washed and combusted glass vials, acidified in the field to pH 2, and analyzed on a Shimadzu TOC-V total organic carbon analyzer via non-purgeable organic carbon method. Unfiltered water samples were collected in sterile glass jars directly from the Ney Springs Creek and primary cistern for water isotope analysis.

Preliminary water and gas samples were also collected from Ney Springs in September of 2016 during a scouting trip to the site. Collection

Table I
Geochemistry of Ney Springs compared to the terrestrial Tablelands ophiolite, terrestrial Samail ophiolite, and marine Lost City hydrothermal field.

Geochemistry	The Lost City	Ney Springs	Tablelands ophiolite	Samail ophiolite[h]
pH	9–11[c]	12.1–12.3	10.7–12.3[e]	7.4–11.4
Temperature (°C)	46–81[c]	18	10.4–24.3[f]	30–35.8
Na^+ (mg/L)	11,017–11,155[b]	10,700	N/A	13.6–406.9
SO_4^{2-} (mg/L)	566–1238[b]	373	0.82–1.3[g]	0.96–93.
S^{2-} (mg/L)	2.05–32.07[d]	516–54	N/A	N/A
$CH_{4(aq)}$ (mg/L)	21.01–31.80[c,a]	17.50	0.03–0.38[e]	0.16–36.9
$H_{2(aq)}$ (mg/L)	2.21–14.39[c,a]	<0.01	0.06–1.20[e]	0.18–2.9
Cells/mL (fluid)	4×10^4–4×10^{5}[c,a]	1.4×10^4	1×10^4–2.2×10^{5}[f]	1.16×10^5–7.28×10^5

[a] Sample ranges reported for the Lost City are from fluid samples only and not those containing carbonates.
[b] Kelley et al., 2005.
[c] Brazelton et al., 2006.
[d] Schrenk et al., 2004.
[e] Szponar et al., 2013.
[f] Brazelton et al., 2013.
[g] Morrill et al., 2013.
[h] Rempfert et al., 2017.

vessels and protocols for water and gas were provided by Thermochem (Thermochem.com; Santa Rosa, CA) and samples were returned to their analysis facility, in person, within 24 h of sampling for subsequent analysis. Briefly, both unfiltered and filtered water samples were collected, with filtered samples being passed through a 0.45 μm polyethersulfone in line filter (25 mm, Whatman, Little Chalfont, Buckinghamshire, UK) prior to storage in sterile LDPE containers. Unfiltered samples for dissolved gas analysis were collected in serum vials and filled completely with water to avoid air in the sample, then sealed with crimped aluminum caps containing PTFE silicone septa. Gas bubbles emanating from Ney Springs were collected into two single stopcock valve gas ("Giggenbach") bottles prepared following internal Thermochem standard protocols (Thermochem, 2013 *unpublished*) the day before sample collection. Sampling followed previously described methods (ASTM International, 2004; Giggenbach and Goguel, 1989), and a complete description of sampling procedures can be found in Supplemental Methods 1.

A comprehensive chemical analysis to determine the cation and anion composition of the Ney Springs samples was performed by Thermochem, Inc., following Environmental Protection Agency (EPA) and American Society for Testing and Materials (ASTM) standards and protocols. The concentration of dissolved gases 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 helium through the septum, and the glass bottle was shaken vigorously for 5 min to facilitate analysis methodology (Capasso and Inguaggiato, 1998) on a Agilent GCMS 7890/5975 and Thermo Scientific Trace 1310 gas chromatograph. As 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.

2.2. Cell enumeration

Fluids from the spring were preserved in a 1:1 ratio with 200 proof ethanol for cell enumeration (Kniggendorf et al., 2011). 40-mL volumes of these fixed cells from two surface samples were filtered onto 0.1 μm black polycarbonate filters (25 mm diameter, Osmonics Inc. Minnetonka, MN, USA) in the lab. Filters were stained with SyBR green nucleic acid stain prior to cell counts. Using a Nikon ECLIPSE TI-E inverted microscope, 30 preset locations with a field of view measuring 118 μm × 118 μm were counted and averaged. This averaged value was then scaled to the total filter size to determine the approximate number of cells per filter and in turn the number of cells/mL.

2.3. Thermodynamic calculations

Species activity, Gibbs free energy, and balanced reactions were calculated using Geochemists Workbench, Aqueous Solutions LLC (Bethke et al., 2021). Aqueous species concentrations were populated from values collected from this work, a summary table of which can be found under Supplemental Table 1. Activity coefficients were determined by extended Debye-Hückel (Bdot) equation. Activities were then applied to a balanced equation in the Rxn program to determine ΔG, or were determined using the methods provided in Canovas et al., 2017 (Canovas et al., 2017) which used the formulas: $\Delta_r G = \Delta_r G^o + RTlnQ_r$ where Q_r is defined as $Q_r = \Pi_i(a_i)^{\mathrm{Vir}}$ and $a_i = m_i\gamma_i$, where activity (a_i) is defined as molality (m_i) times activity coefficient (γ_i). In order to scale energy availability to the limiting reactant as seen in LaRowe and Amend, 2014 (LaRowe and Amend, 2014), energy density was calculated from $\Delta_r G$ using the formula $E_r = \left|\frac{G_r}{\nu_i}\right|[i]$, where $[i]$ represents the limiting electron donor or acceptors and ν_i represents the stoichiometric coefficient of the ith species.

2.4. DNA extraction, sequencing, and analysis of 16S rRNA amplicons

DNA extraction of biomass from the preserved filters for 16S amplicon analysis was done using a Qiagen DNAeasy Powersoil kit with modifications as outlined previously (Suzuki et al., 2013). Samples were then quantified using a Qubit fluorometer (ThermoFisher Scientific, USA) and were sent to Molecular Research (MR) DNA (Shallowater TX, USA) for PCR amplification using the 16S rRNA universal primers 515F/806R as described by the Earth Microbiome Project protocol (Thompson et al., 2017). These were then sequenced by MR DNA (https://www.mrdnalab.com/) using Illumina Mi-seq 300 paired-end sequencing.

Concatenated (515F-806R) raw reads for Ney Springs were received from MR DNA as separate FASTA and qual files. The reads were combined and primers and barcodes were removed using the MR DNA FASTA qual and FASTQ conversion software packages. Ney Springs short read sequences were processed in R using *DADA2* (V 1.14) (Callahan et al., 2016) for trimming, chimera checking, and quality filtering. Qiime 2 was used to generate phylogenetic trees and conduct beta diversity analysis (Bolyen et al., 2019). For comparison with the short reads obtained from Ney Springs, short read (<300 bp) 16S rRNA sequences targeting the V4 region were obtained from NCBI for the Samail Ophiolite (Rempfert et al., 2017), the Tablelands Ophiolite (Brazelton et al., 2013; Dart and Brazelton, 2021), and the Lost City Hydrothermal Field (Brazelton et al., 2022) and processed alongside the Ney Springs reads in the same manner described above. Taxonomy was assigned in *DADA2* for the final unique sequences, henceforth considered amplicon sequence variants (ASVs), using a compatible Naive Bayesian classifier trained using the SILVA_nr99_V138 training set (https://doi.org/10.5281/zenodo.3986799) (McLaren, 2020). Taxonomic bar charts, unifrac PcoA plots, and alpha diversity metrics were generated for the 16S rRNA data sets in Phyloseq (V.1.32.0, Mcmurdie and Holmes, 2013). 16S rRNA gene amplicon sequences are available at the following NCBI BioProject accession numbers: Ney Springs (PRJNA739719), Tablelands (PRJNA672032), and Lost City (PRJNA672129).

2.5. DNA extraction, sequencing, and analysis for metagenomics

To enhance yield of DNA for Metagenomic analysis, DNA was extracted using three rounds of a modified phenol-chloroform extraction method. Filters were incubated with lysozyme (1 mg/mL) in 2 mL lysis buffer (40 mM EDTA, 50 mM Tris-Cl and 0.75 M sucrose, pH 8.0) at 37 °C for 45 min and followed by addition of proteinase K (15 U/mL final) for 2 h at 55 °C. The solution was then subjected to a standard phenol-chloroform extraction. It was noticed after the first proteinase K step that the pH of the final solution remained higher than desired for DNA extraction, presumably from Ney Springs water or minerals with pH buffering capacity remaining on the filters. DNA extractions were performed again as above, but with the additional initial step of adding HCl dropwise until the 2 mL of lysis buffer in the Sterivex™ filters was at pH 8.0 as determined by pH paper. Finally, a third DNA extraction was performed, with an overnight initial incubation with lysis buffer at room temperature. DNA was quantified using a Qubit fluorometer from the three samples and three extraction techniques. In all cases, DNA yields from the first extraction attempt were too low, attributed to the high pH inhibiting enzymatic cell lysis and/or the phenol-chloroform extraction. However, both bottom water samples contained DNA yields high enough (~50 ng total) for sequencing from both the second and third extractions, while the top water sample only had a high enough yield from the third extraction. These five samples were sent to Novogene (https://en.novogene.com/) for Illumina PE 150 sequencing following their Shotgun Metagenomics pipeline for sample QC, library preparation, and sequencing.

Metagenome sequences from all five DNA extractions described above were co-assembled using MEGAHIT v1.2.8 (Li et al., 2015) with k-mers 21, 29, 39, 59, 79, 99, 119, and 141. Metagenome binning was carried out on the Kbase (Arkin et al., 2018) platform using MetaBAT2 (Kang et al., 2019) v1.7 and MaxBin2 (Wu et al., 2016) v.2.2.4 using differential coverage across all five metagenomes. Both sets of Metagenome Assembled Genomes (MAGs) were input into DAS Tool (Sieber et al., 2018) v1.2 which produced a final set of MAGs with higher quality as assessed by CheckM (Parks et al., 2015) v1.0.18 than either the MetaBAT2 or MaxBin2 sets

alone. MAG phylogeny was inferred using the GTDB-Tk v1.1.0 package. The *Tenericutes* MAG was refined manually because the automated binning techniques yielded two low completeness (<40%) MAGs with similar read coverage and GC content that were associated phylogenetically with the *Tenericutes*. These contigs were then combined through a manual approach following the general process described in reference (Albertsen et al., 2013) to generate the final high completeness, low contamination *Tenericutes* MAG. Additional curation was conducted for MAG 31 that was classified as *Gracilibacteria* (Sieber et al., 2019a), as these genomes carry a UGA stop codon reassignment to glycine. Prodigal v2.6.3 (Doug et al., 2010) gene calling software was run on the entire metagenome assembly, and additional potential *Gracilibacteria* contigs were identified by Prodigal's automatic code 4 vs code 11 determination. Predicted code 4 contigs (UGA stop reassignment) were assessed for their likelihood of inclusion into the initial *Gracilibacteria* MAG using GC content and differential coverage. MAGs were annotated by Prokka (Seemann, 2014) v1.13. Metabolic pathway completeness and figure generation were performed using the KEGG-Decoder package v1.2.1 (Graham et al., 2018). Taxonomic classification of raw metagenomic reads was determined using Kaiju v1.7.4 (Menzel et al., 2016) in KBase.

In addition to annotation, a manually curated search method was used to target specific genes in the metagenomes. Predicted protein sequences from the metagenomic assembly were translated and formatted as a BLAST database in Geneious (V2020.2.4) against which bait sequences could be searched using BLASTp. These amino acid bait sequences were obtained from Integrated Microbial Genomes (IMG) (Supplemental Data 1) and included genes encoding for sulfur oxidation (SoxABXYZ), dissimilatory sulfite reductase (DsrAB), dimethyl sulfoxide reductase subunit B (DmsB), polysulfide/thiosulfate reductase (PsrAB), methyl coenzyme M reductase (McrA), particulate methane monooxygenase (PmoA), ammonia monooxygenase (AmoA), ribulose 1,5-bisphosphate carboxylase large subunit (CbbL), respiratory nitrate reductase (NarG), periplasmic nitrate reductase (NapA), NO forming dissimilatory nitrite reductase (NirK), and ammonia-forming nitrite reductase (NrfA). Positive hits in the metagenome were ones that met the criteria of an E-value less than 1×10^{-6} and an alignment with greater than 30% amino acid identities over at least 60% of the length of the bait sequence, as described by Fones and others (Fones et al., 2019). Metagenome data can be found under NCBI BioProject accession number PRJNA739719.

2.6. Microbial isolation and cultivation

A Ney Springs basal medium was designed based on geochemical data and contains, per L of water: 11.7 g NaCl, 0.284 g Na_2SO_4, 0.535 g NH_4Cl, and 0.102 g magnesium chloride hexahydrate ($MgCl_2 * 6H_2O$). The following were prepared as separate stock solutions then added to the basal medium prior to autoclaving at the following concentrations: 1 mM K_2HPO_4, 100 mM $NaCO_3$, 10 mM $C_{10}H_{21}NO_3S$ (CABS), 2 mM CH_3COONa (acetate), and 5 mM NaOH. A pH of 11 was used for liquid media while a pH of 10 was preferred for plates to prevent scorching of the agar (1.5%) during autoclaving. Detailed media instructions can be found at protocols.io (dx.doi.org/10.17504/protocols.io.bqjgmujw).

Environmental samples were collected using sterile disposable loops and scraping alongside the cistern then quadrant streaking for isolation on basal medium plates containing either 62.5 mM methanol, 10 mM acetate/10 mM sulfate, or 5 mM acetate/20 mM polysulfide as electron donors/acceptors for enrichments. Plates streaked from environmental samples were maintained under hypoxic or anoxic conditions at room temperature (20–25 °C) using BD BBL™ GasPak™ jar with Campy™ or anaerobic packs (BD.com, Franklin Lakes NJ, USA) for several weeks until growth appeared. From these plates, colonies were re-streaked onto successive minimal media plates for three rounds until they could be confirmed as successfully isolated with a 16S rRNA sequence obtained via colony PCR. Six of these strains that grew well in aerobic growth conditions and could be preserved in 80% glycerol were chosen for further characterization due to ease of culturing.

Metabolic tests for isolates were confirmed based on the presence or absence of sulfate, nitrate, nitrite, and acetate determined using ion chromatography (IC) (ThermoFisher, Dionex aquion). Sulfide was measured using the Cord-Ruwisch assay (Cord-Ruwisch, 1985). The IC and Cord-Ruwisch methods were used to assess for sulfate reduction, which was quantified from the disappearance of sulfate coinciding with an increase in sulfide. Nitrate reduction was quantified the disappearance of a nitrate peak and subsequent appearance of a nitrite peak on the IC. To test for oxidation of thiosulfate to tetrathionate and eventually sulfate, samples were analyzed using the Cyanolysis assay (Kelly et al., 1969) to measure for the disappearance of thiosulfate, subsequent increase and decrease of tetrathionate, and eventual appearance of sulfate on the IC.

2.7. Isolate sequencing and sequence alignment with 16S rRNA gene survey

Colony PCR was performed using the full length 16S rRNA primers 8F and 1492R, after which products were purified and sent to Genewiz (www.genewiz.com) for Sanger sequencing. The 16S rRNA gene sequences of isolated strains were compared to similar sequences obtained from the 16S rRNA gene survey and metagenome using a MUSCLE alignment in Geneious (Muscle V. 3.8.425, Geneious Prime 2020.2.4) (www.geneious.com). 16S rRNA gene sequences from the isolates were then additionally compared to full length 16S rRNA gene sequences from similar isolates found on NCBI using the SILVA Alignment, Classification and Tree (ACT) service, which uses the SINA aligner (v1.2.11) and RAxML maximum likelihood tree building program (Glöckner et al., 2017). Tree files were further edited using the Interactive Tree of Life web program (Letunic and Bork, 2019).

3. Results and discussion

3.1. The aqueous geochemistry of Ney Springs is consistent over time and distinct from other terrestrial serpentinizing systems

Geochemical data for Ney Springs has been reported for collected samples spanning over a century, starting with a U.S. Geological Survey in 1915. This provides information on the composition and geochemical stability of analytes such as sulfide, sodium, hydroxide, chloride, sulfate, silica, and ammonia over time (Waring, 1915). The reported pH of this system ranges from 10.9–12.4 in the spring cistern and has consistently been declared as one of the highest of all known natural water systems (Barnes, 1972; Feth et al., 1961; Mariner et al., 2003). Our recent analyses show the fluids at Ney Springs contain 18.7–19 g/L total dissolved solids and have a conductivity of 38 mS/cm, making them more akin to seawater (~50 mS/cm) than freshwater. Previously the highest measured conductivity for a terrestrial serpentinizing system was 3.8 mS/cm, which was measured at the Tablelands (P. Morrill, *personal communication*). When comparing our recent measurements to historical ones, sulfur species in particular have remained a consistent notable feature at the spring over time; sulfate (SO_4^{2-}) was initially recorded as 272 mg/L in 1910, 172 mg/L in 1957 and at 267 mg/L in 1958, while sulfide (S^{2-}) was recorded as 264 mg/L, 400 mg/L and 1000 mg/L respectively (Feth et al., 1961). The variation in these measurements may be explained by technique, influence of meteoric water, and/or by changes in microbial metabolic activity over time or season. Notably, sulfide is elevated in Ney Springs when compared to other serpentinizing systems (Table I). Sulfide content is also unusually high at Ney compared to serpentinizing fluids from other locations in geographic proximity that are also associated with similar geology. Specifically, only trace amounts of sulfide (1 μM or 32.07 μg/L) were detected at The Cedars (Morrill et al., 2013), and the highest concentration of sulfide (23.75 μM or 761.66 μg/L; (Sabuda et al., 2020) measured at the CROMO site was approximately three orders of magnitude lower than the levels detected at Ney Springs.

The abundance of sulfide also reflects the anaerobic nature of the spring, though oxygen is detectable at the surface-water interface and was measured at 33 μg/L. Another distinct feature of the spring is the near

constant gas ebullition; measurement of the gas composition of these bubbles revealed them to be comprised of 0.83 atm methane and only 0.02 atm hydrogen by volume, which are consistent with previous gas measurements of the spring measured in 1992 and 1979 (Mariner et al., 2003). In contrast, several pools at The Cedars exsolve gases containing a higher amount of hydrogen compared to methane (e.g. 50.9 atm H_2 to 7.4 atm CH_4 in spring CS1 and 39.2 atm H_2 to 6.5 atm CH_4 in spring BS1) (Morrill et al., 2013). Ney Springs contains 17.4 mg/L of dissolved CH_4 while hydrogen was comparatively low (<0.01 mg/L) (Table I). Dissolved methane in Ney Springs is comparable to fluid samples from Lost City, which contain a greater amount of dissolved methane compared to hydrogen (e.g. marker 7 contains 21.01 mg/L CH_4 compared to 5.54 mg/L H_2) (Brazelton et al., 2006). While hydrogen is much greater within Lost City, similarly low dissolved hydrogen values can be found in other terrestrial systems such as Tablelands (0.06 mg/L in WHC1). The source of the methane at Ney Springs remains unclear as there is evidence for both biogenic or geogenic origins in serpentinizing systems (D. T. Wang et al., 2015), though future investigation into clumped isotopic fractionation patterns (Nothaft et al., 2021; Young et al., 2017) may help resolve this.

With an average DOC of 0.973–1 mg/L, Ney Springs has a comparable DOC content to other exposed pools from terrestrial serpentinizing systems such as Manleluag (0.1–0.42 mg/L), the Tablelands (0.16–2.04 mg/L), and The Cedars (0.02–0.14 mg/L) (Suzuki et al., 2013; Szponar et al., 2013; Woycheese et al., 2015). The water isotopic values of Ney Springs suggest a predominantly non-meteoritic origin deviating significantly from the nearby Ney Springs Creek (Supplemental Fig. 1). Ney Springs is heavily enriched in ^{18}O, which may be explained by slab dehydration and serpentinization of connate waters ("fossilized" seawater) (Boschetti et al., 2017). The distinctive smell of ammonia is also apparent at the spring, though attempts to quantify it in both this work (35.6 ppm) and historic accounts (122–148 ppm) (Feth et al., 1961) are likely inaccurate as the methods used are designed to detect the protonated form (NH_4^+) that is less abundant at higher pH. Though the origins of the ammonia are currently unclear, historic surveys at the spring have drawn on its similarities to connate waters and postulated the high ammonia may result from the fluids interacting with ancient decaying organic matter (Feth et al., 1961; Waring, 1915). Ammonia has also been detected in the Samail Ophiolite at lower concentrations than are seen at Ney Springs (0.03–1.96 ppm) (Canovas et al., 2017) and its origins are equally unclear (Rempfert et al., 2017). Comparatively higher levels of methane, sulfur species, and ammonia highlight some of the differences in the geochemistry of Ney Springs compared to other terrestrial serpentinizing systems. This may offer insight into the metabolic and taxonomic differences observed in the microbial community of this extremely alkaline environment.

3.2. Methane and sulfur could support lithotrophic metabolisms in Ney fluids

Thermodynamic calculations were used to investigate the bioenergetics of microbial metabolisms involving different geochemical species derived from serpentinization, and those quantified at this site. Though organic molecules used as an electron donor in either fermentation or anaerobic respiration are likely to be favorable in this system, the specific composition and bio-availability of DOC are poorly constrained and are the topic of ongoing research. The one exception is acetate, which we were able to quantify directly (500 μM) and use in our thermodynamic modeling. Though the source of acetate is unclear (i.e., fermentation or acetogenesis) its presence provides insight into the favorable energetics of heterotrophic respiration at the site. For example, acetate as an electron donor when coupled with nitrate or sulfate is thermodynamically favorable at Ney Springs (Table II), supporting the potential favorability of these respirations to other organic substrates.

In addition to complex organics, the use of small organic molecules is of interest in these systems, as high amounts of H_2 from serpentinization reactions may drive the production of methane, formate, and short chain organic molecules through Fischer-Tropsch type synthesis (Mccollom, 2013). Methane, in particular is highly abundant in this system, and despite its moderate stoichiometric energy yields, there is ample energy density (energy available per kg water) for anaerobic methane oxidation within Ney Springs (Table II). Aerobic methane oxidation is energy density limited due to oxygen concentrations being extremely low in this highly reduced environment. As described, the source of methane from Ney springs is unelucidated, but based on thermodynamics, both hydrogenotrophic and acetoclastic methanogenesis fall somewhere in between aerobic and anaerobic oxidation of methane with moderate energy densities (Table II). However, these reactions are susceptible to competition by other more favorable reactions that would consume hydrogen or acetate. The same is not true of Anaerobic methane oxidation, which is often associated with serpentinizing systems (Brazelton et al., 2006) (Tiago and Veríssimo, 2013) and has the highest energy density for reactions that would use either methane or sulfate at Ney springs.

The sulfur species sulfide and sulfate serve as important electron donors and acceptors for microorganisms in marine serpentinizing systems such as the Lost City (Brazelton et al., 2006), but these fluid constituents often have limited availability in terrestrial systems. However, on the basis of thermodynamic calculations using geochemical data for fluids from the Samail Ophiolite, sulfide-oxidizing and sulfate-reducing reactions were found to be among the most favorable despite low concentrations (Canovas et al., 2017) and implicit evidence for their use in microbial metabolic activity has been detected in the form of sulfate reduction rates (Glombitza et al., 2021). At Ney Springs, sulfate reduction using methane, hydrogen, or acetate as an electron donor provides only modest energy yields stoichiometrically (per electron) compared to other respiratory reactions using nitrate or oxygen (Table II). However, the increased availability of sulfate compared to oxygen or nitrate in this system results in its greater energy density (Table II). Consequently, sulfate reduction using acetate as an electron donor has the second highest energy density at Ney Springs. In terms of other respiratory processes, it is also important to note that while nitrate is likely limited in terms of inputs to this system (due to the connate origins of these fluids), oxygen is continuously available at the air-water interface and could provide a niche for aerobic or microaerophilic metabolisms.

3.3. Ney Springs 16S rRNA gene surveys reveal a simple microbial community dominated by Izimaplasma spp.

Amplicon sequence analysis revealed that the most abundant ASVs encountered were classified in the genus *Izimaplasma*, which comprised about 52%–70% of the microbial community in each sample (Fig. 2, Supplemental Fig. 2). *Izimaplasma* were first discovered in a methane seep as free-living members of the *Tenericutes*, which lack a cell wall (Skennerton et al., 2016). While many *Tenericutes* are often obligate parasites residing in human or animal guts, members of the *Izimaplasma* have been detected in marine and hypersaline lake sediments (Wang et al., 2020). At the class level, *Clostridia* are also abundant in the Ney Springs samples, as well as in other terrestrial serpentinizing systems (Brazelton et al., 2012; Suzuki et al., 2013; Tiago and Veríssimo, 2013). *Clostridia* belonging to the *Peptostreptococcales-Tissierellales* form the second largest portion of the community, with around 10–28% belonging to this order. Members of this order include *Anoxynatronum* and *Tindalia,* which currently have representatives isolated from alkaline soda lakes capable of amino acid fermentation (Kevbrin et al., 1998; Ryzhmanova et al., 2017). Other notable but less abundant community members are Proteobacteria belonging to the *Halomonas*, *Roseinatronobacter*, and *Dethiobacter* genera, which have been associated with sulfur-cycling in alkaline soda lakes (Sorokin, 2003; Sorokin et al., 2008). Few sequences were detected belonging to *Hydrogenophaga* (i.e., *Serpentinomonas*) in the Ney Springs samples, though members of the genus frequently dominate other terrestrial systems. Less taxonomic evidence exists for methanogenesis in terrestrial serpentinizing systems compared with marine systems, though archaeal sequences related to taxa capable of acetoclastic and hydrogenotrophic methanogenesis as well as methanotrophy have been detected in the Samail ophiolite, The Cedars, Cabeço de Vide, Voltri Massif, and Santa Elena ophiolite (Brazelton et al., 2017; Crespo-Medina et al., 2017; Fones et al., 2020; Kraus et al.,

Table II
Metabolic reactions of interest for Ney Springs. Values and balanced reactions calculated using Geochemist's workbench and organized by decreasing values of energy density (E_r). Energy density is based on the limiting reactant.

Metabolism	Balanced reaction	ΔG_r kJ/mol	ΔG_r kJ/mol e^-	E_r (J/kg H_2O
Anaerobic methane oxidation (ANME) (sulfate)	$CH_{4(aq)} + SO_4^{-2} \rightarrow CO_3^{-2} + S^{-2} + H_2O + 2H^+$	−30.12	−3.77	3.42E+01
Sulfate reduction (acetate)	$CH_3COO^-_{(aq)} + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	−62.24	−7.78	2.76E+01
Nitrate reduction to nitrite (hydrogen)[a]	$NO_3^- + H_{2(aq)} \rightarrow H_2O + NO_2^-$	−255.6	−127.8	1.69E+00
Nitrate reduction to nitrite (acetate)[a]	$CH_3COO^-_{(aq)} + 4NO_3\text{-} \rightarrow 2HCO_3^- + 4NO_2^- + H^+$	−1014	−126.75	1.68E+00
Sulfate reduction (hydrogen)	$SO_4^{2}\text{-} + 4H_{2(aq)} \rightarrow S^{-2} + 4H_2O$	−17.61	−2.2	3.00E-02
Methanogenesis (hydrogen)	$CO_{2\ (aq)} + 4H_{2(aq)} \rightarrow CH_{4(aq)} + 2H_2O$	−40.33	−5.04	2.30E-05
Dissimilatory nitrate reduction to Ammonia (DNRA)[a]	$NO_{3-} + 4H_{2(aq)} + H^+ \rightarrow NH_3 + 3H_2O$	−496.9	−124.23	6.20E-07
Nitrate reduction to nitrogen gas (Hydrogen)[a]	$2NO_3^- + 2H^+ + 5H_{2(aq)} \rightarrow N_2 + 6H_2O$	−471.6	−47.16	2.90E-07
Anaerobic methane oxidation (ANME) (nitrate)[a]	$8NO_3^- + 8H^+ + 5CH_{4\ (aq)} \rightarrow 4\ N_{2(aq)} + 5CO_{2\ (aq)} + 14H_2O$	−714.2	−17.86	1.10E-07
Sulfide oxidation (nitrate)[a]	$5S^{-2} + 8NO_3^- + 8H^+ \rightarrow 5SO_4^{-2} + 4\ N_{2(aq)} + 4H_2O$	−684.1	−85.51	1.10E-07
Nitrate reduction to nitrogen gas (acetate)[a]	$5CH_3COO^-_{(aq)} + 8NO_3^- + 13H^+ \rightarrow 15H_2O + 4\ N_2 + 10CO_{2\ (aq)}$	−466.5	−46.65	4.48E-08
Acetoclastic methanogenesis	$CH_3COO^-_{(aq)} + H^+ \rightarrow CO_{2\ (aq)} + CH_{4(aq)}$	−32.12	−16.06	4.00E-08
Annamox (anaerobic ammonia oxidation)[b]	$NO_2\text{-} + NH_3 + H^+ \rightarrow N_{2(aq)} + 2H_2O$	−190.7	−31.78	1.16E-19
Methanotrophy (aerobic)	$CH_{4(aq)} + 2O_{2(aq)} \rightarrow CO_{2\ (aq)} + 2H_2O$	−270.1	−33.76	2.45E-49
Sulfide oxidation (aerobic)	$S^{-2} + 2O_{2(aq)} \rightarrow SO_4^{2-}$	−251.3	−31.41	2.28E-49
Hydrogen oxidation (Knallgas)	$2H_{2(aq)} + O_{2(aq)} \rightarrow 2\ H_2O$	−74.76	−37.38	1.35E-49
Ammonia oxidation[b]	$NH_3 + 2O_{2(aq)} \rightarrow NO_3\text{-} + H^+ + H_2O$	180.8	30.13	1.64E-49
Nitrite oxidation (aerobic)	$2NO_2^- + O_{2(aq)} \rightarrow 2NO_3^-$	175.1	87.55	3.17E-49

[a] The Nitrate value used for these calculations was obtained via a colorimetric cadmium-reduction method and may be an underestimate, as Ney Springs is filled with highly reducing substances known to inhibit this reaction which we are unable to adjust for.
[b] Ammonia values obtained for this system are likely an underestimate as many of the other dissolved species present in the spring water (e.g. chloride) are inhibitive to this colorimetric method.

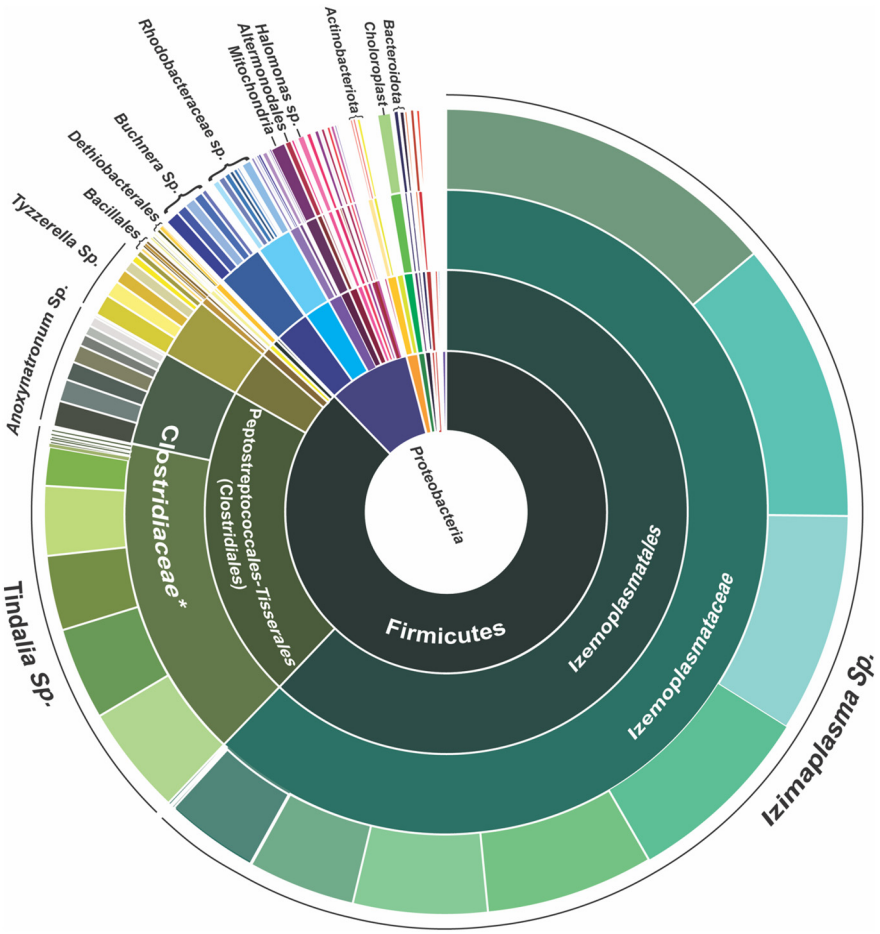


Fig. 2. Sunburst chart of Ney Springs short read 16S rRNA taxonomic data. Chart data illustrate averaged short read (515F-806R) sequences of three samples from the Ney Springs cistern (two from the floor and one from the top near the air-water interface). The rings from center outward correspond to: Phyla, Order, Family, and Species. *Due to unresolved classification for the *Tindalia* and *Anoxynatronum* spp. both the SILVA genetic classification of *Peptostreptococcales-Tisserales* and literature assigned classification of *Clostridiales* are included in the chart.

2021; Nothaft et al., 2021; Rempfert et al., 2017; Suzuki et al., 2013; Tiago and Veríssimo, 2013). Archaea potentially capable of anaerobic methane oxidation have only been observed in only one terrestrial serpentinizing system to date (Tiago and Veríssimo, 2013), and although geochemistry appears very favorable for this metabolic reaction, the known taxa capable of it were not observed in Ney Springs. Overall no archaea were detected within this gene survey, which is a feature shared with fluid samples collected from CROMO (Twing et al., 2017) and Tablelands (Brazelton et al., 2013).

3.4. The Ney Springs microbial community shares taxa with other terrestrial and marine serpentinizing systems but also contains distinct representatives

The microbial community composition of Ney Springs is distinct compared to other terrestrial and marine serpentinizing microbial communities that have been previously characterized (Fig. 3). Both Ney Springs and Tablelands had samples dominated by one genus; Ney Springs community samples were on average 62% *Izimaplasma* spp., while almost 45% of the total sequences from Tablelands belonged to *Hydrogenophaga (*i.e., *Serpentinomonas)*. As a result, Tablelands and Ney Springs data cluster distinctly and apart from Samail Ophiolite and Lost City samples using a weighted, unifrac distance matrix (Fig. 3A). While the abundance of *Serpentinomonas* in Tablelands is consistent with other exposed serpentinizing pools such as Manleluag and The Cedars (Suzuki et al., 2014; Woycheese et al., 2015), *Izimaplasma* has not been detected in any of the other systems analyzed (Supplemental Data 2). Based on unweighted unifrac distance, Ney Springs more closely clusters with the Tablelands and Samail Ophiolite than it does the Lost City (Fig. 3B), despite its more marine-like geochemistry. This trend is likely due to the presence of taxa found only in terrestrial serpentinizing systems that are also observed at Ney, such as *Dethiobacter*, *Rheinheimera*, and *Hydrogenophaga* (Supplemental Fig. 3). Some of the most dominant genera from the Lost City such as *Sulfurovum*, *Halomonas* and *Sulfurospirillum* were found at Ney Springs, but not in the other three terrestrial systems, supporting that the Ney community has marine-like community members. In terms of overall diversity, Alpha diversity indexes for all samples revealed that the Lost City (Shannon 4.57 ± 1.07; Simpson 0.96 ± 0.04) had greater community evenness and diversity when compared to the Samail ophiolite (Shannon 3.74 ± 1.27; Simpson 0.89 ± 0.09), Ney Springs (Shannon 3.03 ± 0.15; Simpson 0.89 ± 0.03) and the Tablelands (Shannon 2.48 ± 0.90; Simpson 0.70 ± 0.20) (values and locations for individual samples listed in Supplementary Data 3). This suggests that Ney Springs has an intermediate diversity compared with other serpentinizing systems. This data demonstrates that the microbial community at Ney Springs appears to have both a distinctive and composite community structure with elements of both marine and terrestrial microbiomes in addition to unique taxa.

3.5. Metagenome results reveal sixteen high quality MAGs with nine belonging to the firmicutes

To investigate the functional potential of the microbial community of Ney Springs, metagenomic sequencing and analyses were performed. Sixteen high quality MAGs were generated from the metagenomic assembly, thirteen of which were ≥90% complete (Table III, Supplemental Fig. 4).

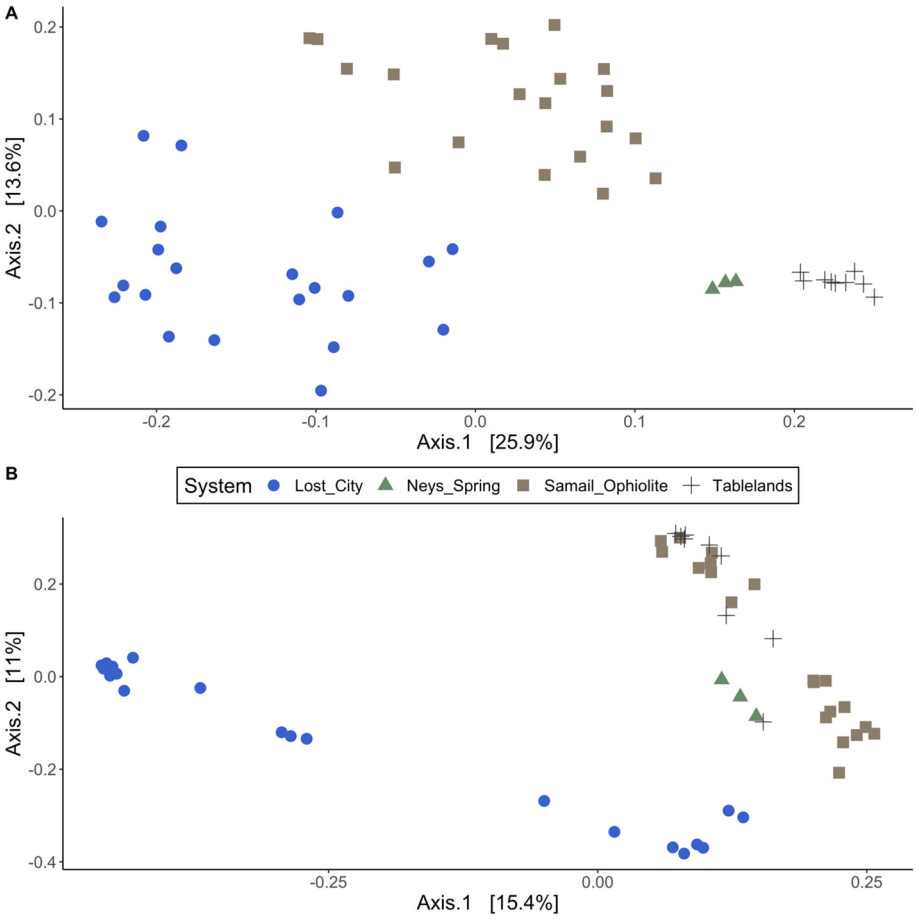


Fig. 3. PCoA plot of 16S samples showing weighted (A) and unweighted (B) unifrac results. Short read (<300 bp) sequences targeting the V4 regions of the 16S rRNA gene were obtained from three well characterized serpentinizing systems (the Tablelands Ophiolite, the Samail Ophiolite, and the Lost City Hydrothermal Field) for comparison to sequences from Ney Springs. Sample locations shown in the legend.

Nine of these belonged to the Firmicutes (MAGs 001, 005, 013, 016, 020, 024, 026, 029 and the *Tenericutes* MAG) while the next most abundant group was the Proteobacteria at four MAGs (MAGs 008, 014, 019, 027). Due to differences in DNA extraction methods and taxonomic databases used for classification (i.e., SILVA taxonomy for 16S rRNA genes vs. NCBI taxonomy for metagenomic sequences classified by Kaiju), care must be taken when broadly comparing the metagenome and 16S rRNA gene survey due to possible DNA extraction and classification biases. While not all MAGs could be directly matched to ASVs because they lacked an assembled 16S rRNA sequence, the six MAGS that did contain 16S rRNA sequences could be matched (>99% similarity) to an ASV found within the gene survey. The *Tenericutes* MAG (*Izemoplasmatales* sp.*)* was 99.7% similar to the most abundant ASV in the gene survey which belonged to the genus *Izimaplasma*. MAG 001 (*Tindalliaceae* sp.) was 99.3% similar to an ASV belonging to next most abundant genus, the *Tindallia*. The remaining MAGs with corresponding ASV matches were MAG 012 (*Nitriliruptoracea* sp.), MAG 005 (*Dethiobacteraceae* sp.), MAG 031 (*Gracilibacteria* sp.) and MAG 019 (*Rhodobacteraceae* sp.). Though MAG 014 (*Halomonas* sp. GFAJ-1) did not contain a 16S sequence, we were able to link our isolated *Halomonas* sp. (also closely related to GFAJ-1) to the ASVs as it was a direct match at the 16S level.

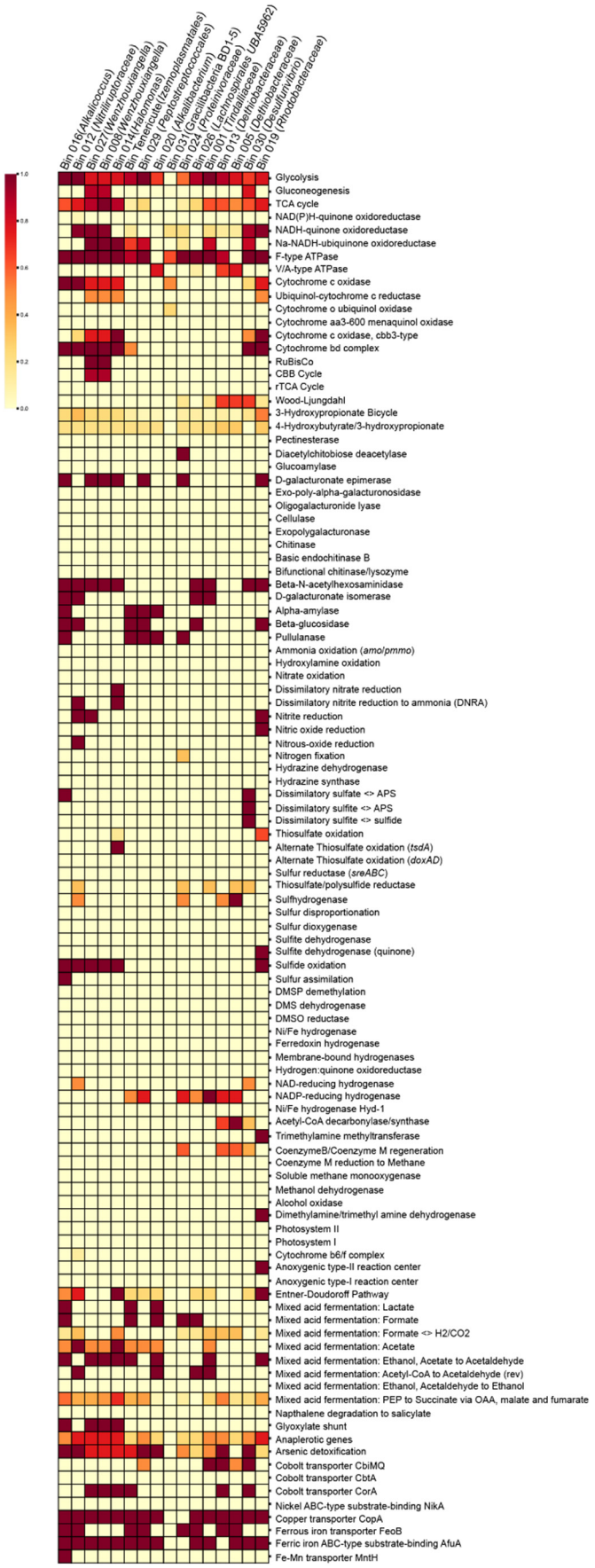
3.6. Metagenome points to a microbial community with metabolisms supported largely by fermentation reactions

Annotation of predicted protein sequences encoded by the MAGs indicated that the majority of the community engages is likely to engage in heterotrophy (Fig. 4). Several MAGs belonging to the *Clostridia* and *Bacilli* classes such as MAGs 001(*Tindallia*ceae sp.), 020(*Alkalibacterium* sp.), 026 (*Lachnospirales* sp.*)*, 029 (*Peptostreptococcales* sp.) and the *Tenericutes* MAG (all 90–97% complete), lack a complete TCA cycle in addition to missing several genes associated with oxidative phosphorylation (e.g., cytochrome *c* oxidase or NADH quinone-oxidoreductase). These same MAGs all contain a complete or mostly complete glycolysis pathway and appear to utilize many forms of mixed acid fermentation for conversion of pyruvate to either acetate, formate, ethanol, and/or lactate. Regarding MAGs corresponding to the most abundant taxa detected in the gene survey, KEGG Orthology (KO) annotation of the *Izemoplasmatales* MAG (*Tenericutes*.bin; 99% complete, Table III) predicted simple sugar utilization similar to other isolates in the order (Skennerton et al., 2016). This MAG contained genes to metabolize glucose, fructose, galactose and ascorbate. As for MAG 001 (*Tindalliaceae* sp.), a complete *hndABCD* operon coding for an NADP-reducing [FeFe] hydrogenase was detected; this enzyme is similar to a [FeFe] hydrogenase that has been characterized as an electron-bifurcating hydrogenase in *Desulfovibrio fructosovorens* (Kpebe et al., 2018). Notably this was the only hydrogenase detected in this metagenome.

Limited evidence for enzymes capable of the breakdown of complex carbohydrates (e.g., cellulase, chitinase, glucoamylase) were detected using KEGG (Fig. 4), but there were several instances of the enzymes for processing intermediates formed after the initial breakdown of these compounds such as D-galacturonate epimerase (pectin), Beta-*N*-acetylhexosaminidase (chitin), and Beta-glucosidase (cellulose). MAGs 020 (*Alkalibacterium* sp.) and 026 (*Lachnospirales* sp.) contain several enzymes involved in fermentation of complex materials such as cellulose (Beta-glucosidase), pectin (D-galacturonate isomerase), starch (alpha amylase), pullulan (pullulanase), and/or chitin (Beta-*N*-acetylehexosaminidase), along with several genes for mixed acid fermentation. MAG 029 (*Peptostreptococcales* sp.) contains similar enzymes to the previous two MAGs but does not contain a complete set of genes for any form of mixed acid fermentation (Fig. 4). Breakdown of complex carbohydrate molecules may occur abiotically due to the high pH of Ney Springs; this hypothesis could explain why nine out of sixteen MAGs have one or more of the enzymes needed for further breaking down these smaller molecules, but none of the initial necessary enzymes. Similar hyperalkaline conditions have been shown to assist microorganisms in metabolizing cellulose and soil organic matter in termite and scarab beetle guts (Lemke et al., 2003).

Table III
Kaiju taxonomic classification of Ney Springs Metagenome Data.

Genome	Phylum	Class	Order	Family	Genera	Completeness	Contamination	Contigs	GC	Mb
Ney.bin.012	Actinobacteriota	Actinobacteria	Nitriliruptorales	Nitriliruptoraceae	–	99	6.8	266	0.72	4.42
Ney.bin.030	Desulfobacterota	Desulfobulbia	Desulfobulbales	Desulfurivibrionaceae	Desulfurivibrio	97	0.9	329	0.60	2.29
Ney.bin.026	Firmicutes_A	Clostridia	Lachnospirales	UBA5962	–	90	0.0	35	0.45	1.82
Ney.bin.029	Firmicutes_A	Clostridia	Peptostreptococcales	–	–	95	2.1	91	0.43	3.15
Ney.bin.001	Firmicutes_A	Clostridia	Peptostreptococcales	Tindalliaceae	–	97	3.0	275	0.48	2.65
Ney.bin.005	Firmicutes_D	Dethiobacteria	Dethiobacterales	Dethiobacteraceae	–	92	1.1	57	0.48	1.80
Ney.bin.013	Firmicutes_D	Dethiobacteria	Dethiobacterales	Dethiobacteraceae	–	94	1.6	185	0.50	2.28
Ney.bin.024	Firmicutes_D	Proteinivoracia	Proteinivoracales	Proteinivoraceae	–	65	1.5	571	0.37	1.70
Ney.bin.016	Firmicutes	Bacilli	Bacillales	Salisediminibacteriaceae	Alkalicoccus	98	0.3	56	0.44	3.83
Ney.bin.*Tenericutes*	Firmicutes	Bacilli	Izemoplasmatales	–	–	99	0	97	0.42	1.84
Ney.bin.020	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Alkalibacterium	88	3.6	173	0.42	2.01
Ney.bin.031	Patescibacteria	Gracilibacteria	BD1–5	UBA6164	–	71	1.1	43	0.34	0.89
Ney.bin.019	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	–	96	1.1	168	0.63	3.35
Ney.bin.014	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Halomonadaceae	Halomonas	99	6.1	229	0.54	3.79
Ney.bin.008	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Wenzhouxiangellaceae	Wenzhouxiangella	96	1.2	56	0.63	3.00
Ney.bin.027	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Wenzhouxiangellaceae	Wenzhouxiangella	98	4.6	71	0.62	3.69



3.7. Several genes for oxidation and reduction of sulfur species detected in metagenome

In addition to organic substrate utilization strategies, many of the MAGs also contained genes for the oxidation of sulfur species. MAGs 008 and 027 (*Wenzhouxiangella* sp.), 012 (*Nitriliruptoraceae* sp.), 014 (*Halomonas* sp.), 016 (*Alkalicoccus* sp.), and 019 (*Rhodobacteraceae* sp.) contain a complete or almost complete TCA cycle and genes for sulfide oxidation (*sqr* sulfide: quinone oxidoreductase and *fccB* sulfide dehydrogenase) and/or thiosulfate oxidation (*sox* system or *tsdA* Thiosulfate dehydrogenase). MAG 016 (*Alkalicoccus* sp.) additionally contains the sulfur assimilation genes *sir* (sulfite reductase) and *cysIJ* (sulfite reductase (NADPH)). Other members of the *Alkalicoccus* genus are aerobic heterotrophs commonly isolated from haloalkaline environments (Zhao et al., 2017). MAG 014 (*Halomonas* sp. GFAJ-1) contains *ttr* (tetrathionate reductase) and *cysP* (thiosulfate/sulfate transport substrate binding protein) suggesting the capacity to use sulfur as an electron donor. It contains a complete set of genes for DNRA (*napAB* and *nirBD*) and two cytochrome oxidases supporting the respiration of nitrogen compounds or oxygen. Type cbb3 cytochrome *c* oxidases have a high affinity for oxygen, but may also improve growth under denitrifying anoxic conditions (Hamada et al., 2014). The bd cytochrome *c* oxidase has a high tolerance to sulfide concentrations, supporting the metabolic flexibility of these organisms and adaptation to sulfidic conditions. Similar features can be seen in MAG 019 (*Rhodobacteraceae* sp.) which possessed genes for nitrite reduction (*nirK* or *nirS*), nitric oxide reduction (*norBC*) and the cbb3 and bd type cytochrome *c* oxidases. MAG 012 (*Nitriliruptoraceae* sp.) partially encodes for *hyd* (sulfhydrogenase), *psr/psh* (polysulfide/thiosulfate reductase) and *sqr* (sulfide:quinone oxidoreductase). Additionally, it contains *nirK* (NO-forming nitrite reductase), *nosZ* (nitrous oxide reductase), and *nrfAH* (ammonia forming cytochrome *c* nitrite reductase). The type strain for the family, *Nitriliruptor alkaliphilus*, is a notable obligate alkaliphilic and aerobic heterotroph that specializes in nitrile degradation (Sorokin et al., 2009), though MAG 012 does not appear to contain the nitrile hydratase enzyme necessary for this process. Collectively these data support the potential for either aerobic or anaerobic oxidation of diverse reduced sulfur species.

While sulfur-species oxidation appears more prevalent, several MAGs also encoded for genes associated with reduction of sulfur-species. MAGs 005 and 030 (*Dethiobacteraceae* spp.) both contain a complete glycolysis pathway, but incomplete TCA cycle. MAG 005 (*Dethiobacteraceae* sp.) and 024 (*Proteinivoraceace* sp.) encode for *psr/psh* (polysulfide/thiosulfate reductase) while 005, 013 and 024 partially encode for *hyd* (sulfhydrogenase), a unique sulfur-reducing hydrogenase found in *Pyroccus furiosus* used to reduce S_0 and polysulfide to H_2S (Ma et al., 1993). Currently the only isolate for the family, *Dethiobacter alkaliphilus* contains the *hyd* (sulfhydrogenase) genes, but lacks the key sulfur-reducing genes *sat* (sulfate reduction), *dsr* (sulfite reduction), *psr* (polysulfide reduction), and *sor* (aerobic sulfur oxygenase/reductase). Despite this it has been shown to disproportionate elemental sulfur and polysulfide in vitro (Melton et al., 2017). *Dethiobacteraceae*-like sequences are regularly detected in other terrestrial serpentinizing systems (Tiago and Veríssimo, 2013) (Suzuki et al., 2013) (Sabuda et al., 2020) making these MAGs particularly beneficial for understanding their role in these systems. MAG 030 (*Desulfurivibrio* sp.) contains a complete set of genes for sulfate reduction to sulfide (*sat*, *aprAB,* and *dsrAB*). Interestingly the type strain of the genus, *Desulfurivibrio alkaliphilus*, in culture grows as a sulfide oxidizer and is unable to reduce sulfate despite having a sulfite reductase (*dsrABC*) genetically indistinguishable from sulfate-reducing microorganisms in the same family (*Desulfobulbaceae*) (Sorokin et al., 2008). Alignment of the DsrAB fragment of MAG 030 with a DsrAB database (Müller et al., 2015) showed this it most closely aligned

Fig. 4. Heatmap of Ney Springs metagenome generated using KeggDecoder output. Heatmap ranging from 0 copies of necessary gene(s) (pale yellow) to all copies of necessary gene(s) (dark red).

with the reductive DsrAB of *Desulfotalea psychrophila* of the *Desulfobulbaceae*. However, without further evidence it is unknown whether this organism may be a true a sulfate-reducer or sulfide oxidizer like the type strain. In addition to partially encoding for the aforementioned sulfur-reduction genes *psr /psh* and *hyd*), MAG 024 (*Proteinivoraceae* sp.) also contains *dac* (diacetylchitobiose deacetylase), an important intermediate in a chitin degradation pathway typically found in archaea (Tanaka et al., 2004). Though it is only estimated to be 65% complete (Table III), it appears similar to the only isolate and type strain of the family thus far; *Proteinivorax tanatarense* is a haloalkaliphilic anaerobe capable of using thiosulfate and S_0 as terminal electron acceptor and will use protein/proteinaceous substrates as carbon, nitrogen, and energy sources (Kevbrin et al., 1998). Additional genes associated with sulfur-metabolism were also detected in the metagenome via BLAST using bait sequences. While these were not included in a specific MAG, sulfur oxidation system SoxABYZ was detected in contigs taxonomically classified as *Rhodobacteraceae* and *Thiomicrospira* spp. and a marker for polysulfide reduction (PsrB) was also detected in contigs classified as belonging to Rhodobacteraceae and *Thioalkalivibrio* spp.

3.8. Metagenomic data suggests oxygen and nitrate are favored over sulfate as terminal electron acceptors

Aerobic terminal cytochrome oxidases were most abundant followed by genes involved in denitrification. Interestingly, the most prevalent cytochrome oxidase present in seven of sixteen MAGs is the cytochrome *bd* complex, which is a copper free cytochrome oxidase not inhibited by sulfide (Forte et al., 2016). Given the high sulfide concentrations seen at Ney's the use of the bd cytochromes is likely favored. As aforementioned, oxygen concentrations are low in the cistern but aerobic organisms may be taking advantage of the continuously available oxygen at the water-surface interface (Section 3.1). Nitrogen species are also detected in the cistern, and denitrification genes are found in several MAGs (019, 012, 027), though only MAG 014 (*Halomonas* sp. GFAJ-1) possessed a complete set of genes for denitrification to ammonia/DNRA (*napAB* in combination with *nirBD*). In terms of nitrogen fixation, only MAG 024 (*Proteinivoraceae* sp.) encoded a partial *nif* (nitrogenesase molybdenum-iron protein) sequence. Though thermodynamic and geochemical data suggest ample energy availability for sulfate reduction (compared with nitrogen and oxygen reduction), only bin 030 (*Desulfurivibrio* sp.) contained a full set of sulfate reduction genes (*sat* in combination with *aprAB* and *dsrAB*).

3.9. Sodium and proton using ATPases detected in Ney metagenome

Currently the ATPases employed by serpentinizing system microbes are not well characterized in the literature, with only studies of The Cedars reporting the types of ATPases observed (Suzuki et al., 2017; Suzuki et al., 2014). ATPases are rotary based molecular machines that couple ion transfer (either H^+ or Na^+) across a membrane for ATP synthesis or hydrolysis. They predominately come in two forms, with the F-type ATPase found in most bacteria, and the A/V type ATPase found in some bacteria, archaea, and the vacuoles of eukaryotic cells (Mulkidjanian et al., 2008). In Ney Springs, F-type ATPases are the predominant ATP synthases found within the metagenome. A/V-type ATPases are found in MAGs 005 and 013 (*Dethiobacteracea* spp.) and MAG 020 (*Alkalibacterium* sp.), which are consistent with the ATP synthases found in isolates from those groups (Ishikawa et al., 2009; Melton et al., 2017). ATPase distribution in The Cedars includes F-type ATPases being observed in shallower groundwater communities and A/V-type ATPases completely dominating deeper groundwater (Suzuki et al., 2017). Of particular interest in serpentinizing systems, is the type of ion used by encoded ATPases, as Na^+ is generally predicted to be more favorable in these environments due to a lack of H^+ ions. To predict if the ATPases encoded were H^+ or Na^+ specific, amino acid composition of the *c* subunit (gene *ntpK* for A/V-type and *aptE* for F-type) was examined, as the Na^+ binding sites are distinct from H^+ motifs and highly conserved (Mulkidjanian et al., 2008). This investigation revealed that while all three A/V-type ATPases were likely Na^+ specific, both H^+ and Na^+ F-type ATPases were encoded among the other MAGs. MAGs 001 (*Tindalliacea* sp.), 024 (*Proteinivoraceae* sp.), 029 (*Peptostreptococcales* sp.), and *Tenericutes*.bin (*Izimaplasma* sp.) all contained Na^+ only F-type ATPases. MAG 013 (*Dethiobacteracea* spp.) contained an Na^+ F-ATPase in addition to an A/V-type. The remaining F-ATPases observed bind protons. At present, only *Serpentinomonas* spp. From The Cedars have had their ATPases characterized, and are shown to utilize H^+ for ATP synthesis (Suzuki et al., 2014). While Na^+ driven gradients are almost always used by alkaliphiles for solute uptake and motility, the value of using an H^+ binding ATPase may be in maintaining pH homeostasis rather than ATP generation (Hicks et al., 2012).

3.10. Unique geochemistry of Ney Springs may place selective pressure favoring heterotrophs adapted to high ammonia and arsenic concentrations

Autotrophy is of interest in serpentinizing systems due to the frequent abundance of inorganic electron donors found in these systems and the potential role of these environments in the origin of life on Earth. Most forms of carbon fixation utilize carbon dioxide, which can be limiting in these high pH environments due to the precipitation of carbonates. Additionally, mechanisms of carbon fixation at high pH are not well studied. Evidence of heterotrophy is much more predominant within the metagenome, though some evidence of carbon fixation may be observed. No MAGs were found to contain evidence of a reverse TCA cycle, but MAGs 005 and 013 (*Dethiobacteraceae* spp.), along with MAG 030 (*Desulfurivibrio* sp.) contained a partial Wood-Ljungdahl carbon fixation pathway. The type strain for *Desulfurivibrio, D. alkaliphilus* AHT2^T, as well as the type strain for the *Dethiobacteraceae* family, *Dethiobacter alkaliphilus* AHT1^T, are classified as facultative autotrophs that utilize the Wood-Ljungdahl pathway (Melton et al., 2017, Melton et al., 2016). However, MAGs classified as *Dethiobacteraceae* from haloalkaline environments are suspected to use the reverse Wood-Ljungdahl pathway for syntrophic acetate oxidation (Dyksma et al., 2020; Timmers et al., 2018). MAGs 008 and 027 (*Wenzhouxiangella* spp.) contained a complete Calvin-Benson-Bassham (CBB) cycle in addition to RuBisCO (Fig. 4). The only isolates with a complete genome in this genus are *W. marina* and *Candidatus* W. alkaliphila AB-CW3, neither of which contains a complete set of genes for a carbon fixation pathway (Sorokin et al., 2020; G. Wang et al., 2015). An alkaliphilic *Wenzhouxiangella* sp. has also been shown to be bacterivorous (Sorokin et al., 2020), making the potential for autotrophy in these MAGs quite peculiar. On the other hand, other MAGs show adaptations to suggest the favorability of heterotrophy and even parasitism over autotrophy. MAG 019 (*Rhodobacteraceae* sp.) does not appear to contain a complete set of genes for the photosynthesis pathway, but the presence of an anoxygenic photosystem II gene (*pufLM)* suggests it may follow the same trend of other closely related alkaliphilic *Rhodobacteraceae* species that have evolved to become heterotrophic in exchange for the loss of photosynthetic capabilities (Kopejtka et al., 2017). Meanwhile, MAG 031 (*Gracilibacteria* (BD1–5) lacks even a glycolysis pathway, and members of this candidate phylum are predicted to be dependent on a host bacterium due to their limited metabolic capabilities (Sieber et al., 2019b). An apparent host for MAG 031 is not yet discernable, although future studies will investigate matching surface protein motifs that may indicate its attachment to another member of the microbial community (Sieber et al., 2019b). Lastly, although not assigned to a specific MAG, several copies of the ribulose 1,5-bisphosphate carboxylase large subunit gene (*cbbL*) were detected in the metagenome that were taxonomically classified as belonging to members of *Thiomicrospira* and *Nitrospira* further supporting the potential for autotrophy in this system, despite the predominance of genes for heterotrophic metabolisms.

Another feature seen across the Ney Springs metagenome is an adaptation to arsenic. Arsenic reduction genes for toxicity mitigation (*arsABCR*) are found complete or almost complete in ten of the sixteen MAGs. Using ICP/MS Arsenic was revealed to be elevated within the system (0.107 mg/kg), a feature shared with many alkaline lakes in the region

including Mono Lake (Oremland et al., 2004) Additionally, in despite of geochemical and thermodynamic predictions, none of the MAGs or unbinned portions of the metagenome contained marker genes related to ammonia/methane oxidation (*amoA/pmoA*) or methane production (*mcrA)*. The presence of ammonia in the cistern may explain why, as it is shown to disrupt aerobic methane oxidation and methanogenesis (Bedard and Knowles, 1989; Gallert et al., 1998) and is inhibitory even to most ammonia oxidizers as they primarily use ammonium (NH_4^+) (Lehtovirta-morley, 2018). The added selective pressure of these geochemical constituents may aid in explaining why the microbial community composition is dominated by a few members not detected in other serpentinizing systems.

3.11. Halomonas and Rhodobacteraceae isolates show evidence of sulfur oxidation at high pH

Six strains of heterotrophic bacteria were isolated on minimal media 5 mM acetate/20 mM polysulfide plates under hypoxic conditions. Sequencing of their full length 16S rRNA genes (8F/1492R) showed successful isolation of two strains from the genus *Halomonas* and four strains from the family *Rhodobacteraceae.* Isolate 16S rRNA genes were compared to 16S rRNA sequences found in the amplicon and metagenome surveys. The 16S rRNA genes of the two isolated *Halomonas* strains were 95% similar (i.e., percent identities) to each other and 92–95% similar to other *Halomonas* sequences found in the short read 16S rRNA amplicon survey. MAG 014 was taxonomically classified as *Halomonas* sp. GFAJ-1, which is also the closest relative to both *Halomonas* strains and was originally isolated from Mono Lake (Kim and Rensing, 2012) (Supplementary Fig. 4). The four *Rhodobacteraceae* isolates had greater variation (91–92% similarity) but fell within the variation seen among other *Rhodobacteraceae* spp. in the 16S rRNA amplicon survey (84–99.6%). Additionally, all four *Rhodobacteraceae* isolates' 16S rRNA gene sequences were 96–97% similar to MAG 019 (*Rhodobacteracea*) of the metagenome assembly and were most closely related to an uncharacterized *Rhodobaca* sp. HJB301 isolated from soda soil (GenBank: MT892652.1, 95–97% similarity, Supplementary File 8).

Under anaerobic conditions all four *Rhodobacteraceae* strains were found capable of reducing nitrate to nitrite, while neither *Halomonas* strain could despite evidence in MAG 014 (*Halomonas)* of nitrate reduction genes and the genes for DNRA. Because these MAGs (*Halomonas* MAG 014 and *Rhodobacteraceae* MAG 019) also encoded genes for sulfide and thiosulfate oxidation, the isolates were tested for utilization of thiosulfate under aerobic conditions. As shown by representative strains of the *Halomonas* (strain M1) and *Rhodobacteracea* (strain S2), the isolates were found to oxidize thiosulfate to tetrathionate and eventually sulfate over a nine-day period in the presence of acetate in pH 11.7 minimal media (Supplementary File 9). Both strains were observed taking up acetate and thiosulfate as optical density (OD) increased, but tetrathionate and sulfate were not detected until all the acetate had been used up and OD had begun to decrease. This suggests that while the cells are unable to grow without a carbon source such as acetate, they can continue oxidizing thiosulfate. Chemolithoheterotrophic *Halomonas* and *Rhodobacteraceae* isolates from alkaline soda lakes have been shown to oxidize thiosulfate and sulfide, albeit these previously characterized *Halomonas* strains did not completely oxidize to sulfate and stopped at tetrathionate (Sorokin, 2003). Rather than using thiosulfate as an electron donor, the oxidation of thiosulfate is suspected to act as a supplementary aid for lithoheterotrophy and may be utilized as a stimulant for anaplerotic CO_2 assimilation and ATP synthesis (Sorokin, 2003), both of which are particularly challenging for life in hyperalkaline systems.

To determine a pH range for growth of the isolates, the presence or absence of growth was evaluated at discrete pH conditions between 7 and 12.4. This test revealed that the *Halomonas* strains were capable of growth at pH 7 and up to 12.05, while the *Rhodobacteraceae* strains were capable of growth between pH 9.2 and 12.4. The *Rhodobacteraceae* strains in particular have potential to grow at or above the current record of pH 12.5 (Takai et al., 2001), and more rigorous testing (Sorokin, 2005) is needed to determine the exact limit of their growth. Nonetheless, these isolates are the second microorganisms to be obtained from a serpentinizing system with a pH optimum above 9 (Postec et al., 2015; Rowe et al., 2017; Suzuki et al., 2014). Further characterization of these isolates will provide information on the physiology and metabolic strategies of microorganisms associated with serpentinization, particularly in regard to oxidation of sulfur intermediates such as thiosulfate, polysulfide, and tetrathionate at high pH.

4. Conclusions

Ney Springs is the most marine-like terrestrial serpentinizing system characterized to date. It has unique geochemical features including a high methane concentration relative to hydrogen and the highest sulfide concentration reported for a terrestrial serpentinizing system. The *Izemoplasmatales* MAG and the *Tindalliaceae* MAG that corresponded with the two most abundant genera from the 16S rRNA gene survey are likely to engage in fermentative and/or heterotrophic metabolic reactions. While only sulfide and sulfate were explicitly measured in this system, metagenomic and experimental evidence from cultivars suggests that intermediate sulfur species (e.g. thiosulfate, polysulfide, tetrathionate) may also play a large role in this community. Evidence for utilization of sulfur species was detected in several MAGs corresponding with abundant taxa from the 16S rRNA gene survey. The *Dethiobacteracea* MAGs are suspected to engage in sulfur disproportionation, while the *Halomonas* and *Rhodobacteraceae* spp. isolated were confirmed to oxidize thiosulfate in vitro.

Interestingly, no evidence was detected for methanotrophic or methanogenic microbes, despite the potential energy available for these reactions. The absence of ANME and/or methanogenic lineages also makes Ney Springs distinct from the marine serpentinizing counterparts that have similarly high methane concentrations and ample sulfate. While the aforementioned inhibitory effect ammonia species (NH_3 and NH_4^+) (Section 3.1) have on these particular lineages may explain their absence, the increasing concentration of the more toxic NH_3 species at higher pH may be placing strong selective pressures on the overall microbial community as well (Leejeerajumnean et al., 2000). A total lack of Archaea is noted in the 16S rRNA gene surveys and metagenome from Ney Springs, though these organisms are thought to dominate extreme environments (Valentine, 2007). Although the EMP 16S rRNA primers are designed to be universal, they are often biased against Crenarchaeota, Nanoarchaeota, and many unclassified lineages (Hugerth et al., 2014). Future 16S rRNA gene surveys using primers to target archaea specifically may reveal if this is a true absence or not.

Currently the sources of sulfide and methane in the spring, and whether they are biogenic in nature, remain unclear. It is possible that these molecules are sourced from microbial activity deeper in the subsurface, or that they are a geogenic product caused by the extremely reducing conditions this system offers. Distinguishing between these options is a focus of ongoing work at the site. Additional cultivation and activity measurements will focus on confirming the presence of sulfate reduction in Ney Springs, as both metagenomic and 16S rRNA amplicon data suggest the presence of sulfate-reducing taxa. Other isolate cultivation and characterization efforts will focus on investigating how organisms persist in this extremely high pH environment. This will include further investigations into culturing the dominant community member, *Izimaplasma*, that is unique to this serpentinizing system, and experiments to determine if the isolated *Rhodobacteraceae* and *Halomonas* strains can oxidize a wider variety of sulfur species under varying conditions.

CRediT authorship contribution statement

LT, GC, BK, JB, and AR participated in sample collection and data analysis from Ney Springs. WB, ED collected samples from Table Lands and Lost City and supplied 16S rRNA data for comparative analyses. LT, BK, and JB generated geochemical data. LT performed thermodynamic calculations and 16S rRNA based analyses. GC generated and analyzed metagenomic data. AR and LT are the primary authors of this manuscript, with editorial

comments from GC, JB, and WB. Funding for this work was obtained by AR, JB, and BK.

Declaration of competing interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

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