

ARTICLE

Freshwater Ecology

Synecological response of spring benthic prokaryotes and macroinvertebrates to Paleozoic roof pendant-derived calcium

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Abstract

Despite the ecological significance of desert springs, little is known about relationships between spring hydrogeochemistry and ecology, particularly over multiple trophic levels. Here, we surveyed microbial communities (bacteria and archaea) and benthic macroinvertebrate (BMI) communities in springs that recharge through granitoid bedrock in Owens Valley, CA, to determine whether subtle geochemical differences imparted by metamorphosed roof pendant weathering in the source area affect spring ecosystems. Relative to other springs, roof-pendant-recharged springs were characterized by elevated (1) $\text{Ca}^{2+}/\text{Na}^{+}$, $\text{Ca}^{2+}/\text{Mg}^{2+}$, and divalent/monovalent cation ratios, (2) relative abundance of taxa inferred to be benthic aerobes and prosthecate/stalked bacteria, and (3) abundance and diversity of shredder and collector gatherer BMIs. These BMI feeding groups graze on biofilm communities and stimulate bacterial degradation of particulate organic matter, consistent with extensive bacteria–BMI connections in co-occurrence networks in these springs. Springs not impacted by roof pendants were instead enriched with taxa inferred to be anaerobes, chemolithotrophs, and low-diversity BMI communities with poor

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bacteria–BMI network connectivity. We speculate that excess Ca^{2+} derived from roof pendant weathering plays a key role in biofilm formation on coarse granite substrates, with subsequent trophic interactions between benthic microbial biofilms and diverse BMI shredders/collector gatherers.

KEYWORDS

biofilm, $\text{Ca}^{2+}/\text{Na}^{+}$ ratio, desert springs, ecohydrogeology, Sierra Nevada, synecology

INTRODUCTION

Springs emanate where groundwater encounters geologic features, such as faults or contacts, that direct groundwater to the surface (Meinzer, 1923). A combination of factors determines spring hydrogeochemistry, including the geology of the recharge zone/aquifer, source geomorphology, and climate (Cantonati, Segadelli, et al., 2020). Variable spring hydrogeochemistry, physicochemically distinct microenvironments, and the often isolated nature of springs contribute to the uniqueness and complexity of these ecosystems (Cantonati, Segadelli, et al., 2020; Stevens et al., 2021). Despite their ecological significance, springs are historically understudied, and the connections between spring hydrogeochemistry and ecology are not well understood. Ecohydrogeology is a new, interdisciplinary approach to spring research that emphasizes integration of geology, chemistry, and biology (Cantonati, Segadelli, et al., 2020; Cantonati, Stevens, et al., 2020; Stevens et al., 2021), with an ultimate goal of improving management strategies to protect springs from threats such as groundwater extraction and climate change (Cantonati, Stevens, et al., 2020; Davis et al., 2017). These threats are particularly pronounced for desert springs, which serve as critical habitat in desert systems as they are the predominant freshwater features in this biome (Myers & Resh, 2002; Shepard, 1993). Climate change is predicted to disproportionately affect desert springs by decreasing the magnitude of mountain system recharge that supports their flow (Meixner et al., 2016). Although there is a dire need for integrated ecohydrogeological studies of desert springs, few studies have explored these ecosystems within that framework.

Bacteria and archaea are important in any ecosystem due to their abundance, diversity, and critical roles in biogeochemical cycles (Bodelier, 2011; Lennon & Locey, 2020), yet these communities remain understudied in desert springs. Several studies have focused on bacteria and archaea in desert springs within the Cuatro Ciénegas Basin (Coahuila, Mexico), which are supported by oligotrophic geothermal groundwater (Breitbart et al., 2009; Souza et al., 2006, 2018). These studies revealed diverse water and benthic communities comprised of

Proteobacteria, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, and *Planctomycetes*, among other phyla (Breitbart et al., 2009; Souza et al., 2006, 2018). In these systems, extremely low P content results in a stoichiometrically unfavorable C:P ratio in photoautotrophic benthic mats, limiting grazing by benthic macroinvertebrates (BMIs) (Elser et al., 2005). Limited BMI grazing facilitates the development of microbialites with dense matrices of extracellular polymeric substances (EPS) (Elser et al., 2005), allowing for increased nutrient cycling between colocalized photoautotrophic *Cyanobacteria* and heterotrophic bacteria (Breitbart et al., 2009). Contrastingly, studies of other desert surface water systems have linked microbial diversity to groundwater flow, salinity, and pH, but not nutrient chemistry (Kaestli et al., 2019; Paulson & Martin, 2019; Zeglin et al., 2019). These patterns are consistent with the known importance of salinity and pH in structuring microbial communities across multiple biomes (Fierer & Jackson, 2006; Lozupone & Knight, 2007; Power et al., 2018; Thompson et al., 2017). Nevertheless, the effects of these and other physicochemical factors on microbial community structure and dynamics in desert springs, and their hydrogeological controls, are not well understood.

Comparatively more research has focused on macroscopic organisms in springs, including plants, fish, and BMIs. In desert springs, BMI communities include both insects such as the aquatic larvae of caddisflies, stoneflies, and true flies, and noninsect taxa such as amphipods, gastropods, ostracods, and mollusks (Myers & Resh, 2002; Shepard, 1993). A variety of physicochemical parameters are correlated with BMI community richness and composition in springs, including temperature, salinity, dissolved oxygen, pH, and alkalinity (Barquín & Death, 2006; Glazier, 2012; Myers & Resh, 2002; Seidel et al., 2010). In related aquatic ecosystems, such as rivers and streams, additional factors like substrate particle size (Duan et al., 2009; Quinn & Hickey, 1990) and current velocity (Mérigoux & Dolédec, 2004; Rempel et al., 2000) are also important, as many BMIs prefer specific substrate types, which can be controlled by current velocity. Importantly, BMIs are central to various ecosystem functions and serve as integral members of the food web in aquatic ecosystems (Covich et al., 1999).

Biological communities in aquatic ecosystems are influenced by both top-down (e.g., grazing and predation of lower trophic levels) and bottom-up (e.g., rate of primary productivity or prey availability) processes that work together to shape community structure and food web interactions over multiple trophic levels (Taylor et al., 2015). Microorganisms are important members of the food web in aquatic communities, playing critical roles in primary production and all stages of decomposition of detritus (Battin et al., 2016; Krumins et al., 2013). BMIs can graze microbial biomass, consume other BMIs, or facilitate detritus decomposition and nutrient cycling through feeding or burrowing activities (Covich et al., 1999). As such, individual BMI taxa are assigned to functional feeding groups (FFGs) based on their preferred feeding behaviors: (1) shredders, which consume microbially conditioned plant litter and other coarse particulate organic matter (CPOM); (2) collectors (gatherers and filterers), which feed on fine particulate organic matter (FPOM) colonized by microbes; (3) scrapers, which graze on periphyton; and (4) predators, which capture and ingest live prey (Cummins, 1974; Cummins & Klug, 1979). BMIs can also serve as prey for vertebrates, transferring energy and nutrients to higher trophic levels (Covich et al., 1999). Despite the ecological importance of both microorganisms and BMIs, few studies have explored community ecology and food web interactions over multiple domains in springs within a comparative framework of spring hydrogeochemistry.

Owens Valley is a cold semiarid-to-arid desert valley between the Sierra Nevada and White-Inyo Mountains of California, which contains numerous springs that are recharged predominantly via precipitation in the Sierra Nevada (Hollett et al., 1991). The dominant rock types in the Sierra Nevada are diverse Mesozoic-age granitoid rocks along with a patchy distribution of Paleozoic metasedimentary and metavolcanic roof pendants and septa (Bateman et al., 1965; Moore, 1963). The Paleozoic sedimentary and volcanic precursors of the roof pendants and septa were metamorphosed by the intrusion of granitic plutons during the Mesozoic Era (Hollett et al., 1991; Stevens & Greene, 1999). The metamorphosed rock originally formed a continuous “roof” over the plutons but was subsequently eroded since the late Cretaceous, leaving only patches of the now-metamorphosed roof pendants. The granitoid rocks have an average of 3.3 weight percent (wt.%) CaO and 3.55 wt.% Na₂O (Bateman et al., 1965). In contrast, the CaO concentration in Paleozoic roof pendant rocks, such as marble and calc-hornfels, is >40 wt.% and 19.0 wt.%, respectively, with Na₂O concentrations of 0.2–0.9 wt.% in these same rocks (Bateman et al., 1965).

The objective of our study was to determine whether recharge and rock–water interaction through Sierra

Nevada roof pendants affects spring geochemistry and aquatic communities across multiple trophic levels in springs that emerge thousands of meters downslope from the pendants. To address this objective, we collected ~60 physicochemical parameters, 843,111 bacterial and archaeal V4 16S rRNA gene amplicons, and 2660 individual BMIs from 10 mountain-front springs in the Owens Valley that were partially recharged through the roof pendants or only through granitoid rocks and compared their geochemistry and community ecology.

MATERIALS AND METHODS

Study area and spring sites

The Owens Valley (~1400 m above mean sea level [masl]) is an endorheic basin in the southwestern Great Basin between the Sierra Nevada (maximum elevation 4421 masl) and White-Inyo Mountains (maximum elevation 4344 masl). Average precipitation in the Sierra Nevada is >76 cm/year, whereas much lower precipitation in the Owens Valley (13 cm/year) and White-Inyo Mountains (18–36 cm/year) illustrates the effect of the Sierra Nevada rain shadow (Hollett et al., 1991). Precipitation and surface runoff from the surrounding mountains collect in the Owens River and flow to Owens Lake (dry since 1927). Annually, Owens Valley average temperatures range from 4 to 35°C, which explains its cold arid to cold desert climatic classification (Duell, 1990).

Ten rheocene-type (Stevens et al., 2021) reference springs were sampled at the foot of the eastern Sierra Nevada in the Owens Valley. These springs are broadly distributed from the northern to southern end of the valley and were selected in order to capture geologically heterogeneous recharge zones. Geochemical and microbial samples were collected during March 2016, May 2016, and October 2017. BMI samples and associated aquatic habitat parameters were collected from all of the springs in a single sampling trip in summer 2017. The slope at the spring sources was calculated using ArcGIS from 1/3 arc second digital elevation models. Field notes, sampling dates, and geographic coordinates are included in the data tables 1 and 2 archived at <https://doi.org/10.5281/zenodo.7603891>.

Field measurements and hydrogeochemistry sampling

Physicochemical characteristics were recorded for each spring source concurrently with the collection of microbial samples, as outlined previously (Miller-Coleman et al., 2012). A YSI Professional Plus Quatro multiparameter

probe (YSI, Yellow Springs, OH) was used for measuring electrical conductance (EC), dissolved oxygen, pH, and temperature. Specific conductance (SPC; in microsiemens per centimeter) was calculated by the YSI from: $SPC = EC \times 1.91$, where 1.91 is the temperature coefficient for waters at 25°C. Aquatic habitat parameters (current velocity, water depth, wetted width, bank cover, head cover, and substrate characteristics) were recorded for each spring while BMI samples were collected. Current velocity was measured using a Marsh-McBirney Flo-Mate 2000 in-stream flow meter (Field Environmental Instruments, Pittsburgh, PA). Both current velocity and water depth represent averaged values of five measurements collected at the center of quadrats where BMIs were sampled. Wetted width represents an averaged value of five width measurements along evenly spaced transects perpendicular to the spring channel. Bank cover and head cover were qualitatively estimated. Benthic substrate was characterized in percentages as silt, sand, gravel, cobble, boulder, bedrock, and muck within the BMI sampling area using the Wolman pebble count method. These benthic substrate characteristics represent an averaged value of five measurements of percent substrate present. Quaternary ammonium and 50% ethanol were utilized on sampling equipment between locations to prevent cross-contamination.

Spring water was collected with a Geopump peristaltic pump (Geotech, Denver, CO) directly from the spring source or as close to the source as possible using Masterflex platinum-cured silicone tubing (Cole-Parmer, Vernon Hills, IL) and filtered using 0.2- μ m polyethersulfone membrane Sterivex-GP pressure filters (Millipore Sigma, Burlington, MA). Filtered water samples for measurement of major ions were collected in 250-mL high-density polyethylene (HDPE) bottles that were rinsed three times with NanoPure deionized water (DI), soaked in DI for a minimum of 24 h, and rinsed with filtered spring water three times. Filtered water for trace elements was collected in 60-mL HDPE bottles that were soaked in 10% nitric acid (EMD, Omni Trace) for a minimum of 72 h, rinsed three times with NanoPure DI, and dried in a laminar flow hood with a high efficiency particulate air filter. Four hundred microliters of ultrapure nitric acid (EMD, Omni Trace Ultra) was added prior to fieldwork to prevent precipitation of metals. Unfiltered water samples were collected for tritium (^3H) and radiocarbon (^{14}C) analyses, whereas filtered water samples were collected for chlorine-36 (^{36}Cl) analysis. ^3H , ^{14}C , and ^{36}Cl samples were collected with minimal headspace in 1-L HDPE bottles and the caps were sealed with electrical tape to minimize atmospheric exchange.

Major ions were measured at the New Mexico Bureau of Geology and Mineral Resources Chemistry Lab. Cations were measured using a PerkinElmer Optima 5300 DV

inductively coupled plasma optical emission spectrometry (ICP-OES), according to EPA 200.7. Anions were measured using ion chromatography (IC), according to EPA 300.0. Alkalinity and hardness were measured according to EPA 310.1 and SM 2340B, respectively. Trace elements were measured for most samples at MM Lab (Las Vegas, NV), but the trace element sample for one spring (Lubkin Canyon Spring 2—IES054) was measured at the New Mexico Bureau of Geology and Mineral Resources Chemistry Lab. Trace elements were measured using an Agilent 7500 inductively coupled plasma mass spectrometry (ICP-MS), according to EPA 200.8. Tungsten was measured using an Agilent 7900 ICP-MS system, according to EPA 200.8. ^3H was measured at the Miami Tritium Lab using low-level electrolytic enrichment techniques (<https://tritium.rsmas.miami.edu/>), ^{14}C was measured at the University of Arizona AMS Laboratory (<https://ams.arizona.edu/radiocarbon>), and ^{36}Cl was measured at the Purdue PRIME Lab (<http://www.physics.purdue.edu/primelab>).

Analysis of spring hydrogeochemistry data

Prior to hierarchical clustering analysis (HCA) or plotting of Piper diagrams, milliequivalents/kilogram (meq/kg) major ion data (Ca^{2+} , Mg^{2+} , K^+ , Na^+ , Cl^- , SO_4^{2-} , and HCO_3^-) were normalized by dividing each cation by the sum of all the cations in the sample and each anion by the sum of all the anions to get the final units of % meq/kg. Values for specific conductance and Si included in the HCA were normalized prior to the analysis. Specific conductance was log-transformed initially, and then both the specific conductance and Si values were normalized to a value between zero and one using the R package SuperPCA version 0.3.0 (Li et al., 2020). To identify the hydrogeochemical groups, a HCA was performed using a Euclidean-based dissimilarity matrix of the transformed data with the R package stats version 4.0.3 using Ward's Linkage. To identify major spring water types and geochemical differences between the hydrogeochemical groups, Piper diagrams were generated using Geochemist's Workbench Community Edition 15.0 (<https://community.gwb.com/>). Additionally, a two-sample Student's *t* test was conducted using Microsoft Excel version 2204 on the physicochemical data (units as shown in data table 1 archived at <https://doi.org/10.5281/zenodo.7603891>) to determine which parameters were significantly different between the hydrogeochemical groups. An expanded discussion of the hydrogeochemical framework and analysis is available in another study (Meyers et al., 2022).

Major ion or trace element values below the detection limits (DLs) were set to DL/2 if they were included in an analysis. Analytes with >50% of the concentrations below

the DL were excluded from analyses. DLs and analytical uncertainties are included in data table 1 archived at <https://doi.org/10.5281/zenodo.7603891>.

Analysis of groundwater residence time

Spring waters were dated using a sequential approach using ^3H , ^{14}C , and ^{36}Cl in that order (Gleason et al., 2020). First, ^3H dating was used to identify the youngest spring waters (e.g., the shortest residence times). Samples with ^3H activities >0.80 TU were dated using TracerLPM (Jurgens et al., 2012). A time series of atmospheric ^3H from Modesto, CA, was chosen as the input function to the convolution integral since it represents the closest and most complete times series for the study area. A dispersion model was selected as the response function because it accurately represents mixing of different groundwater flow paths in spring discharge (Jurgens et al., 2012). The lower and upper age constraints in the dispersion model were kept constant at 0 and 400 years, and the dispersion parameter was initialized to 0.50. Because TracerLPM minimizes error as it seeks a solution within the specified boundaries, the analytical error reported for each water sample by the lab was used to assess numerical uncertainty in the model. The results from TracerLPM were compared with residence times calculated using a recharge-weighted, steady-state, backward-in-time tritium mixing model (Gleason et al., 2020) to identify discrepancies in residence times. The raw data, analytical uncertainty, estimated residence time, and numerical uncertainty associated with ^3H dating are reported in data tables 1 and 3 archived at <https://doi.org/10.5281/zenodo.7603891>.

Second, ^{14}C dating was used to date spring water samples having ^3H activities <0.80 TU, and ^{14}C percent modern carbon (pMC) and $\delta^{13}\text{C}$ values suitable for ^{14}C corrections. A graphical method (Han et al., 2012) was used to gain insight into the geochemical reactions affecting the $\delta^{13}\text{C}$ and ^{14}C pMC and to inform the selection of radiocarbon correction models. ^{14}C corrections were completed using Netpath XL (Parkhurst & Charlton, 2008) by (1) inputting the measured $\delta^{13}\text{C}$ and ^{14}C pMC of each water sample, (2) specifying the $\delta^{13}\text{C}$ and ^{14}C pMC of carbonate in soil and bedrock, and CO_2 in the soil, and (3) testing different ^{14}C correction models (Fontes & Garnier, 1979; Han & Plummer, 2013, 2016; Pearson & Hanshaw, 1970; Vogel, 1970). Hand calculations were performed by allowing the carbonate end-members to vary substantially and then seeking suitable residence times within the potential solutions. For this step, $\delta^{13}\text{C}_{\text{rec}}$ (the $\delta^{13}\text{C}$ of recharge) was allowed to vary from 0‰ to -22 ‰ and $\delta^{13}\text{C}_{\text{carb}}$ (the $\delta^{13}\text{C}$ of bedrock calcite) was allowed to

vary from 0‰ to -10 ‰. However, the $\delta^{13}\text{C}_{\text{carb}}$ values of roof pendant marble and disseminated calcite in the granitic plutonic bedrock likely fall within the range of 0‰ to -5 ‰, but disseminated calcite precipitated in fractures by groundwater could be relatively modern and hence more negative. The raw data, analytical uncertainty, estimated residence time, and numerical uncertainty associated with ^{14}C dating are reported in data tables 1 and 3 archived at <https://doi.org/10.5281/zenodo.7603891>.

Finally, a chlorine-36 chronometer ($^{36}\text{Cl}/\text{Cl}$ chronometer) was utilized to estimate the residence times of spring water samples that were not suitable for either ^3H or ^{14}C dating (Gleason et al., 2020). The $^{36}\text{Cl}/\text{Cl}$ chronometer was produced by fitting a trend line between the $^{36}\text{Cl}/\text{Cl}$ ratio and the contributing groundwater residence time of springs that had young residence times (estimated using ^3H) and springs that had old residence times (estimated using ^{14}C). The power-law trend line ($r^2 = 0.76$) of the chronometer was found to be: $\text{RT} = 700,670 \times (^{36}\text{Cl}/\text{Cl})^{-1.06}$, where RT is residence time in years and $^{36}\text{Cl}/\text{Cl}$ is the chlorine-36 ratio of the spring water sample. An envelope of uncertainty surrounds the chronometer since there is analytical uncertainty in the measured ^3H and $^{36}\text{Cl}/\text{Cl}$ compositions of the spring water and numerical uncertainty in the estimation of ^3H and ^{14}C residence times. Uncertainty in the $^{36}\text{Cl}/\text{Cl}$ chronometer residence times was determined by linearizing the trend line (Gleason et al., 2020). The raw data, analytical uncertainty, estimated residence time, and numerical uncertainty associated with ^{36}Cl dating are reported in data tables 1 and 3 archived at <https://doi.org/10.5281/zenodo.7603891>.

Organic and inorganic nutrient analysis of benthic material and CPOM

Benthic samples collected at the time of microbial sampling were utilized to measure total organic carbon (TOC), total nitrogen (TN), and total sulfur (TS) in benthic material and CPOM. After thawing, benthic samples for CPOM analysis were sieved through a 1-mm sieve, rinsed with DI, and objects >100 μm were removed with tweezers. Next, benthic material and CPOM samples were dried at 60°C in an incubator until at a constant mass. Dried material was then ground with a steel mortar and pestle, sieved (88- μm sieve), and analyzed using a Costech ECS-4010 CHNSO analyzer (Costech Analytical Technologies, Valencia, CA) at the Arizona Laboratory for Emerging Contaminants (ALEC). All data are included in data tables 1 and 4 archived at <https://doi.org/10.5281/zenodo.7603891>.

Microbial field sampling

Water microbial communities were sampled by pumping spring water (≥ 2 L total) onto replicate 0.2- μ m polyethersulfone membrane Sterivex-GP pressure filters (four filters per spring), as described in the “*Field Measurements and hydrogeochemistry sampling*” section. Excess water was cleared from each filter after sampling. Benthic samples were gathered using a sterilized shovel (top > 2 cm) to fully represent the substrate diversity (e.g., fine-grained benthic material, coarse-grained benthic material, or microbial mat) of each spring to maximize sampling of unique habitats at every spring. All samples were frozen immediately after sampling, transported on dry ice, and stored in a -80°C freezer. Field notes, including descriptions of benthic samples, are detailed in data table 2 archived at <https://doi.org/10.5281/zenodo.7603891>.

Sample preparation and sequence processing for 16S rRNA gene sequencing

Microbial DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. The concentration and purity ratios of microbial DNA extracts were measured with a Nanodrop 1000 full-spectrum spectrophotometer (Thermo Scientific, Waltham, MA) and are included in data table 5 archived at <https://doi.org/10.5281/zenodo.7603891>. The V4 region of the 16S rRNA gene was amplified and sequenced using the updated bacterial- and archaeal-specific 515F/806R primer set (Apprill et al., 2015; Parada et al., 2016; Thompson et al., 2017). Library prep and paired-end sequencing was conducted at Argonne National Laboratory (Lemont, IL) using an Illumina MiSeq platform (2×151 bp) per the Earth Microbiome Project pipeline (<https://earthmicrobiome.org/protocols-and-standards/16s/>). Analysis of the 16S rRNA gene sequences was carried out using QIIME2 version 2019.4 (Bolyen et al., 2019). Paired-end reads were joined, quality filtered, and assigned to amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). Taxonomy was assigned using a naïve-Bayesian classifier trained on the V4 region of the Silva NR99 132 alignment (Pruesse et al., 2007) via the q2-feature-classifier plugin (Bokulich et al., 2018). Sequence variants were aligned using Mafft via the q2-alignment plugin at the default settings. Phylogenetic trees were constructed from this alignment using FastTree (Price et al., 2010) in QIIME2 version 2019.10. ASVs classified as chloroplast or mitochondria were removed.

BMI field sampling and sample processing

BMIs were sampled no more than 15 m from each spring source because the source represents the most stable temperature and chemical environment for these communities (Glazier, 1991; McCabe, 1998). A composite BMI community sample was collected from five subsamples at each spring. Subsamples were collected by placing 120-cm² quadrats and 10 $\times$ 12 cm 500- μ m mesh D-frame netting along the same sequence of five equally spaced transects positioned 3 m apart: center, right bank, center, left bank, and center. Benthic substrate was gently agitated using a quadrat to release BMIs from the substrate and allow them to float downstream into the netting. Samples were preserved in the field using 90% ethyl alcohol. Processing, identification, and enumeration via microscopic examination were conducted at Rhithron Analytical Laboratory (Missoula, MT). A random subsample of at least 300 BMI individuals was identified and enumerated to determine the structure of BMI communities; this number has been shown to provide adequate coverage of BMI community diversity (Vinson & Hawkins, 1996). Crenobionts were identified to the species level, whereas other aquatic insects and mites were identified to the genus level. Triclad (order *Tricladida*), oligochaetes (subclass *Oligochaeta*), ostracods (class *Ostracoda*), and nematodes (phylum *Nematoda*) were not identified any further. BMI community abundance data and taxonomic information are included in data table 6 archived at <https://doi.org/10.5281/zenodo.7603891>.

α - and β -diversity analysis of microbial and macroinvertebrate communities

For microbial samples, rarefaction curves were constructed and plotted with the R package vegan version 2.5-6 (Dixon, 2003). Faith's phylogenetic diversity (Faith's PD) values were calculated for microbial samples using the R package picante version 1.8 (Kembel et al., 2010). Three other α -diversity metrics (Observed, Shannon Index, and Gini-Simpson Index) were calculated for both microbial and BMI communities using the R package phyloseq version 1.30.0 and all metrics were plotted using ggplot2 version 3.3.0 (McMurdie & Holmes, 2013; Wickham, 2016). All α -diversity data were tested for normality with the Shapiro-Wilks test using R package stats. Data that passed the normality check were then tested for homoscedasticity using the Levene's test of equality of variance (implemented from R package car version 3.0-10) to ensure that the assumption of homogeneity of variance necessary to run ANOVA was

not violated (Fox & Weisberg, 2019). Data that failed normality and homogeneity of variance assumptions were tested with a Kruskal–Wallis test.

Three approaches were used to explore and compare the patterns in benthic microbial and BMI Bray–Curtis community dissimilarity: (1) nonmetric multidimensional scaling (NMDS) analysis, (2) principal coordinate analysis (PCoA), and (3) analysis of similarity (ANOSIM). For both benthic microbes and macroinvertebrates, NMDS ordinations were used to visualize community patterns between the samples and spring groups. Then, ANOSIM was utilized to determine whether the community structures in the spring groups were significantly different. For water microbes only, a PCoA ordination was used instead of NMDS due to the nearly zero stress value associated with the water NMDS ordination. These analyses were carried out using the R packages phyloseq and vegan. Environmental vectors were generated from major ion and trace metals data (in mole per liter) and physicochemical parameters via the envfit function of R package vegan. The Mantel test was used to determine if the benthic microbial and BMI Bray–Curtis community dissimilarity was associated with geographic distance. The distance between springs was calculated using the R package geodist version 0.0.7 (Padgham & Sumner, 2021). Spearman's rank-order correlation Mantel tests (9999 permutations) were conducted on (1) unaveraged benthic microbial samples, (2) averaged benthic microbial samples, and (3) BMI samples using the package vegan. Prior to the Mantel test, benthic microbial community data in each spring were averaged to remove zero-distance comparisons.

The taxonomic composition of the water and benthic microbial communities were assessed by agglomerating microbial taxa at the phylum, order, and genus levels and BMI taxa at the order and genus levels. Microbial ASV and BMI count data were then converted to percent relative abundance and a cutoff was applied to merge low abundance taxa. Taxonomic agglomeration and data transformation for the biological communities were carried out using phyloseq and taxonomic bar plots were plotted using ggplot2. To identify differentially enriched benthic microbial taxa in granitic and roof pendant springs, a linear discriminant analysis effect size (LEfSe) analysis was conducted using the R package microbiomeMarker version 0.0.1.9000 (Yang, 2020). As suggested, benthic microbial relative abundance data were normalized using a counts per million (CPM) transformation prior to conducting the LEfSe analysis (Yang, 2020).

Predictions regarding microbial metabolisms and attachment were generated for family- and genus-level LEfSe bar plots via a thorough literature review focusing on taxonomically verified reports published in Bergey's Manual of Systematics of Archaea and Bacteria and the

effective publications for these taxa as listed on the List of Prokaryotic Names with Standing in Nomenclature (Parte, 2014). To complement these literature-based inferences, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) version 2.5.0 was implemented (Douglas et al., 2020). The standard PICRUSt2 python script (picrust2_pipeline.py), available on the PICRUSt2 GitHub page (<https://github.com/picrust/picrust2/wiki>), was run on default settings to generate putative functional profiles. Next, the PICRUSt2 output of KEGG ORTHOLOGY (KO) gene counts were totaled by module with a custom script. A differential abundance analysis of the total counts per KEGG module was conducted using the R package DESeq2 version 1.34.0 to identify modules that were differentially abundant in either hydrogeochemical group, as summarized in data table 7 archived at <https://doi.org/10.5281/zenodo.7603891>.

Triplicate microbial water samples were originally sequenced for two springs: Grover Anton Spring (IES 028) and Elderberry Canyon Spring (IES 033), as part of an effort to constrain the variability in microbial community structure between replicate water samples. Given the within-spring similarity of the triplicate water samples, the microbial ASV counts of these samples were averaged together for the two springs individually. ASV counts were averaged (“=AVERAGE” function) and rounded to the nearest integer (“=ROUND” function) using Excel. These averaged ASV counts were utilized for most analyses, except for the rarefaction curves and Faith's PD analysis, which were run prior to averaging the triplicate water samples. Several plots were generated for the water microbes and included in the supplement with supporting text; however, the water microbial community plots were not analyzed in detail in the text due to the weak relationships observed with the geochemical groups (Appendix S1: Figures S1–S6).

Co-occurrence analysis of microbial and BMI communities

The relationship between the structure and relative abundances of benthic microbes and BMIs was visualized using a correlation co-occurrence network map. First, benthic microbial and BMI taxa were taxonomically agglomerated at the genus level and converted to percent relative abundance. Taxa representing <7.5% (benthic microbes) or <5% (BMIs) across the datasets were then merged. Next, Spearman's rank-order correlation coefficients and *p* values were calculated between the community relative abundance matrices using the R package Hmisc version 4-4.0 (Harrell, 2020). Correlation co-occurrence network

maps were constructed using the R package network version 1.16.1 (Butts, 2008, 2020) and then plotted using the R package ggnet version 0.1.0 (Briatte, 2021). Genera were color-coded on the maps by BMI stress tolerance/microbial metabolism or BMI behavior/microbial attachment. Inferences regarding microbial metabolisms and attachment were generated as detailed in the previous section. Stress tolerance values (range: 0–10) were assigned to BMI taxa as previously characterized with intolerant taxa denoted by low stress tolerance values and tolerant taxa denoted by high stress tolerance values (Mandaville, 2002). Additional Spearman's rank-order correlation testing was conducted to evaluate the relationship between the abundance of BMIs and total microbial DNA using Hmisc (Harrell, 2020). Prior to testing, BMI abundance counts were summed by spring and total microbial DNA concentration was averaged per spring using only benthic samples with A260/280 ratios between 1.6 and 2.

RESULTS

Paleozoic roof pendants impart subtle geochemical differences in similar springs

The 10 springs along the foot of the eastern Sierra Nevada provide a broad geographic and geologic representation of springs along the west side of Owens Valley (Figure 1A). These rheocrane-type springs were physicochemically similar, being characterized by cool water (temperature: 11.2–18.7°C), low specific conductance (66.1–470.7 $\mu\text{S}/\text{cm}$), circumneutral pH (6.94–8.16), and oxygen undersaturation (dissolved oxygen: 2.9–8.7 mg/L). In all springs, dissolved nutrient concentrations were below method DLs, except for nitrate, which was detected in 9 of the 10 springs at 0.11–2.71 mg/L. Detailed physicochemical data and representative photos of each spring are included in data tables 1–4

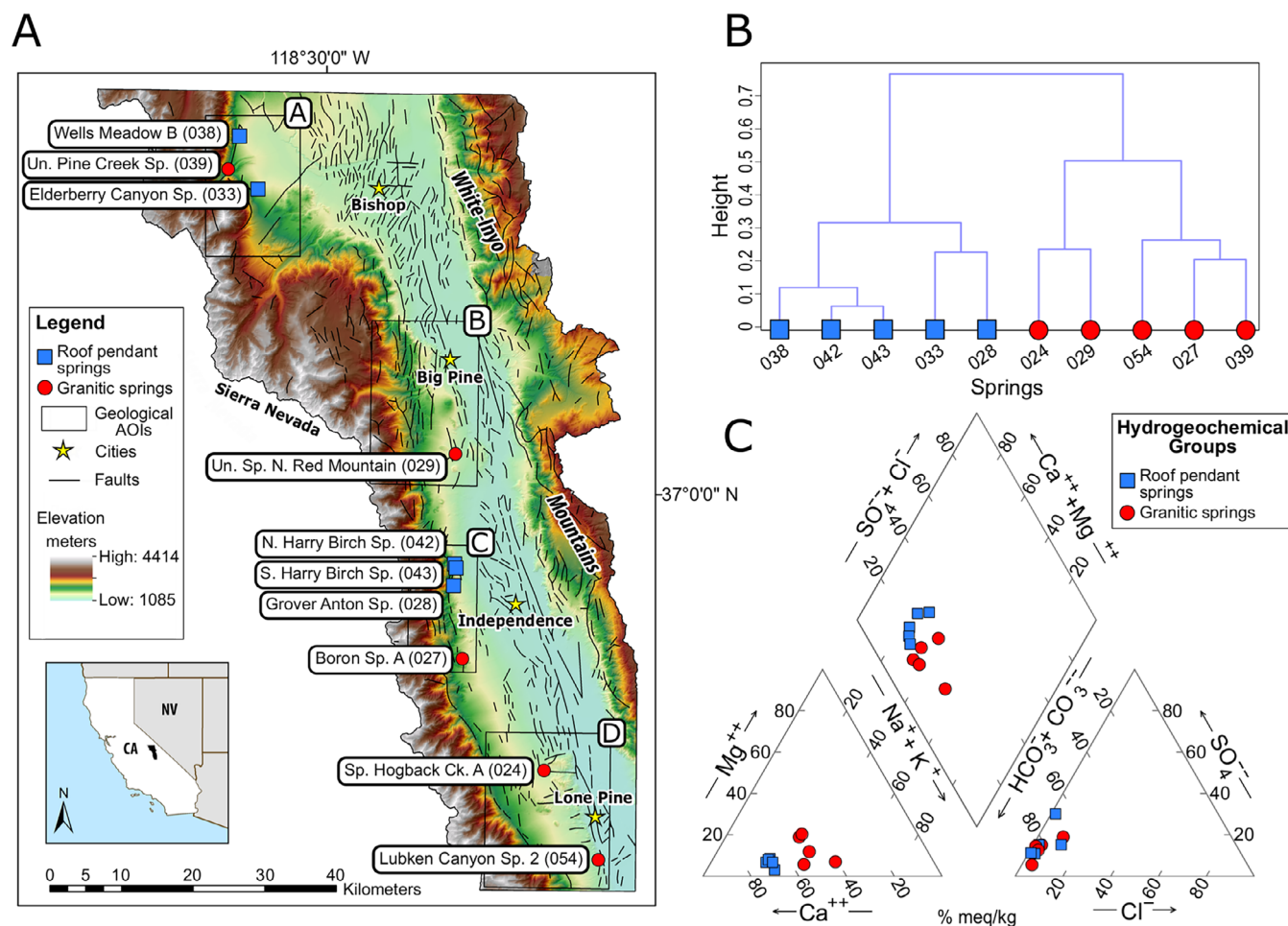


FIGURE 1 Location and geochemical signatures of two distinct spring groups in Owens Valley. (A) Study area map of Owens Valley shaded by elevation (range: 1085–4414 m). Major faults and geological areas of interest (AOIs) are marked on the map with black lines and rectangles. A simplified geological setting map detailing the major geological units and petrologic profiles of the geological AOIs is shown in Appendix S1: Figure S8. (B) Dendrogram from the hierarchical clustering analysis (HCA) of the seven major ions in the 10 study springs. The HCA was conducted using % meq/kg for each ion. (C) Piper diagram displaying geochemical composition of spring waters.

archived at <https://doi.org/10.5281/zenodo.7603891> and Appendix S1: Figure S7.

A HCA incorporating major ions, Si, and specific conductance data divided spring waters into the two major geochemical groups (Figure 1B). Student's *t* tests were utilized to assess geochemical differences between the groups for ~60 physical and chemical analytes. This revealed that the two spring groups possessed significantly different $\text{Ca}^{2+}/\text{Na}^{+}$ molar ratios ($p = 3.1 \times 10^{-5}$), $\text{Ca}^{2+}/\text{Mg}^{2+}$ molar ratios ($p = 0.05$), divalent to monovalent cation ion ratios (D/M; $p = 0.001$), and slopes at the spring sources ($p = 0.004$) (data table 1 archived at <https://doi.org/10.5281/zenodo.7603891>). Spring geochemistry in both groups reflected recharge through granitoid rock, which predominates the high-elevation recharge zones in the Sierra Nevada. All spring waters also contained an excess of calcium ($\text{Ca}^{2+}/\text{Na}^{+} > 0.6$) over that expected for plagioclase weathering alone ($\text{Ca}^{2+}/\text{Na}^{+}$ of 0.4–0.6; Figure 1C), consistent with dissolution of disseminated calcite during recharge (Meyers et al., 2022). However, only one spring group contained an “excess of excess calcium,” with significantly higher $\text{Ca}^{2+}/\text{Na}^{+}$ ratios ($\text{Ca}^{2+}/\text{Na}^{+} > 1.5$). This “excess of excess calcium” signature has been interpreted to reflect roof pendant weathering, which is consistent with the location of the five roof pendant springs and their elevated $^{87}\text{Sr}/^{86}\text{Sr}$ ratios (Meyers et al., 2022). Two emerge downgradient from the Pine Creek Pendant units near Mt. Tom (Elderberry Canyon spring, IES-033) and Wheeler Crest (Wells Meadow B spring, IES-038), whereas the three remaining springs emerge downgradient of unnamed roof pendants near Lookout Point (Appendix S1: Figure S8). For simplicity, the spring group with “excess of excess calcium” signatures is hereafter referred to as “roof pendant springs,” whereas those with lower $\text{Ca}^{2+}/\text{Na}^{+}$ ratios are hereafter referred to as “granitic springs.”

Roof pendant springs host more diverse benthic macroinvertebrate communities

Following quality filtering, 843,111 total V4 16S rRNA gene amplicon sequences from bacteria and archaea were recovered from 39 benthic samples, with an average of 21,618 reads per sample. A total of 14,523 unique ASVs were recovered, approximating the species richness of prokaryotes in these samples, and accounting for 52 bacterial phyla and 9 archaeal phyla. Rarefaction curves for all benthic microbial community samples reached an asymptote between 10,000 and 15,000 sequences, indicating adequate sequencing depth for robust comparisons among springs and spring groups (Appendix S1: Figure S9). A total of 2671 individual BMIs were identified across 10 composite benthic samples with an average

of 267 BMIs per sample. From the BMI community data, 5 phyla, 8 classes, 16 orders, 47 families, and 87 genera were identified.

Several α -diversity metrics were calculated to determine whether geochemical grouping influenced diversity (Figure 2; data tables 8–10 archived at <https://doi.org/10.5281/zenodo.7603891>). No significant differences in benthic microbial diversity were observed between roof pendant and granitic springs (Figure 2A–D; data tables 8 and 10 archived at <https://doi.org/10.5281/zenodo.7603891>). In contrast, BMI α -diversity was higher in roof pendant springs for richness (observed count ANOVA, $p = 0.009$) and diversity (Gini–Simpson Index, Kruskal–Wallis test, $p = 0.01$; Shannon Index, Kruskal–Wallis test, $p = 0.01$) (Figure 2E–G; data tables 9 and 10 archived at <https://doi.org/10.5281/zenodo.7603891>).

Subtle geochemical differences alter community composition of benthic microbes and macroinvertebrates

NMDS ordinations based on Bray–Curtis dissimilarity for both prokaryotes and BMIs demonstrated that spring groups hosted unique communities (Figure 3). Significant differences were observed in both microbial ($R = 0.28$, $p = 0.001$) and BMI ($R = 0.65$, $p = 0.009$) communities, with samples clustering on the ordination according to geochemical grouping. Geographic distance was not correlated with Bray–Curtis community dissimilarity of microbial (Mantel test, $p = 0.11$) or macroinvertebrate communities (Mantel test, $p = 0.08$) (data table 11 archived at <https://doi.org/10.5281/zenodo.7603891>).

Covariates of community dissimilarity ($p \leq 0.05$) identified among the ~60 physical and chemical analytes were fitted to the NMDS ordinations to visualize the major environmental factors structuring spring biological communities (Figure 3). $\text{Ca}^{2+}/\text{Na}^{+}$ and D/M ratios correlated with microbial and BMI community dissimilarity in roof pendant springs, reflecting weathering of the roof pendants during recharge. Additional vectors corresponding to spring characteristics and landscape placement were also significant. In roof pendant springs, the slopes at the spring sources, $\text{Ca}^{2+}/\text{Mg}^{2+}$ ratio, current velocity, and cobble percentage correlated only with microbial community dissimilarity and gravel percentage only with BMI community dissimilarity. In granitic springs, spring water residence time and muck percentage correlated with both communities. Neither soluble nutrients nor TOC, TN, or TS measurements (benthic material or CPOM) correlated with community dissimilarity for microorganisms or BMIs.

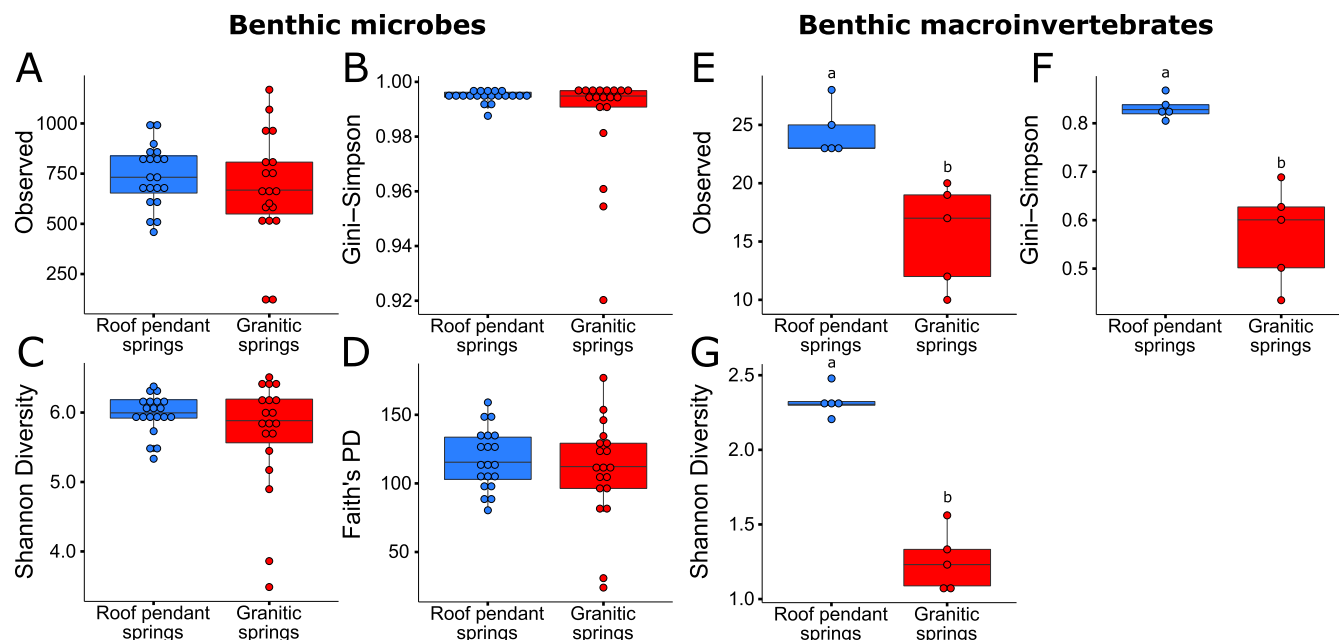


FIGURE 2 Benthic macroinvertebrate (BMI) diversity, but not microbial diversity, is higher in roof pendant springs. Box plots of (A) observed sequence variants, (B) Gini-Simpson Index, (C) Shannon Diversity Index, and (D) Faith's phylogenetic diversity (Faith's PD) values for benthic microbial communities. Box plots were also made of (E) observed counts, (F) Gini-Simpson Index, and (G) Shannon Diversity Index for BMI communities. All boxes are colored according to geochemical grouping: roof pendant springs (blue) and granitic springs (red). Interquartile ranges, medians, and data points are shown. For the benthic microbial community, no statistically significant differences were observed between the hydrogeochemical groups. For the BMI community, richness and diversity were higher ($p < 0.05$) in roof pendant springs.

Many analytes correlated only with microbial community dissimilarity (Figure 3A). Temperature and various ions/trace elements (vectors number 12–27) correlated with the microbial community dissimilarity of samples from the two oldest springs, Spring along Hogback Ck. A (IES-024, 1850 years in age) and Lubken Canyon Spring 2 (IES-054, 1270 years in age) of the granitic springs group. Mo, Al, and sand percentage correlated with microbial community dissimilarity of samples from the granitic spring Unnamed Pine Creek Spring (IES-039). Wetted width and V correlated with microbial community dissimilarity of samples from the granitic spring Unnamed Spring north of Red Mountain (IES-029).

Roof pendant springs are enriched with physiologically distinct biological communities

Benthic microbial communities were compromised predominantly of *Proteobacteria* (46.1% of the microbial community on average), *Bacteroidetes* (10.5%), *Acidobacteria* (6.9%), *Actinobacteria* (5.1%), *Verrucomicrobia* (5.1%), *Chloroflexi* (4.1%), *Planctomycetes* (3.8%), *Nitrospirae* (3.7%),

and *Cyanobacteria* (3.6%) (Appendix S1: Figure S10). Relative abundance bar plots representing benthic microbial communities at various taxonomic levels are included in the supplement (Appendix S1: Figures S11–S13).

LEfSe analysis of benthic microbial communities revealed 408 taxa (8 phyla, 33 classes, 69 orders, 111 families, and 187 genera) that were significantly ($p \leq 0.05$) enriched in either roof pendant springs or granitic springs (Figure 4; data table 12 archived at <https://doi.org/10.5281/zenodo.7603891>). Several phyla and one class with multiple child taxa were enriched in roof pendant springs: the phyla *Acidobacteria* (four classes and three orders), *Planctomycetes* (three classes and four orders), *Gemmatimonadetes* (three classes and three orders), and *Actinobacteria* (one class and four orders), and the class *Alphaproteobacteria* (six orders) (Figure 4A). In granitic springs, only the archaeal phylum *Euryarchaeota* (two classes and three orders) had multiple classes or orders enriched. Granitic springs were also enriched with several bacterial classes with fewer child taxa, namely *Clostridia* (one order), *Endomicrobia* (one order), and *Gracilibacteria* (one order), and bacterial orders *Bacteroidales*, *Beggiatoales*, *Chromatiales*, *Desulfobacterales*, *Desulfuromonadales*, and *Methylococcales*.

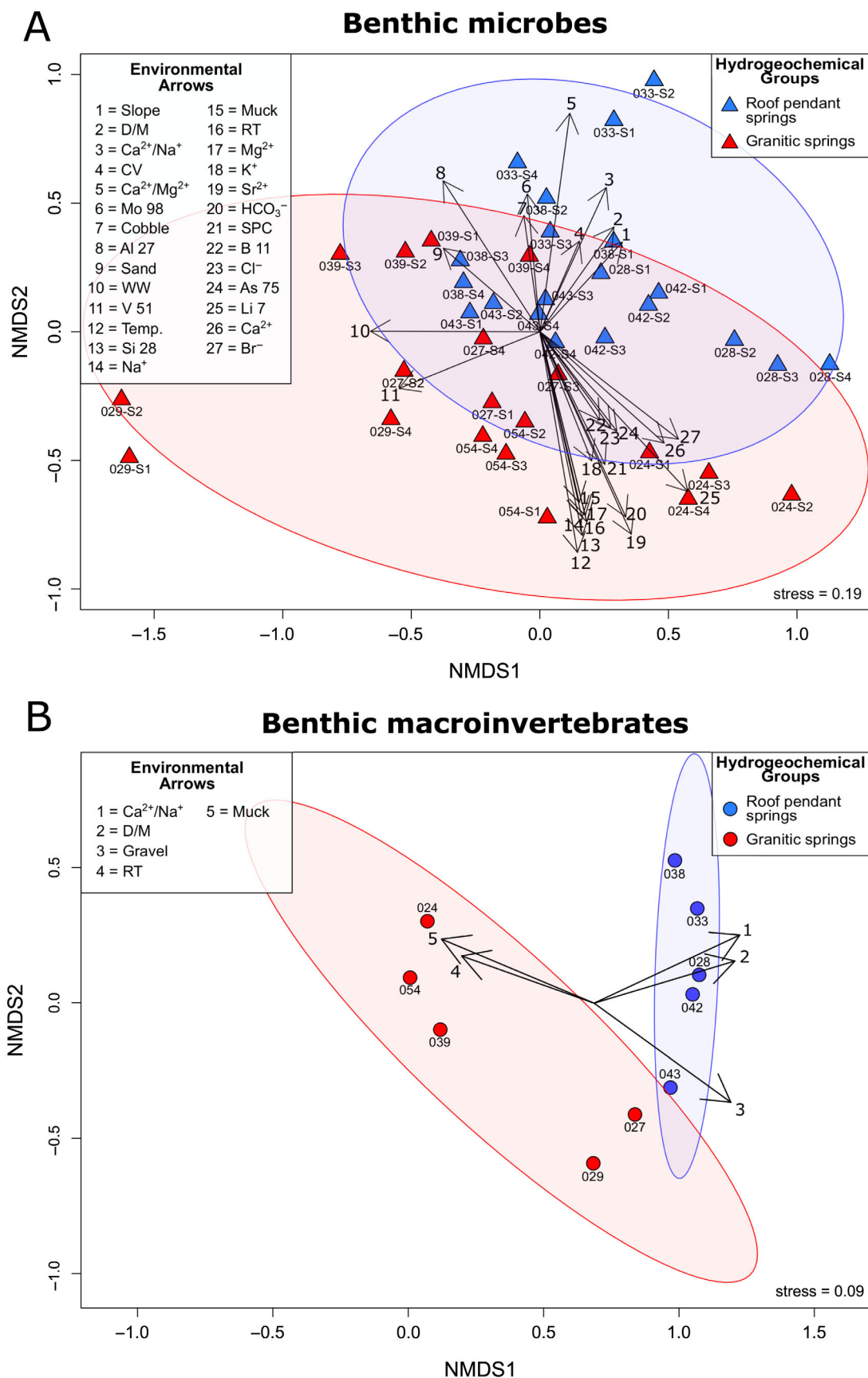


FIGURE 3 Legend on next page.

The enriched families and genera suggest a unique set of physiological traits associated with each geochemical group, which was also supported through the differential abundance of KEGG modules in simulated metagenomes from the two spring groups (Figure 4B,C; data table 7 archived at <https://doi.org/10.5281/zenodo.7603891>). Roof pendant springs were enriched with taxa inferred to be aerobic/facultatively anaerobic predominantly from *Alphaproteobacteria* (8 families and 11 genera), *Actinobacteria* (eight families and eight genera), *Planctomycetes* (three families and two genera), and *Acidobacteria* (two families and two genera), and the abundance of the module encoding biosynthesis of the cytochrome aa3-600 heme-copper menaquinol oxidase (M00416). These taxa also include those known to produce prosthecae/stalks and holdfasts in the *Alphaproteobacteria* and *Planctomycetes*, such as *Bauldia*, *Hirschia*, *Gemmata*, *Pedomicrobium*, Pir4 lineage (family: *Pirellulaceae*), *Schlesneriaceae*, *Planctomyces*, *Pedomicrobium*, *Hyphomicrobiaceae*, *Micropepsaceae*, SWB02 (family: *Hyphomonadaceae*), and *Rhodomicrobium*.

In contrast, granitic springs were enriched with taxa inferred to be anaerobes, including fermenters in the *Clostridia* (one family and four genera), sulfate reducers in the *Deltaproteobacteria* (five families and eight genera), and methanogenic archaea within the *Euryarchaeota* (three families and three genera). Similarly, simulated metagenomes enriched in granitic springs included modules for acetogenesis (M00579), methylotrophic methanogenesis (M00563), V/A-type ATPases (M00159), and denitrification (M00529). Aerobic bacteria included methylotrophic bacteria in the *Gammaproteobacteria* (six families and four genera) and similarly a module for methanotrophy (M00174) was enriched (Figure 4C). A full list of significant results from the DESeq analysis of KEGG modules and LEfSe analysis of 16S rRNA gene community abundance data can be found in the supplement (data tables 7 and 12 archived at <https://doi.org/10.5281/zenodo.7603891>).

BMI communities differ in composition at multiple taxonomic levels (Figure 5; Appendix S1: Figure S14). At the genus level, communities in roof pendant springs were relatively even and dominated by vagile insect taxa such as *Lepidostoma*, *Optioservus*, *Gumaga*, *Malenka*,

Ironodes, *Baetis*, and *Argia*, and the annelid taxon *Enchytraeidae*. These taxa are primarily shredders, collector gatherers, and predators. *Pyrgulopsis* was present in only one roof pendant spring, Elderberry Canyon Spring (IES-033), where it was the most abundant taxon. In contrast, granitic spring BMI communities were dominated by *Pyrgulopsis* and stress-tolerant, noninsect taxa, mainly *Pisidium* and *Hyaella*, with less abundant populations of the insect taxa *Argia*, *Optioservus*, and *Tricladida*.

Extensive network connectivity between microbes and BMIs in roof pendant springs

A co-occurrence network revealed 112 significant ($p < 0.05$) positive correlations between microbes and BMIs (Figure 6). Cluster 1 is a large network highlighting positive correlations between microbial and BMI genera in roof pendant springs. BMI genera belonging to shredder, collector gatherer, or predator feeding groups comprised central nodes with high connectivity to microbial taxa, inferring key roles for these taxa in trophic interactions (Figure 6A): *Ironodes* (14 network connections), *Gumaga* (13), *Baetis* (11), *Malenka* (7), and *Pentaneura* (7). Prosthecae/stalked bacteria in Cluster 1 included *Hyphomicrobium*, *Pedomicrobium*, *Pirellula/Pirellulaceae*, *Gemmataceae*, and SWB02 (*Hyphomonadaceae*). Almost all bacteria in this cluster are inferred to be aerobic or facultatively anaerobic, while most BMIs were vagile insects with low to moderate stress tolerance values (Figure 6B).

Much less connectivity between microbes and BMIs was observed in granitic spring communities (Clusters 2–4), inferring looser trophic associations (Figure 6). BMI genera in Cluster 2 included three FFGs, collector gatherers (*Hyaella*), collector filterers (*Pisidium*), and predators (*Tricladida*); both Cluster 3 and Cluster 4 were represented by the scrapers *Pyrgulopsis* and *Zaitzevia*. No shredders were present in the granitic spring clusters. The largest network was Cluster 2, with correlations between the stress-tolerant BMI genera *Pisidium*, *Hyaella*, and *Tricladida* and a mixture of taxa inferred to be aerobic/facultatively anaerobic (*Dechloromonas* and *Lacunisphaera*), methylotrophic (*Crenothrix* and *Methyloglobulus*), and anaerobic (*Anaeromyxobacter* and *Geobacter*) bacteria (Figure 6B).

FIGURE 3 Both microbes and benthic macroinvertebrates (BMIs) roof pendant spring communities are unique. Nonmetric multidimensional scaling (NMDS) ordination of Bray–Curtis dissimilarity between 10 springs for both (A) benthic microbes and (B) BMIs. Each point is colored by geochemical grouping: roof pendant springs (blue) and granitic springs (red). Shading represents 95% confidence intervals. Physicochemical factors deemed to be significantly ($p < 0.05$) correlated with community dissimilarity were overlaid on the NMDS plot. Names of some environmental parameters have been abbreviated on the plot: current velocity (CV), wetted width of the spring run (WW), the divalent to monovalent cation ratio (D/M), residence time (RT), and specific conductance (SPC). The influence of hydrogeochemical grouping on β -diversity was found to be significant via ANOSIM for both benthic microbial communities ($R = 0.28$, $p = 0.001$) and BMI communities ($R = 0.65$, $p = 0.009$).

In Cluster 3, *Zaitzevia* was positively associated with the aerobic, ammonia-oxidizing archaeon *Candidatus Nitrosoarchaeum* and an unclassified genus in the *Anaerolineaceae*. Cluster 4 included *Pyrgulopsis* and the cyanobacterium *Pleurocapsa* PCC-7319 and an unclassified genus of *Chitinophagaceae*.

DISCUSSION

We documented distinct communities of benthic microorganisms and macroinvertebrates in springs that recharge through Paleozoic roof pendants thousands of meters upslope from the spring sources. Weathering of these roof pendants imparts an “excess of excess calcium” and an increased proportion of dissolved calcium compared with other ions (Meyers et al., 2022) that may promote initial microbial attachment to the negatively charged granitic substrate and enhance biofilm maturation within the spring benthos. Biofilms are multispecies surface-attached microbial communities embedded in a three-dimensional matrix of endogenously produced EPS composed of polysaccharides, proteins, extracellular DNA, and lipids (Berne et al., 2018; Flemming & Wingender, 2010). Divalent cations are known to reduce electrostatic repulsion by forming cross bridges between negatively charged surfaces and negatively charged cell surface structures, such as phospholipids, glycolipids, and teichoic acids (Geesey et al., 2000; Wang et al., 2019). The presence of divalent cations can also promote the expression and functionality of specific adhesins produced by a variety of bacterial species (Geesey et al., 2000; Wang et al., 2019). During biofilm maturation, the formation of nonspecific cation cross bridges between cells and EPS enhances the thickness and structural stability of the biofilm and traps nutrients (Sobeck & Higgins, 2002; Wang et al., 2019). Most research on the role of Ca^{2+} or other divalent cations in microbial attachment and biofilm formation focuses on ionic strength, yet neither Ca^{2+} concentration nor total ionic strength accounted for the community structures observed in the roof pendant springs. Instead, these patterns were attributed to differences in valence, specifically $\text{Ca}^{2+}/\text{Na}^+$ ratios.

A past study showed that $\text{Ca}^{2+}/\text{Na}^+$ ratios >1.0 resulted in larger microbial flocs in activated sludge compared with those with ratios <1.0 (Cousin & Ganczarczyk, 1999). Although our study focuses on benthic biofilms rather than suspended aggregates, we also identified $\text{Ca}^{2+}/\text{Na}^+$ ratios as the strongest correlate of community structure and propose that high $\text{Ca}^{2+}/\text{Na}^+$ ratios promote microbial attachment to granite in these springs. Only springs in the roof pendant group possessed $\text{Ca}^{2+}/\text{Na}^+$ ratios above 1.0 (average: 1.5 ± 0.12), and these springs were enriched

with unrelated lineages of bacteria often associated with biofilms, including members of the phyla *Acidobacteria*, *Planctomycetes*, *Gemmatimonadetes*, and *Actinobacteria*, and the class *Alphaproteobacteria* (Besemer, 2015; Figure 4). Several of the enriched *Alphaproteobacteria* and *Planctomycetes* also produce prosthecae and/or stalks that are often tipped with holdfast adhesins, which are secreted polysaccharides used for attachment and biofilm formation (Berne et al., 2015; Fritts et al., 2017; Jogler & Jogler, 2013). Previous work with a purified polysaccharide holdfast adhesin (fr2ps) from *Hyphomonas* MHS-3 demonstrated a critical role for Ca^{2+} cross-bridging in the initial binding of this adhesin to germanium oxide surfaces (Bhosle et al., 1998). We postulate that the higher abundance of biofilm-associated and prosthecae/stalked bacteria in roof pendant springs is a response to the molar excess of Ca^{2+} , which may promote microbial surface attachment and biofilm development. In flowing aquatic systems such as spring runs, surface attachment and biofilm formation are necessary to prevent benthic microorganisms from flushing downstream. As cells attach and settle in the system, endogenous nutrient cycling is increased, promoting the development of metabolically diverse assemblages and enhances trophic interactions (Battin et al., 2008; Besemer, 2015).

Calcium may also be important for structuring BMI communities, as observed in other aquatic systems (Bere et al., 2016; Lento et al., 2022). We propose that Ca^{2+} may structure these BMI communities indirectly through trophic interactions with microbial biofilms (as discussed below) or directly by influencing BMI physiology. For example, Ca^{2+} is present at a 1:1 molar ratio with phosphoserines in the major silk adhesin of caddisfly larvae, where it forms stabilizing cation cross bridges between antiparallel β -sheets (Ashton & Stewart, 2019). This adhesin is initially secreted from the silk gland as a fluid that is then solidified and stabilized into metallofibers by Ca^{2+} absorbed from the surrounding environment (Ashton & Stewart, 2019). When Ca^{2+} is replaced with Na^+ , the β -sheets in the stabilized silk adhesin revert to random coils, destroying the structure and adhesive properties of the silk (Ashton et al., 2013). Ca^{2+} -stabilized silk adhesins are necessary for substrate attachment and construction of composite cases comprised of stones and detritus that are utilized by the larvae for protection and in food gathering (Ashton et al., 2016). In our study, the caddisflies *Gumaga* and *Lepidostoma* were dominant BMI taxa in roof pendant springs but were nearly absent from granitic springs, which may be a direct response to $\text{Ca}^{2+}/\text{Na}^+$ ratios. Given their roles as shredders, *Gumaga*, *Lepidostoma*, and other enriched taxa, such as the stonefly *Malenka*, likely interact extensively for the synergistic degradation of biofilms or allochthonous organic matter, explaining the well-connected microbe–BMI network in roof pendant springs (Figure 6).

In the majority of springs and streams, allochthonous organic matter is the dominant source of organic carbon (Marks, 2019; Tichá et al., 2012; Wilson & Blinn, 2007) and is typically derived from the debris of riparian vegetation, collectively referred to as CPOM (>1 mm in particle size) (Cummins, 1974). BMI shredders and microorganisms are responsible for the synergistic breakdown of CPOM into FPOM (<1 mm in particle size), which is then ingested by BMI collector gatherers (Cummins, 1974; Marks, 2019). Both shredders and collector gatherers preferentially feed on organic matter that has been colonized by microbial biofilms (Bärlocher & Kendrick, 1975; Cummins, 1974) due to the stoichiometrically favorable nutrient ratios

(low C:N and C:P) of microorganisms over plant biomass (Cummins, 1974; Marks, 2019). EPS, which dominates over cells in mature biofilms, is an additional food source for BMIs, further facilitating the energy transfer to higher trophic levels (Couch et al., 1996; Hall & Meyer, 1998). Thus, we propose that Ca^{2+} -enriched populations of biofilm-associated and stalked/holdfast bacteria in roof pendant springs exert a bottom-up control on shredders and collector gatherers as a food source and metabolic partner. Biofilms might be particularly important in selecting for the abundant populations of shredders and collector gatherers in the roof pendant springs because these springs do not have higher total TOC or CPOM

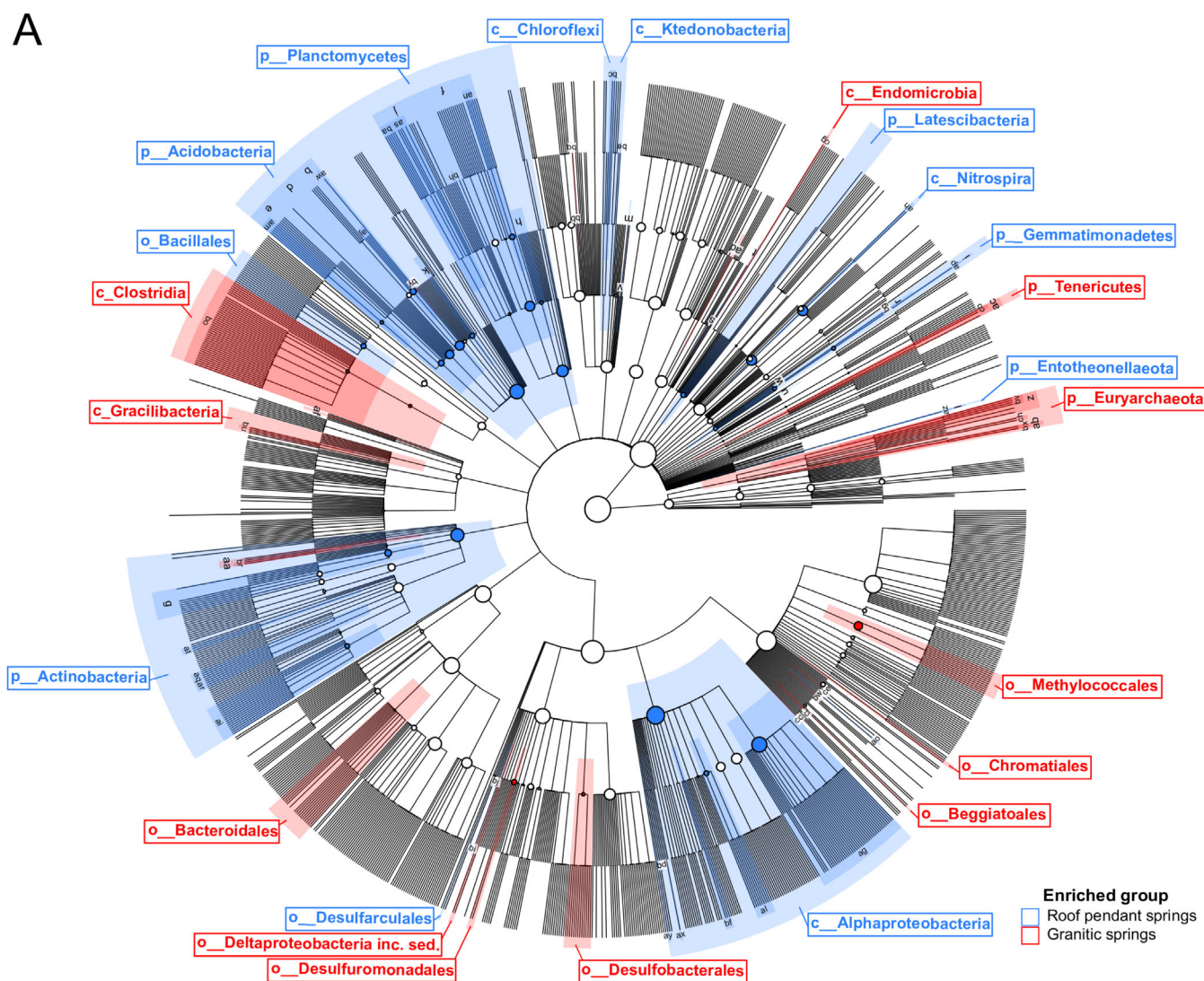


FIGURE 4 Linear discriminant analysis effect size (LEfSe) analysis reveals physiological differences between microbial communities in the spring groups. Linear discriminant analysis (LDA) effect size (LEfSe) (A) cladogram and (B + C) bar plots depicting microbial taxa enriched ($p < 0.05$) in either (B) roof pendant springs (blue) or (C) granitic springs (red). Results shown in the bar plots are plotted at the family and genus levels. Relevant physiological and ecological traits for families and genera are depicted with colored triangles. Taxa with alpha-numeric names that had no inferred physiology/ecology and unclassified taxa were removed at all taxonomic levels for both the cladogram and bar plots; the full list of significantly enriched taxa can be found in data table 12 archived at <https://doi.org/10.5281/zenodo.7603891>.

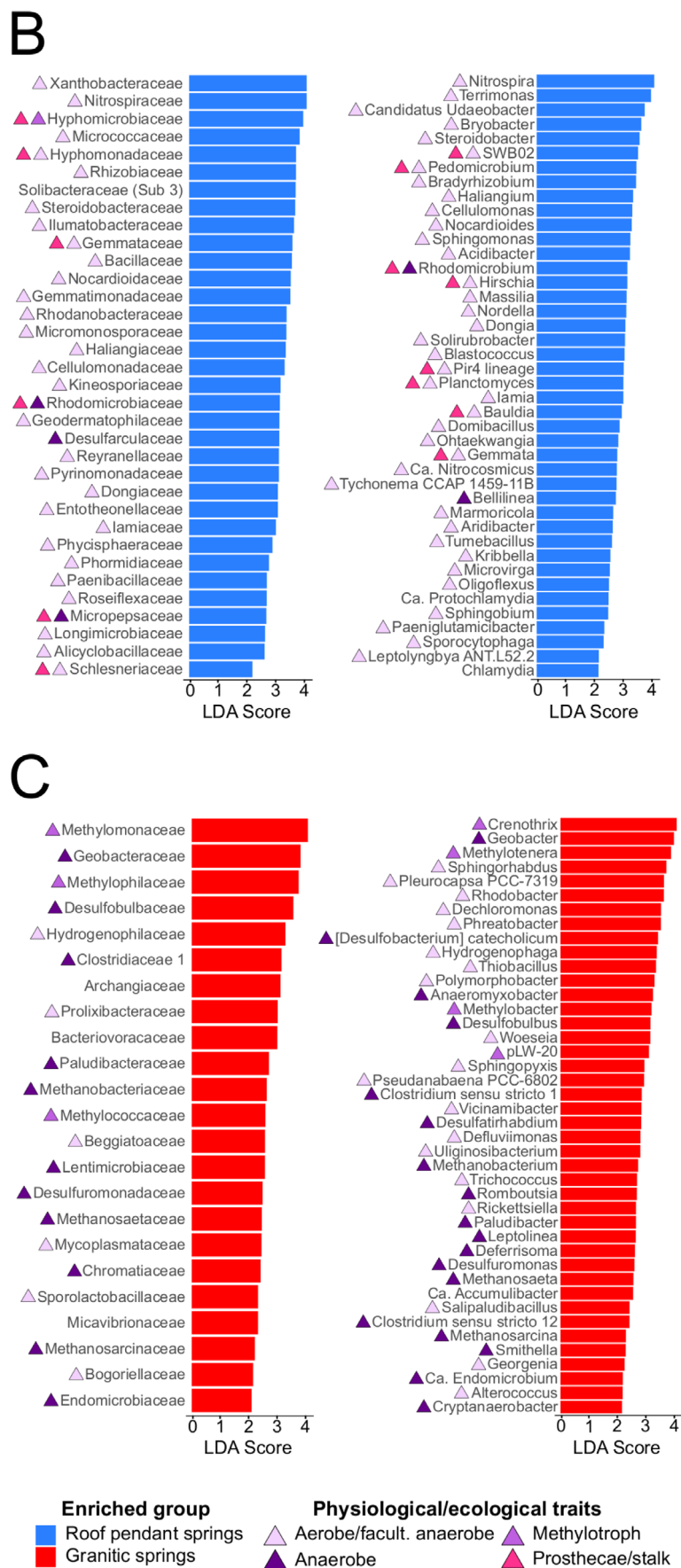


FIGURE 4 (Continued)

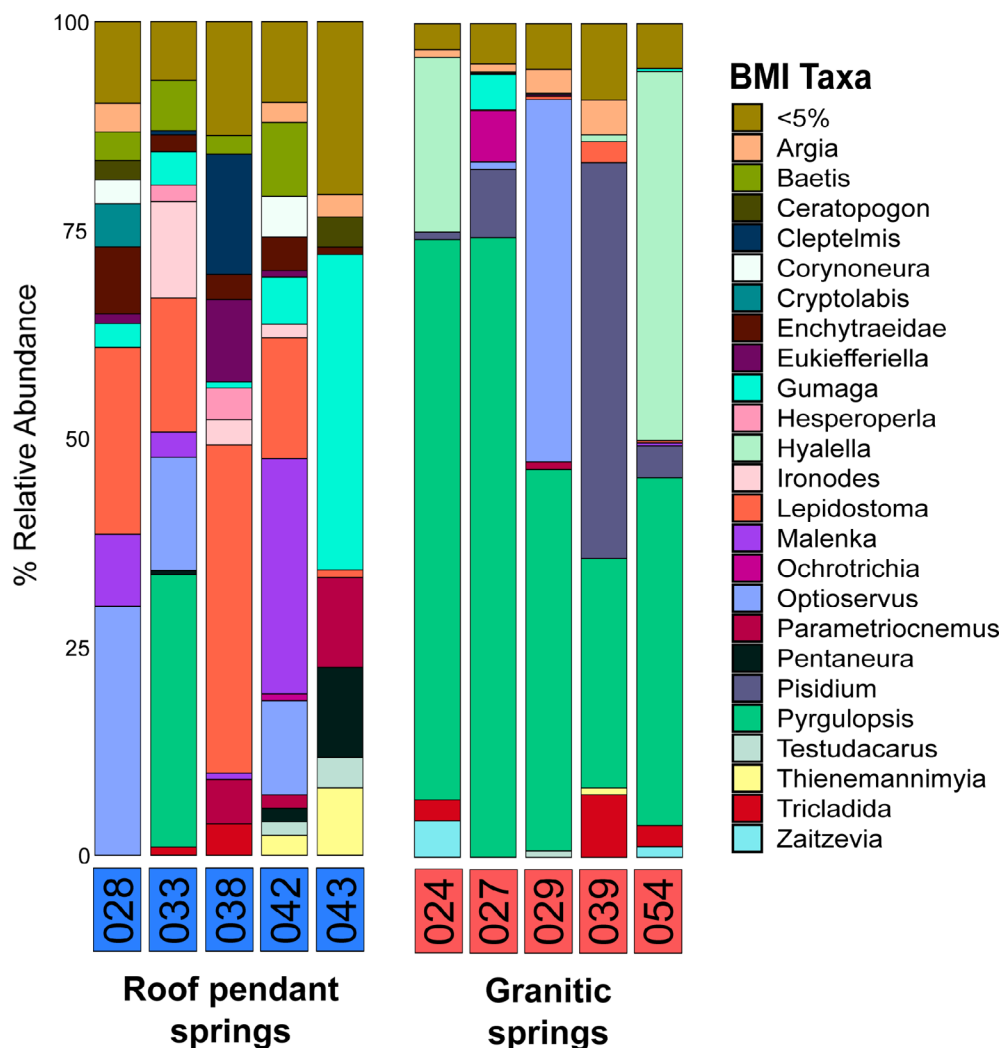


FIGURE 5 Benthic macroinvertebrate (BMI) communities are distinct between geochemical groups. Relative abundance of BMI communities identified microscopically. Roof pendant springs host diverse insect taxa, whereas granitic springs are dominated by *Pyrgulopsis*. Taxa calculated to be <5% across the dataset were agglomerated and plotted as such. Most taxa were identified at the genus level, with the exception of *Enchytraeidae* (family) and *Tricladida* (order).

compared with the granitic springs. As such, neither TOC nor CPOM explain the differential abundance of these feeding groups in these springs, which is in contrast to strong relationships between CPOM and these functional groups observed elsewhere (Wallace et al., 1999). Yet, despite our interpretation of strong trophic interactions between prokaryotes and BMIs in the roof pendant springs, BMI abundance and total microbial DNA were not correlated in either the roof pendant springs (Spearman's rho: $R = -0.80$, $p = 0.13$) or in all springs combined (Spearman's rho: $R = 0.06$, $p = 0.86$). This result might represent an uncoupling of this interaction in some springs or the importance of additional, unaccounted for, controls on abundance/biomass.

Due to their high population density in all granitic springs and their feeding behavior as scrapers, hydrobiid *Pyrgulopsis* spring snails could possibly exert an alternative

top-down control on benthic microbial biofilms. However, our results do not support this hypothesis. Only the roof pendant Elderberry Canyon Spring (IES-033) hosted *Pyrgulopsis* (Figure 5). Despite the presence of *Pyrgulopsis*, microbial and BMI communities in Elderberry Canyon Spring clustered with the other roof pendant spring communities on the NMDS plots (Figure 3). Furthermore, microbial communities in springs with or without *Pyrgulopsis* were not significantly different (ANOSIM: $R = 0.06$, $p = 0.12$). Additionally, Elderberry Canyon Spring hosts enriched populations of biofilm-associated and stalked/holdfast bacteria, like other roof pendant springs. Altogether, we did not identify any evidence consistent with top-down control of biofilm-associated and stalked/holdfast bacteria through grazing by *Pyrgulopsis*.

In aquatic environments, current velocity is one of the most important factors determining the grain size of

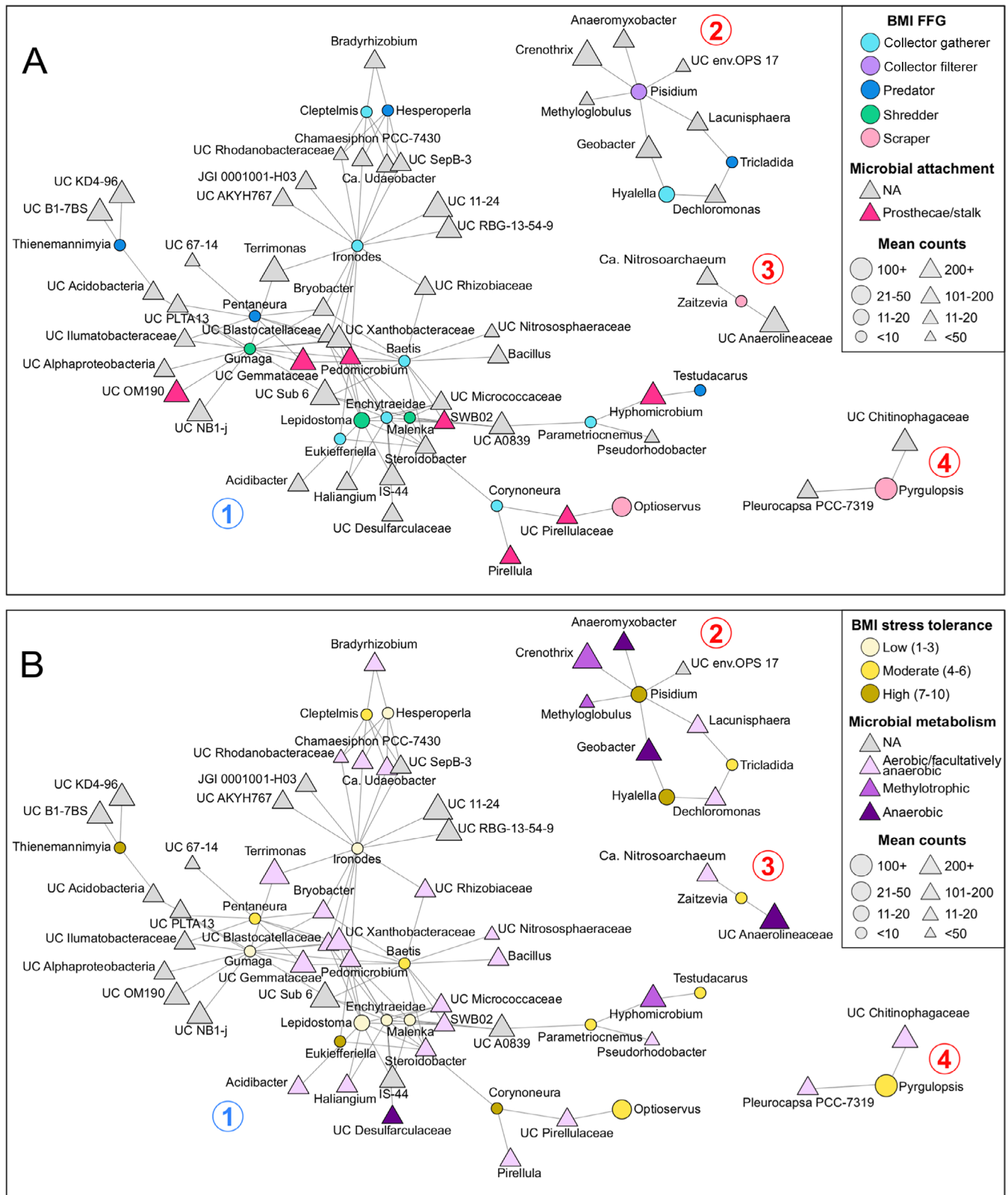


FIGURE 6 High connectivity of a benthic macroinvertebrate (BMI)-microbe network in roof pendant springs. A co-occurrence network map depicting the positive correlations identified following the Spearman's rank-order correlation analysis performed on relative abundance data from BMIs (circles) and microbes (triangles) in the 10 springs. Nodes are colored by (A) BMI functional feeding group (FFG) and microbial attachment or (B) BMI stress tolerance and microbial metabolism. Only significant ($p < 0.05$) comparisons and nodes with more than one edge are displayed. Major network clusters have been marked with numbers that have been colored to demonstrate the corresponding geochemical grouping.

benthic substrate (Davis & Barmuta, 1989) in addition to structuring both microbial (Battin et al., 2003) and BMI (Mérigoux & Dolédec, 2004; Rempel et al., 2000) communities. Fine grain sizes that dominate in low-flow springs slow oxygen diffusion, selecting for anaerobic microorganisms and spatially controlled redox cycles of C, N, S, and other elements (Nogaro et al., 2010). The roof-pendant-recharged springs tended to have higher current velocities and were appropriately enriched with bacteria inferred to be aerobic and facultatively anaerobic. This spring group also contained diverse, low or moderately stress-tolerant insect taxa, in line with the coarser substrates found in these springs. In contrast, the generally slower flowing granite-recharged springs were enriched with anaerobes, such as obligate fermenters, sulfate reducers, and methanogens, as well as the corresponding chemolithotrophs—sulfur oxidizers and methylotrophs (Figure 4). The granite-recharged springs also hosted highly uneven BMI communities dominated by moderate or high stress-tolerant noninsect taxa. Despite these trends, it seems unlikely that differences in current velocity are causally related to recharge through roof pendant rocks. Rather, these differences are characteristic of the individual springs and controlled by a combination of the slope of the spring source and the depth and location of associated faults. This explanation is consistent with exceptions to the stated relationship between slope/current velocity and spring type. For example, the current velocity at South Harry Birch Spring (IES-043, roof pendant spring) was among the slowest of any of the springs, whereas Unnamed Spring north of Red Mountain (IES-029, granitic spring) was among the fastest, as it emerges at a fault. Even with their exceptional current velocities, both microbial and BMI communities in these springs clustered with their respective geochemical group (Figure 3), underscoring our interpretation that the roof pendant-driven molar excess of Ca^{2+} is the primary factor structuring these communities.

A third alternative environmental control was spring water residence time, or the time elapsed between recharge and discharge. In general, older residence times are associated with warmer, more geochemically evolved water (Gascoyne, 2004; Manga, 2001). The specific physicochemical effects associated with increased residence time are highlighted as important factors structuring microbial communities in groundwater-fed ecosystems, such as streams (Zeglin et al., 2019) and aquifers (Ben Maamar et al., 2015; Yan et al., 2020). Additionally, temperature and salinity are well-known drivers of microbial community ecology across multiple biomes (Fierer & Jackson, 2006; Lozupone & Knight, 2007; Power et al., 2018; Thompson et al., 2017). In our study, spring residence time estimates varied from ~40 to

200 years for most springs, to over 1200 years for Lubken Canyon Spring 2 (IES-054) and Spring along Hogback Creek A (IES-024). Residence time correlated with the community dissimilarity of benthic microbes and macro-invertebrates in granitic springs, trending with vectors for specific conductivity and many individual ions diagnostic of increased water–rock interactions (Figure 3). These relationships, however, were subordinate to the geochemical groupings. Ultimately, although residence time appears to play some role in structuring the ecology of these communities, the molar excess of Ca^{2+} imparted by the roof pendants is the dominant factor.

CONCLUSIONS

This study answers the call for implementation of an integrated ecohydrogeology approach to better understand spring ecosystems (Cantonati, Segadelli, et al., 2020; Cantonati, Stevens, et al., 2020; Stevens et al., 2021). By using this approach, we demonstrated a strong ecological response of desert spring benthic communities across multiple trophic levels to subtle changes in spring geochemistry imparted by recharge through metamorphic roof pendants. This response is striking given the overall physicochemical similarity of these springs, as the flow path for both groups is predominantly through granitoid bedrock. We identified tightly connected community responses to roof pendant-derived elevated $\text{Ca}^{2+}/\text{Na}^{+}$ ratios, namely the enrichment of biofilm-associated and stalked/holdfast bacterial taxa alongside abundant, diverse populations of shredder and collector gatherer insects, in roof pendant springs. These interactions could potentially impact biomass and energy flux through the benthos and beyond to higher trophic levels in these springs and associated riparian areas. To further refine our understanding of the connections between spring hydrogeochemistry and ecology in Sierra Nevada springs and beyond, future studies could assess these ecological responses directly through experimental manipulation.

AUTHOR CONTRIBUTIONS

This study was conceived by Ariel D. Friel, Khaled Pordel, Zachary Meyers, Brian P. Hedlund, and Donald W. Sada. Samples for geochemistry measurements were collected and processed by Ariel D. Friel, Brian P. Hedlund, Zachary Meyers, Laura Rademacher, and Marty Frisbee. Microbial samples were collected and processed by Ariel D. Friel, Nicole J. Thomas, and Brian P. Hedlund, whereas Khaled Pordel and Donald W. Sada collected and processed BMI samples. Fred M. Phillips and Jeffrey R. Knott provided geological and hydrogeochemical interpretation of the data. Ariel D. Friel and Brian P. Hedlund

worked together to design the figures, but Ariel D. Friel was responsible for generating and editing all figures for the manuscript with assistance from Cale O. Seymour. Ariel D. Friel created all tables, which were edited by Brian P. Hedlund. All statistical analyses were run by Ariel D. Friel with assistance from Cale O. Seymour. Ariel D. Friel wrote the first draft of this manuscript, which was initially edited by Brian P. Hedlund. Subsequent drafts were edited by all authors.

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CONFLICT OF INTEREST STATEMENT

The authors of this manuscript declare that they have no commercial or financial relationships that could serve as a potential conflict of interest to this work.

DATA AVAILABILITY STATEMENT

Unprocessed microbial 16S rRNA gene sequences are available in the NCBI Sequence Read Archive (SRA) under the BioProject accession PRJNA788156. Novel R code and supporting data (Hedlund *et al.*, 2023) are available from Zenodo: <https://doi.org/10.5281/zenodo.7603891>.

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