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Bacterial Diversity in Replicated Hydrogen Sulfide-Rich Streams

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Abstract

Extreme environments typically require costly adaptations for survival, an attribute that often translates to an elevated influence of habitat conditions on biotic communities. Microbes, primarily bacteria, are successful colonizers of extreme environments worldwide, yet in many instances, the interplay between harsh conditions, dispersal, and microbial biogeography remains unclear. This lack of clarity is particularly true for habitats where extreme temperature is not the overarching stressor, highlighting a need for studies that focus on the role other primary stressors (e.g., toxicants) play in shaping biogeographic patterns. In this study, we leveraged a naturally paired stream system in southern Mexico to explore how elevated hydrogen sulfide (H_2S) influences microbial diversity. We sequenced a portion of the 16S rRNA gene using bacterial primers for water sampled from three geographically proximate pairings of streams with high (> 20 μM) or low (~ 0 μM) H_2S concentrations. After exploring bacterial diversity within and among sites, we compared our results to a previous study of macroinvertebrates and fish for the same sites. By spanning multiple organismal groups, we were able to illuminate how H_2S may differentially affect biodiversity. The presence of elevated H_2S had no effect on overall bacterial diversity ($p = 0.21$), a large effect on community composition (25.8% of variation explained, $p < 0.0001$), and variable influence depending upon the group—whether fish, macroinvertebrates, or bacteria—being considered. For bacterial diversity, we recovered nine abundant operational taxonomic units (OTUs) that comprised a core H_2S-rich stream microbiome in the region. Many H_2S-associated OTUs were members of the Epsilonproteobacteria and Gammaproteobacteria, which both have been implicated in endosymbiotic relationships between sulfur-oxidizing bacteria and eukaryotes, suggesting the potential for symbioses that remain to be discovered in these habitats.

Keywords 16S sequencing · Microbial ecology · Toxicity · Sulfur oxidation · Biogeography · Mexico

Introduction

Understanding the role of environmental conditions in shaping the distribution of organisms and the composition of communities is a prominent focus in ecological research. For plants and animals, clear biogeographic patterns arise from varied dispersal capacities and/or ecological selection that limits species persistence [1]. Biogeographic patterns are also evident for microbial life [2–5], contradicting the historical premise that microbes are globally distributed with no limits to their dispersal capacity [6]. Still, the degree to which microbes disperse and/or are environmentally selected, both in terms of individual taxa and communities, remains far from resolved [2, 3]. Perhaps nowhere is the influence of environmental variation on communities more apparent than in extreme habitats, where strong, temporally consistent forces (e.g., cold or toxicity) can drive significant variation in community composition across spatial scales [7]. Environmental variation among sites, however, tends to vary with geographic distance which can complicate our understanding of how environmental factors affect microbial communities, particularly when attempting to control for myriad other factors (including dispersal capacity). In this study, we took

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advantage of naturally paired streams within drainages that differ in their presence or absence of hydrogen sulfide (H_2S) to understand the influence of extreme conditions on diversity from bacteria to fishes.

Hydrogen sulfide is a naturally occurring, globally distributed environmental toxicant that significantly affects biogeographic patterns of microorganisms [8], macroinvertebrates [9], and fish [10] living in streams [11], caves [7], and deep-sea hydrothermal vents [4]. For eukaryotes, H_2S is highly toxic because it inhibits the mitochondrial respiratory chain [12, 13], and therefore, can substantially influence both total diversity and community assemblage patterns [9, 10, 14]. In contrast, H_2S can be a significant source of energy for prokaryotes [15], and the diverse metabolic plasticity of bacteria, particularly several genera in the Epsilonproteobacteria, have allowed them to colonize an array of H_2S-rich environments [7, 12, 16–18]. Though temperature, and not energy source, may ultimately set the limits for prokaryotic life [12, 19], the effects of elevated H_2S on prokaryotic communities in stream environments remains unclear, particularly over small spatial scales (e.g., within and between drainages). It has been hypothesized that geochemistry should act as a strong control on microbial communities in sulfidic springs [8], more so than general biogeographic processes like dispersion by water [20], wind [21], or hitchhiking on larger organisms [22].

Previous studies of microbial diversity in H_2S-rich streams have identified a core microbiome that appears largely free of biogeographic influence [8, 23, 24]. As expected, this core microbiome includes many sulfur-oxidizing, bacterial genera—*Hydrogenimonas*, *Sulfrovum*, *Thiotrix*, *Thiofaba*, and *Sulfurimonas*—belonging to two classes: Epsilonproteobacteria and Gammaproteobacteria [8]. However, these prior efforts primarily focused on the thick, filamentous microbial mats that form in stable spring environments (sulfidic or otherwise) instead of flowing water, a transient but important source of microbial cells to stream ecosystems [25]. The same previous investigations also focused on two ends of a broad geographic continuum—local, single springs [e.g., 23] and continental-scale comparisons of hydrologically distinct sites [8]. An intermediate scale remained to be explored: paired springs in hydrologically connected drainages, where the presence or absence of H_2S, and not geographic distance, is the overarching factor differentiating sites.

In this study, we characterized bacterial diversity of streamwater in three geographically proximate pairs of streams in the Rio Grijalva basin of southern Mexico which differed substantially in dissolved H_2S over small spatial scales (< 10 km). Each stream pairing included one stream with elevated H_2S and one without. While myriad things can vary in natural stream systems, we considered each sulfidic or non-sulfidic stream to be experimental replicates of one another because they were spatially and hydrologically constrained yet consistently differed in their concentrations of H_2S [26–28]. If bacterial communities differed consistently on such a small geographic scale, we could infer that differences are likely due to the effect of increased H_2S (and associated conditions: reduced pH, decreased dissolved oxygen, elevated solutes [26]) rather than the multitude of factors that vary regionally (e.g., over hundreds of kilometers). The influence of H_2S on fish and invertebrate communities has also been assessed for the same sites [9, 10], making it an ideal system for comparing the effects of H_2S on biodiversity across multiple organismal groups.

We addressed three questions: (1) Does total bacterial diversity and community makeup differ between adjacent sulfidic and non-sulfidic streams? (2) Does the influence of elevated H_2S vary among taxonomic groups (i.e., bacteria, macroinvertebrates, and fish)? (3) Is there a shared bacterial microbiome across sulfidic streams in southern Mexico? We hypothesized that H_2S-rich streams would be substantially less diverse and include a significantly altered bacterial community when compared to their non-sulfidic counterparts. This hypothesis stems from previous studies of microbial diversity in harsh stream environments (e.g., cold, alpine streams, [29]) and the effects of elevated H_2S on fish and invertebrates in the same streams [9]. If distance among sites (and not elevated H_2S) was driving patterns of community assemblage, we expected sites within drainages to be more similar to one another than sites in other drainages. We also predicted that elevated H_2S would affect organismal groups differently, perhaps due to variation in metabolic strategies. Finally, we expected the common harshness of elevated H_2S to yield a core bacterial microbiome across our study streams that generally aligned with previous studies (e.g., [8]) but perhaps included differences due to either microhabitats sampled (i.e., biofilm mats versus streamwater) or an effect of biogeographic regionalization. To address these questions, we characterized the bacterial community of filtered streamwater by sequencing a variable region of 16S rRNA genes and comparing our results to previous studies of macroinvertebrate and fish communities across the same sites. Taken together, this study addresses important gaps in our collective understanding of sulfidic stream biogeography, particularly in terms of how communities change across and within drainages that vary in H_2S concentrations, and how this variation differs among organismal groups.

Methods

Study Sites and Sample Collection

In May 2015, streamwater was sampled from three river drainages—Pichucalco (Pich), Puyacatengo (Puy), and Tacotalpa (Tac)—in the Rio Grijalva basin of southern Mexico (Table S1). We sampled two sites in each drainage: a sulfidic spring

with a high concentration of H_2S ($H_2S > 20$ μM) and a nearby non-sulfidic stream ($H_2S \approx 0$ μM). Elevated H_2S in the study region is most likely the product of bacterial sulfate reduction [28] but some contribution of volcanism from the Chichón Volcano may also be present [30]. All sulfidic sites were part of first-order springs and were sampled within ~200 m of where the springs discharge to the surface. Non-sulfidic sites were distributed along larger, likely third-order streams. Care was taken to avoid sampling non-sulfidic sites that were influenced upstream by sulfidic streams.

Measurements of H_2S concentrations, as well as four other environmental variables (temperature, pH, specific conductivity, and dissolved oxygen), were taken from Tobler et al., [26] (Table S1). To minimize temporal variability, environmental data was averaged across 2, 3, or 4 years depending on site [26]. Since previous studies have observed a pattern of continuously high H_2S concentrations through time for sulfidic streams in the Rio Grijalva basin (e.g. [27]), we are confident that our sulfidic/non-sulfidic classifications reflect temporally stable groupings. Environmental variation among sites was compared with a principal component analysis (PCA) of the five variables using the R function princomp after our data set was converted to a correlation matrix (Fig. S2). The average geographic distance between sites within a drainage was 6.8 km and the maximum distance between any two sites was 37.7 km (Fig. 1).

At each site, streamwater was collected from a depth of ~10 cm in the main channel. We took care to not dislodge any upstream benthic organisms or sediment while sampling. Two 3-L samples were separately collected at each site into sterilized, pre-rinsed (with water from the site) containers. Samples were immediately transferred to the local field station for filtering. We designed our filtering scheme based on [31] and used a modified bicycle pump system that could be easily disassembled and sterilized with ~20% bleach solution (Fig. S1). To capture the breadth of microbial diversity living in the water column, each 3-L sample was sequentially passed through three filter sizes: 10-μm (Pall Corporation, Ann Arbor, MI); 1.2-μm (GE Healthcare, Buckinghamshire, UK); and 0.45-μm (Merck Millipore Ltd., Cork, Ireland). Filters were folded and stored in 2-mL PowerWater DNA Bead Tubes (MO BIO Laboratories, Carlsbad, CA) at room temperature until DNA was extracted. This sampling scheme yielded 36 samples: 6 per site (three filter sizes with two field replicates each) for each of the six sites.

DNA Extraction, PCR Amplification, and Sequencing

DNA was extracted from sample filters using a DNeasy PowerWater Kit (MO BIO Laboratories, Carlsbad, CA) following the manufacturer protocol with one exception; we added a 10-min heating step post-lysis (65 °C). DNA concentrations were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) and extracted DNA was stored at − 80 °C until PCR was performed. For PCR, we used primer sets that targeted the variables V1–V3 regions of bacterial 16S rRNA genes (*Escherichia coli* positions 27F-534R; Table S2)

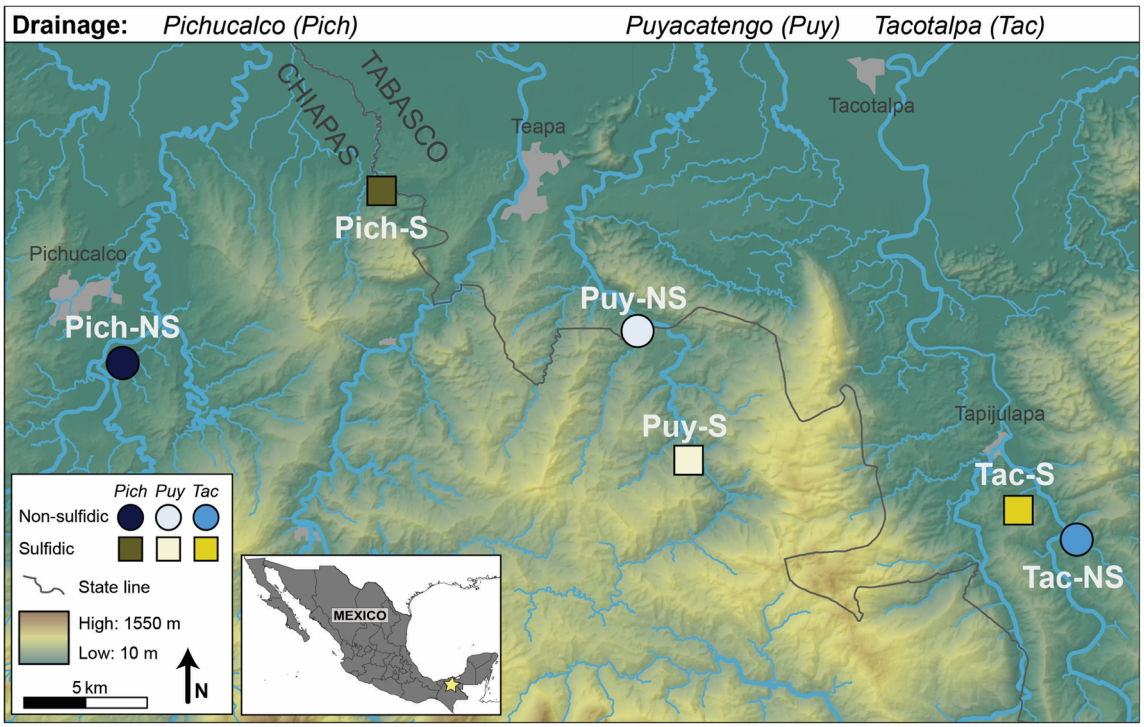

Fig. 1 Sampling locations within the Rio Grijalva drainage of southern Mexico. Sulfidic (S) and non-sulfidic (NS) sites were paired within drainages. The location of the study area is shown by the star in the inset map of Mexico

[32]. We employed a two-step PCR protocol provided by the Institute for Bioinformatics and Evolutionary Studies (IBEST) Genomics Core at the University of Idaho. The first step was a standard PCR reaction to amplify the target 16S rRNA region and included a single cycle of 60 s at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 51 °C, and 60 s at 68 °C, followed by a final 300-s cycle at 68 °C. This was followed by a second reaction to add dual-index barcodes and Illumina-specific sequencing adapters, which included a single cycle of 60 s at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 60 s at 68 °C, followed by a final 300-s cycle at 68 °C. Successful PCR reactions were confirmed by gel electrophoresis and pooled based on band visibility (i.e., less DNA was added to the final pool for amplicons with brighter bands). The final, pooled library was bead-cleaned with Agencourt AMPure XP beads (Beckman Coulter Inc., Brea, CA) and sequenced on a single Illumina MiSeq flowcell with 300-bp paired-end chemistry at Washington State University. Sequences were demultiplexed using the Illumina MiSeq pipeline.

Raw Sequence Processing and OTU Picking

Forward and reverse reads were merged in FLASH [33] with a minimum and maximum of 20- and 250-bp of overlap allowed, respectively. Reads that did not merge were not included in downstream analyses. Merged reads were filtered for quality using the Fastx-Toolkit [34] with reads retained only if their average quality score was ≥ 21 across 75% of the read and they contained no more than four low-quality (< 21) bases in a row. After read merging and quality filtering, all other analytical steps were performed within the QIIME v.1.8.0 [35] pipeline unless otherwise noted. Sequence files were converted from FASTQ to FASTA (convert_fastaqual_fastq.py), labeled for QIIME, and combined for downstream processing (add_qiime_labels.py). Operational taxonomic units (OTUs) were identified using the default 97% similarity threshold (pick_de_novo_otus.py), aligned sequences (align_seqs.py), and assigned taxonomy (assign_taxonomy.py) by comparing aligned sequences to the Greengenes reference database (version 13_8) [36]. After OTU picking, our database was filtered to remove singleton OTUs (i.e., those OTUs which had only one read mapped to them). Throughout the analytical process, we combined field replicates (or sites) to the highest level needed for a given analysis. For instance, if biodiversity among drainages was being calculated, site and field replicates were combined within drainages for those analyses.

Statistical Analyses

To assess biodiversity differences within and among groups, we calculated within site (α) diversity using the Shannon diversity index (H) [37] and taxonomic evenness with the QIIME metric "equitability." We also estimated differences in bacterial communities (β diversity) between habitat types (presence or absence of H_2S) and among drainages using Bray-Curtis distances (B-C). We constructed plots to visualize β diversity across samples using beta_diversity_through_plots.py. For all biodiversity metrics, we rarefied samples to 21,281 sequences, the lowest number of reads recovered for all libraries after quality filtering and singleton removal. We summarized samples by taxonomic groups (phyla to species) using summarize_diversity_through_plots.py. To compare total α diversity among groups, we conducted a two-sample, non-parametric t test with H as our diversity metric and 1000 test permutations.

To determine if bacterial communities differed with the presence or absence of H_2S, we performed adonis analyses (analogous to a PERMANOVA) using the R package "vegan" [38]. We designed our statistical model to incorporate individual factors sequentially so that their relative effects (i.e., the amount of total variation they explain) could be compared. Habitat type, drainage, and filter size were included as interaction terms. In a separate test, we assessed whether individual field replicates at a given site differed from one another by only allowing permutations to occur within sites (using strata) thereby focusing the test on within site replication. For all adonis analyses, a non-parametric approach was used to partition B-C distance among groups and we assessed the strength and significance of groupings with 1000 permutations.

We quantified β diversity dispersion (the distance to the centroid of defined clusters when visualized as dissimilarity distances) by first converting our OTU matrix to B-C distances with the function vegdist followed by dispersion calculation with betadisper. Both functions are part of the R package vegan [38]. Dispersal distances provide a proxy for how distinct samples are from one another within groups of interest. We tested the null hypothesis—that the average distances to the centroid of two groups are the same (meaning samples within each group are equally different from one another)—using 1000 permutations of a non-parametric test. We compared mean dispersal distances for three groups: habitat type, drainage, and sites. To identify taxa that had an outsized influence over observed patterns of β diversity dissimilarity between sulfidic and non-sulfidic sites, we used the function simper, another component of the vegan package [38]. Simper decomposes B-C dissimilarity matrices to quantify the contribution of individual taxa (in this case, OTUs) to overall patterns of dissimilarity.

A phylogenetic clustering approach allowed us to assess the degree to which species in sulfidic or non-sulfidic streams are more or less related to one another relative to the other stream type. To do this, a phylogeny of the 10,000 most abundant OTUs in our data set was constructed using FastTree [39]. Next, we compared phylogenetic distances among the

250 most abundant OTUs in sulfidic and non-sulfidic streams, respectively. Two summary metrics were calculated: the net relatedness index (NRI) and nearest taxon index (NTI). NRI indicates how clustered dominant OTUs are in each habitat type, while NTI is an indicator of how much terminal clustering is present for given set of taxa on a phylogenetic tree [40]. These analyses were performed for 1000 iterations using the QIIME script relatedness.py.

Comparing Diversity across Organismal Groups

To investigate how our results aligned with similar studies of fish and macroinvertebrate diversity in the same streams, we incorporated relevant results from Greenway et al. [9]. Briefly, the relevant methods from that study were as follows. Macroinvertebrate communities were quantitatively sampled using a Surber sampler at multiple locations for each stream site. A subset of sites were re-sampled later in the same year and values were averaged across time points. Specimens were identified to the lowest taxonomic level possible (typically genus) and Shannon diversity (*H*) was calculated for each site [9]. Fish communities were assessed by seine sampling and also identified to the lowest taxonomic level possible (typically species). Fishes were sampled 1–13 times from 2004 to 2013 and totals (species, genera, etc.) were summed through time for each site [9]. To make comparisons across all three groups (fish, macroinvertebrates, and bacteria), we used number of genera observed for each group. We also compared *H* for macroinvertebrates and bacteria only. For all comparisons, only data from the same sites surveyed in both studies were used.

Identifying Dominant Taxa and a Core Bacteriome in H_2S-Rich Streams

To visualize how taxon abundance and frequency corresponded with groupings (in this case, habitat type or drainage), we constructed either ternary plots (three groups) or line plots (two groups) with the R package ggtern [41]. For ternary plots, we used a 20% threshold to specify whether a taxonomic grouping was unique to one or two groups or shared among all three. We focused on the most abundant phyla, orders, and families, for three comparisons: (1) Phyla, orders, and families in sulfidic versus non-sulfidic sites as a proxy for the taxa most strongly associated with elevated H_2S, irrespective of drainage or site. (2) We then compared abundance of the most common families across drainages within groups (with or without H_2S). (3) To investigate the influence of our sequential filtering scheme on recovered diversity, we compared the abundance of the most common families for samples grouped by filter size (10, 1.2, and 0.45 μm). For an OTU to be included in the core H_2S-rich stream bacterial microbiome, it had to be at greater than 1% frequency overall and present in all three sulfidic sites.

Results

We generated 11,629,959 raw sequences for 36 libraries. After read merging, quality filtering, and the removal of singleton OTUs, we retained 1,935,870 reads with an average of 53,774 reads per library (min. = 3314, max. = 150,264; Table S4). Raw sequence data have been archived in GenBank under the BioProject #PRJNA419935. One library (20) was removed from downstream analyses because the number of reads retained (3314) was more than two standard deviations below the mean for all other libraries (Table S4). We determined sequencing depth to be sufficient for appropriately resolving the amount of diversity present based upon rarefaction curves of Shannon diversity (*H*), which showed little to no additional diversity discovered after ~ 10,000 sequences per sample. Overall, we identified 147,566 OTUs that were classified into 40 phyla, 122 classes, 256 families, and 859 genera (Table S5).

Study sites clearly partitioned into two environmental groups that aligned with the presence or absence of H_2S, which varied from 23.7 and 26.2 μM in Tac and Puy, respectively, to 190.4 μM in Pich (Table S3; Fig. S2). Sulfidic streams were also more acidic ($pH_{NS} = 7.9$ versus $pH_S = 7.1$), had higher specific conductivity ($SPC_{NS} = 1.14$ mS/cm versus $SPC_S = 3.07$ mS/cm), and contained less dissolved oxygen than non-sulfidic sites ($O_{2,NS} = 7.13$ mg/L versus $O_{2,S} = 1.3$ mg/L; Table S3). Only temperature appeared unrelated to the sulfidic/non-sulfidic groupings (with co-varying pH, O_2, and SPC). However, with no clear temperature break separating groups, and a relatively small temperature gradient among sites (5.1 °C between the warmest and coldest sites), we did not pursue temperature-associated bacterial partitioning any further (Table S3; Fig. S2). Our sequential filtering scheme captured a wide breadth of diversity, with many abundant families observed across all three filter sizes. However, at the large end, the initial 10-μm filtering removed almost all of the diversity associated with two families: *Peptostreptococcaceae* and *Chromatiaceae*. Three families—*Campylobacteraceae*, *Microbacteriaceae*, *Bradyrhizobiaceae*—were primarily recovered with only the 0.45-μm filter (Fig. S3).

Bacterial Diversity

We observed no difference in total bacterial diversity for sulfidic (mean $H_S = 8.64$) versus non-sulfidic sites (mean $H_{NS} = 9.48$; $p = 0.21$; Fig. 2a). There was also no difference in taxonomic evenness (0.71 versus 0.76; Table S5). The amount of diversity collected by the first round of filtering

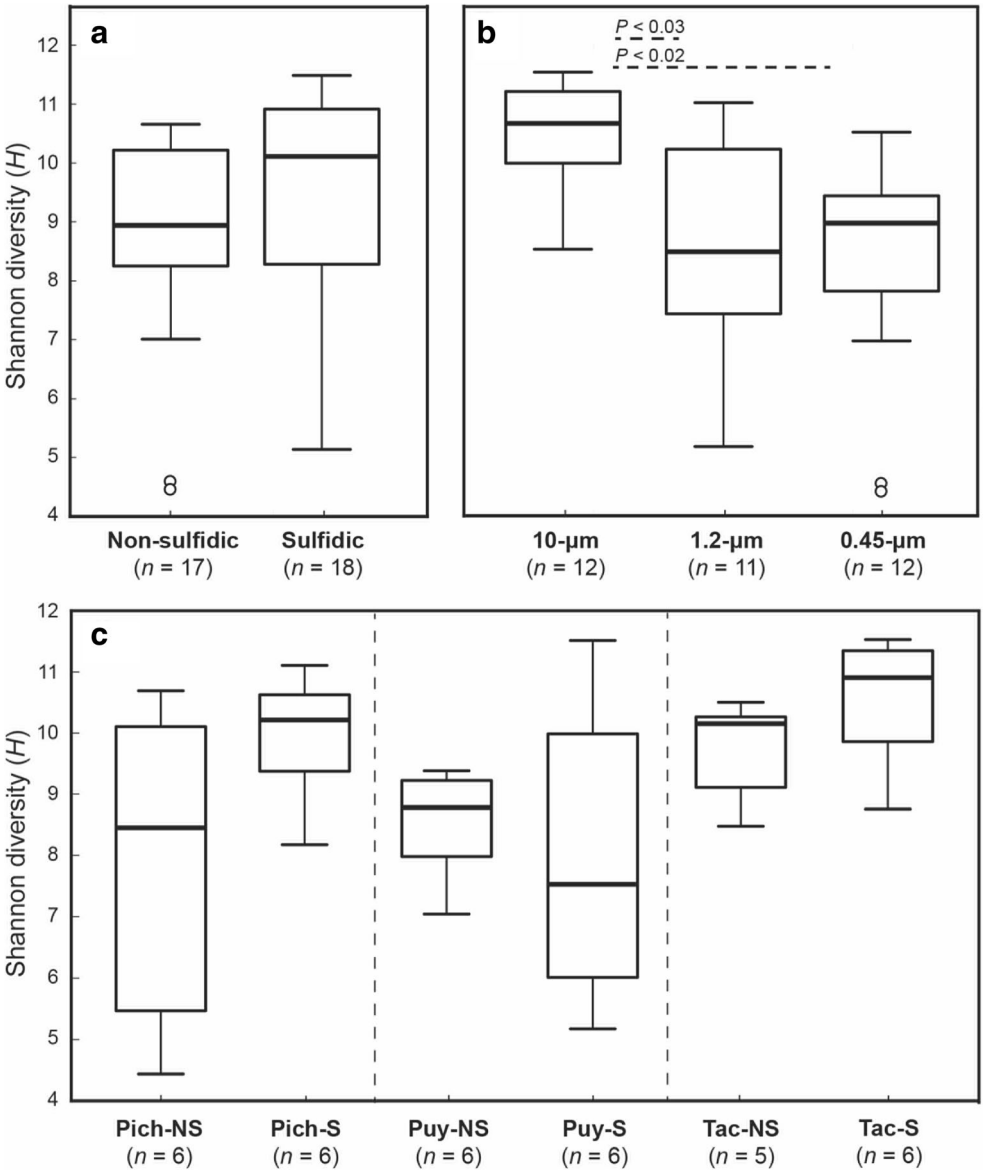

Fig. 2 Box-and-whisker plots of alpha diversity (Shannon diversity index, *H*) calculated for **a** habitat type, **b** filter size, and **c** site. Sample size is listed below each entry. Significance is denoted when values were less than $P = 0.05$. Outliers are denoted by small circles

(10 µm) was significantly higher than the diversity obtained from either other size: 1.2 µm ($p = 0.03$) and 0.45 µm ($p = 0.02$; Fig. 2b). Among sites, we observed some variation in total diversity, ranging from the highest diversity in Tac-NS ($H = 10.51$) to the lowest in Pich-S ($H = 7.86$), but no between-site comparisons of total bacterial diversity amounted to significant differentiation (Table S5, Fig. 2c).

We observed a clear influence of H_2S on bacterial community composition (Fig. 3) with the presence of H_2S explaining the highest proportion of variation among sites (25.8%, $p < 0.0001$). Drainage ($p = 0.0003$, 9.1% of variation) and filter size ($p = 0.0042$, 7% of variation) were also substantial sources of variation (Table 1). Field replicates (diversity recovered from all three filter sizes combined and compared for each site) were not significantly different from one another ($p = 0.943$). When viewed in principal coordinate space, patterns of β diversity aligned with these statistical conclusions with samples clearly partitioning into sulfidic and non-sulfidic clusters (Fig. 3). No significant differences were observed for mean dispersion (a proxy for how different samples are within groups) among samples regardless of grouping, whether habitat type (mean B-C = 0.40–0.42), drainage (mean B-C = 0.44–0.47), or site (mean B-C = 0.25–0.44; Table S6).

Taxa that were most abundant in sulfidic streams were overdispersed (not clumped closely with one another) across a phylogeny of the 10,000 most abundant OTUs (NRI < 0) and more clustered at the tips (NTI > 0). In non-sulfidic streams, taxa were clustered at deeper phylogenetic scales, with no evidence for overdispersion (NRI and NTI > 0; Table 2).

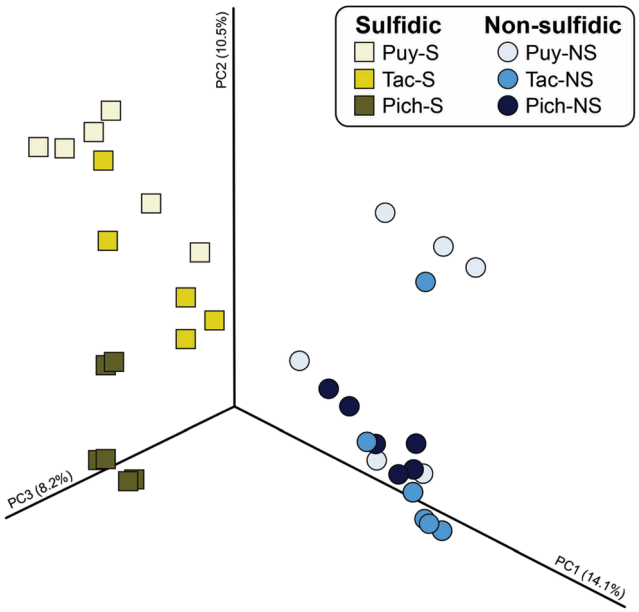

Fig. 3 Beta diversity for all samples in three-dimensional principal coordinates space for the first three axes. Samples are coded for each habitat type by shape (square = sulfidic; circle = non-sulfidic) and by drainage with color (light = Puy, medium = Tac, dark = Pich). Samples were rarefied to 21,281 reads. Input data were OTUs and dissimilarity distances between samples are Bray-Curtis

Dominant Taxa

Regardless of habitat type, drainage, or site, the bacterial community was dominated by *Proteobacteria* (Fig. 4a), but clear differences emerged within this phylum. For sulfidic sites, ~ 60% of sequences assigned to three orders: *Campylobacterales* (43.3%), *Acidithiobacillales* (10.7%), and *Burkholderiales* (5.1%; Fig. 4b). Conversely, the top three orders found in non-sulfidic sites accounted for just 25.4% of total sequences: *Pseudomonadales* (9.9%), *Sphingomonodales* (9.5%), and *Actinomycetales* (5.9%; Fig. 4b). At the family level, 35.5% of sequences obtained from sulfidic sites belonged to the *Helicobacteraceae* (including 27.1% assigning to an unclassified OTU; Fig. 4c). In non-sulfidic sites, there was a more equitable distribution of family-level sequence assignments with 9% assigned to *Pseudomonadaceae* and 7% to *Sphingomonadaceae* (Fig. 4c). There was also a substantial difference among stream types in the percentage of sequences that did not map to a known OTU in the Greengenes database and were classified as "Other": sulfidic (18.5%) and non-sulfidic (38.6%; Figs. 4 and 5).

When abundances of the most common families in each habitat type were directly compared, dramatic differences were evident. For instance, we observed ~ 4700 times more *Acidithiobacillaceae*-associated sequences in sulfidic versus non-sulfidic sites. Other families enriched in sulfidic sites included *Aeromonadaceae* (56.5×), *Halothiobacillaceae* (42×), *Helicobacteraceae* (30×), and *Campylobacteraceae* (28.8×; Fig. 4). A different subset of families were enriched in non-sulfidic sites: *Erythrobacteraceae* (343.8×), *Cytophagaceae* (248.2×), and *Microbacteriaceae* (233.6×; Fig. 4). When sulfidic and non-sulfidic communities were compared at the genus level, just 10 (of 859) OTUs explained 56% of the dissimilarity between the two groups (Table 3).

Using our 20% threshold for classifying taxonomic groups as either specific to a given drainage (or drainages) or shared among all drainages, we identified a number of families that were drainage-specific (e.g., *Sinobacteraceae*, Pich; Fig. 5a or *Chlorobiaceae*, Puy; Fig. 5b). We also identified many examples of families shared by two drainages, including *Acidithibacillaceae*, which was almost exclusively found in sulfidic sites within the Puyacatengo and Tacotalpa drainages (Fig. 5b).

Table 1 Results of a permutational multivariate analysis of variance (PERMANOVA) used to infer the amount of observed variation among samples as explained by a given term

Term	Variation explained (%)	*p* value
Habitat type	25.8	< 0.0001
Drainage	9.1	0.0003
Filter	7.0	0.0042
Habitat type/drainage	9.1	0.0002
Habitat type/filter	5.9	0.011
Drainage/filter	9.9	0.014
Habitat type/drainage/filter	9.5	0.018

Also included are tests of interactions among terms. Terms were added in the order they appear in the table, which corresponded with the levels of our experimental design. Dissimilarity among samples was quantified with Bray-Curtis distances and significance was assessed with 10,000 permutations. Model residuals explained the remaining 23.7% of total variation

Comparisons to Fish and Macroinvertebrates

When bacterial diversity was compared to similar estimates of fish and macroinvertebrate diversity across the same sites, substantial differences in how the presence of elevated H_2S affects total biodiversity were evident. Elevated H_2S reduces the richness of fish species and invertebrate genera by 88.1 and 95.4%, respectively (Table 4; [9, 10]). For bacterial diversity, however, elevated H_2S only translates to a 32% decline in the number of genera for the same comparison (Table 4). For *H*, macroinvertebrate communities in sulfidic sites were 95.5% less diverse than their non-sulfidic counterparts whereas bacterial communities were only 8.9% less diverse for the same comparison ($p = 0.21$; Table S7).

Sulfidic Stream Bacterial Microbiome

We were particularly interested in taxa shared among all sulfidic sites (but absent or present at very low frequencies

Table 2 Phylogenetic structure of the 250 most abundant taxa in sulfidic and non-sulfidic streams

Habitat type	NRI	NTI
Sulfidic	− 4.09	7.44
Non-sulfidic	7.53	3.98

NRI indicates the degree to which taxa are clustered, while NTI is an indicator of how much terminal clustering is present for focal taxa on the phylogenetic tree. Both metrics become negative with overdispersion (or repulsion) when taxa are more distant from one another than expected

NRI net relatedness index, *NTI* nearest taxon index

in non-sulfidic sites), as these taxa may represent the core members of a common bacteriome in sulfidic springs of southern Mexico. This putative core group included four phyla (Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes); six classes (*Alphaproteobacteria*, *Betaproteobacteria*, *Epsilonproteobacteria*, *Gammaproteobacteria*, *Clostridia*, *Actinobacteria*), 12 families, 11 genera, and 13 species (Table 5), each of which could only be classified to the taxonomic level provided. Five families (*Xanthomonadaceae*, *Comamonadaceae*, *Rhodobacteraceae*, *Moraxellaceae*, *Pseudomonadaceae*) were shared among all non-sulfidic sites, suggesting they are not specific to H_2S-rich springs, leaving seven families in our core sulfidic-site bacteriome: *Helicobacteraceae*, *Campylobacteraceae*, *Sphingomonadaceae*, *Clostridiaceae*, *Enterobacteriaceaea*, *Halothiobacillaceae*, and *Neisseriaceae*. Of these, five genera or species were at greater than 1% frequency: an undescribed OTU within *Helicobactetacae* (27.1% of all reads), another undescribed OTU within *Enterobacteriaceaea* (1.5%), *Sulfuricurvum kujiense* (4.2%), and two putatively species-level OTUs, *Sulfurimonas* sp. (4.2%) and *Arcobacter* sp. (2.1%).

Discussion

Understanding the degree to which environmental pressures and dispersal capacities shape biotic communities is an important focus of biogeographic research [3, 42]. Extreme environments provide natural models for studying these effects as they simplify the myriad of factors controlling the distributions of organisms—whether macro- or microscopic—and allow for conclusions to be drawn about specific, often clear, environmental influences. In this study, we investigated how elevated H_2S affects microbial communities in H_2S-rich streams of southern Mexico. This system provided a naturally replicated model for comparing biodiversity of sulfidic and non-sulfidic streams across a small spatial scale. A comparable experimental design for the sites has also been used to study the effects of H_2S on macroinvertebrate and fish communities [9, 10], which allowed us to extend previous knowledge of the system to include patterns of community structure across multiple organismal groups. We focused our efforts on the biotic effects of H_2S, rather than other environmental variables, because a suite of other factors (pH, O_2, SPC) vary substantially (and consistently) with the presence or absence of H_2S [26].

The Influence of H_2S on Biotic Communities

We found no support for our prediction that elevated H_2S would reduce bacterial diversity in sulfidic streams. This runs counter to other studies of microbial diversity in harsh

Table 3 Contribution of the most influential bacterial taxa to overall patterns of Bray-Curtis dissimilarity observed between sulfidic and non-sulfidic sites

Order	Family	Genus	Dissimilarity explained (%)
Campylobacterales	*Helicobacteraceae*		16
Other			11
Acidithiobacillales	*Acidithiobacillaceae*	*Acidithiobacillus*	7
Pseudomonadales	*Pseudomonadaceae*	*Pseudomonas*	7
Campylobacterales	*Campylobacteraceae*		3
Campylobacterales	*Helicobacteraceae*	*Sulfurimonas*	3
Actinomycetales	*Microbacteriaceae*		3
Campylobacterales	*Helicobacteraceae*	*Sulfuricurvum*	3
Burkholderiales	*Comamonadaceae*	*Thiomonas*	2
Stramenopiles			2
		Total	56

"Other" indicates a catch-all taxonomic category for OTUs that do not match an existing OTU in the GreenGenes database at >97% similarity. Each line in the table refers to a specific putative cluster and the lowest taxonomic classification is given. For instance, the first entry refers to a cluster in the family *Helicobacteraceae* with no lower taxonomic classification information available

Table 4 Taxonomic diversity across study sites and habitat types included in this study

Site	Fish species	Invertebrate genera	Bacterial genera
Tac-NS	16	8.6	567
Tac-S	1	0.4	366
Pich-NS	15	6.6	544
Pich-S	2	N/A	478
Puy-NS	11	N/A	605
Puy-S	2	0.3	323
Mean NS	14	7.6	572
Mean S	1.67	0.35	389
Difference	− 88.1%	− 95.4%	− 32.0%

Fish and invertebrate were data taken from Greenway et al. [9]. Number of invertebrate genera per site is the average observed per square meter across sampling events

environments, where diversity tends to decline with escalating environmental stress (e.g., glacier-fed streams, [29, 43]). Hence, H_2S may not be particularly toxic for bacteria, or there are at least enough species tolerating (or exploiting) H_2S that its presence does not greatly reduce total diversity. Elevated H_2S does, however, drastically alter community composition. We found strong support for our prediction that elevated H_2S would be the most influential factor explaining bacterial community composition, rather than drainage (a proxy for geographic distance and thereby dispersal potential) or any other factors (e.g., filter size). Other examples from a variety of habitats and levels of taxonomic focus support the hypothesis that harsh environmental conditions (e.g., cold temperature, high toxicity) translate to substantial differences in community composition [2, 29, 44, 45], including communities of deep-sea hydrothermal vents that also exhibit substantial variation in H_2S concentration over small spatial scales (e.g., [4]).

Elevated H_2S clearly affects animal (fish, macroinvertebrates) and bacterial communities differently. For fish and macroinvertebrates, elevated H_2S translates to > 88% declines in both the number of species (fish), genera (macroinvertebrates), and *H* (macroinvertebrates) with correspondingly modest or non-existent declines for bacteria (Table S7). Though percent declines across organismal groupings are not perfectly comparable across groups because species concepts developed for eukaryotes apply poorly to prokaryotic taxa [46], *H* is a more directly comparable metric, and has been used previously for similar eukaryote-prokaryote comparisons [47]. Such starkly different effects of elevated H_2S may be twofold in origin: first, while microbes may not be the absolute dispersers once hypothesized, their means for dispersing are still much greater than those available to macroinvertebrates and fish [48], and as such, H_2S-tolerant taxa can spread across broad geographic scales to colonize locally restricted H_2S-rich streams. However, this hypothesis should be considered with caution in light of how body size and dispersal capacity may interact. A previous study suggested that the

Table 5 The core sulfidic spring bacterial microbiome identified in this study

Phyla	Classes	Families	Genera
Proteobacteria	*Epsilonproteobacteria*	*Helicobacteraceae*	Undescribed
			Sulfurimonas
			Sulfuricurvum
		Campylobacteraceae	Undescribed
			Arcobacter
	Gammaproteobacteria	*Acidithiobacillaceae*	*Acidithiobacillus*
		Pseudomonadaceae	*Pseudomonas*
		Enterobacteriaceae	Undescribed
		Aeromonadaceae	
		Halothiobacillaceae	
		Moraxellaceae	
	Betaproteobacteria	*Comamonadaceae*	*Thiomonas*
	Alphaproteobacteria	*Acetobacteraceae*	
Firmicutes	*Clostridia*	*Clostridiaceae*	
		Peptostreptococcaceae	Undescribed
Actinobacteria	*Actinobacteria*	*Mycobacteriaceae*	Undescribed
Bacteroidetes			

We included groups at each taxonomic level that fit two criteria: (1) present at greater than 1% frequency and (2) observed at all three sulfidic sites. In cases such as *Bacteroidetes*, nothing lower than the phyla is given because while the phyla was present at greater than 1% frequency, no class, family, or genus in *Bacteroidetes* also exceeded the 1% frequency. "Undescribed" refers to a single genus

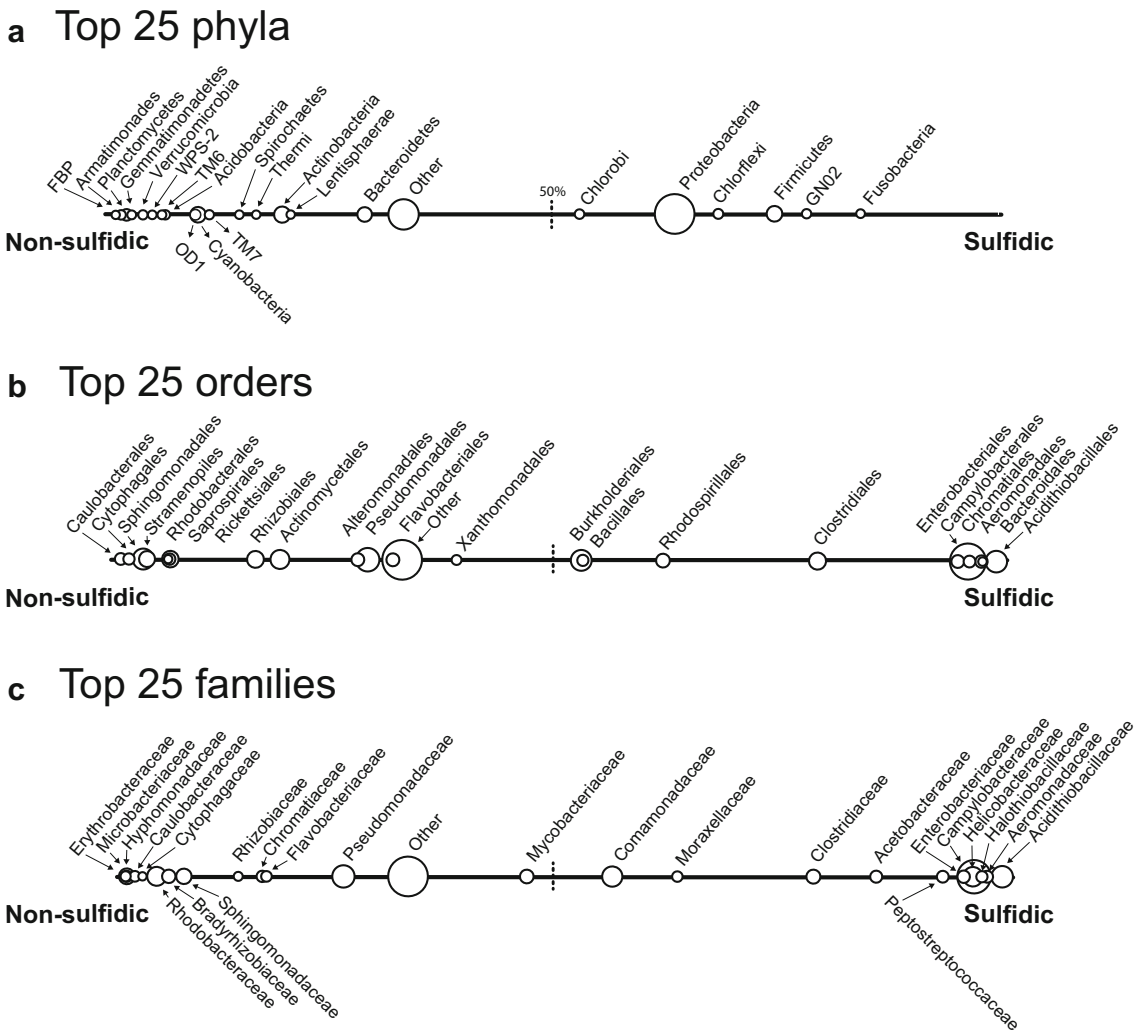

Fig. 4 Distribution of the top 25 most common taxa for **a** phyla, **b** orders, and **c** families by habitat type [non-sulfidic or sulfidic]. Position along the line is based upon taxon abundance in each habitat. For instance, circles near 50% are equally represented in both sulfidic and non-sulfidic streams. Circle size indicates the relative abundance of each taxon overall

breakpoint where body size greatly limits dispersal is actually between invertebrates and vertebrates rather than between unicellular and multicellular organisms as generally assumed [49]. Second, unlike eukaryotes, many bacteria actively metabolize H_2S and contain respiratory oxidases that are resistant to H_2S inhibition [12]—thus making the presence of elevated H_2S a resource, rather than a limitation. Therefore, the metabolic flexibility of prokaryotes paired with the diversity of microbial taxa worldwide are likely the key factors driving the substantial differences in total diversity of bacteria versus fish and macroinvertebrates in H_2S-rich streams.

The H_2S-Rich Spring Bacterial Microbiome

The flowing water of sulfidic springs in southern Mexico support a diverse, spatially consistent bacterial community with a significant portion of resident diversity exclusive to these habitats (or at least not present beyond trace occurrences in nearby non-sulfidic streams). Headd and Engel [8] posited that the assemblage of microbiota in sulfidic spring habitats should be controlled by a combination of spring geochemistry and surface/sub-surface transport mechanisms, rather than geography. Our findings support this, as geographic proximity among sites had little effect on community structure. The dominance of Proteobacteria in H_2S-rich environments is also not surprising as many sulfur-oxidizing bacteria belong to the class *Epsilonproteobacteria* [8, 17, 18, 50–54]. Outside of Proteobacteria, we observed comparatively small, but non-negligible (~ 1% of total diversity) populations of two unclassified OTUs: one in the family *Peptostreptococcaceae* (phylum Firmicutes) and a *Mycobacterium* sp. (phylum Actinobacteria). While both phyla are common in a wide array of environments, neither has any well-established connection with sulfidic streams [55] aside from thick-walled mycobacteria being

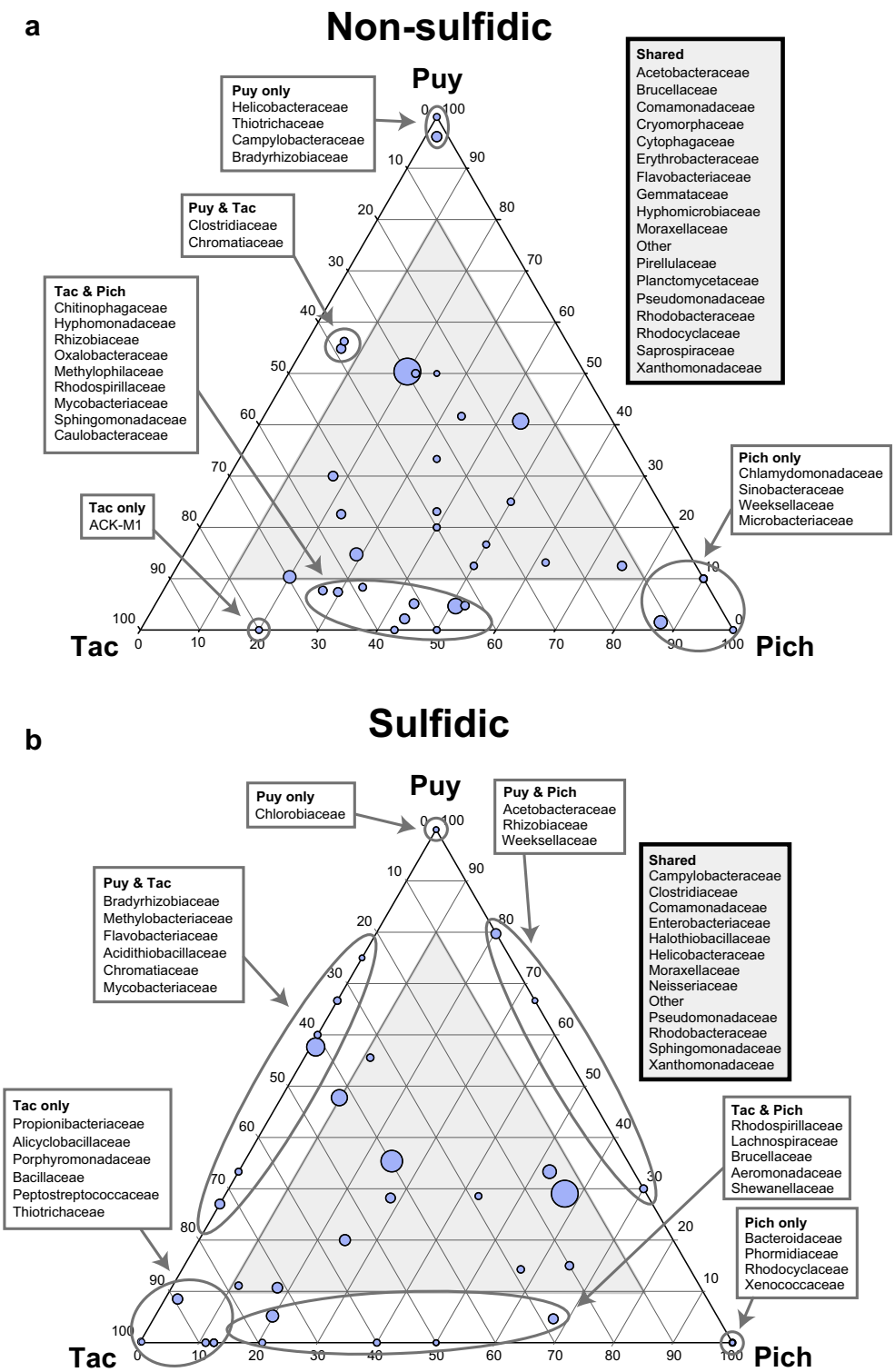

Fig. 5 Distribution of the top 40 most abundant families among drainages for **a** non-sulfidic and **b** sulfidic sites. The relative position of a given point indicates the percentage of the reads associated with each taxon from a given drainage. Circle size represents the relative abundance of a specific taxon overall. The light gray triangle indicates "shared" taxa (i.e., those taxa with no less than 20% representation in all three drainages)

commonly associated with acidic conditions [56, 57], a typical characteristic (pH < ~7) of sulfidic streams [26].

Within the Proteobacteria, we identified three enriched, but unclassified OTUs: one each in the families *Helicobacteraceae* (27.1% overall), *Campylobacteraceae* (5.3%), and *Enterobacteraceae* (1.5%). Sulfur-oxidizing species can be found in all three families, and many Helicobacteraceae taxa exhibit endosymbiotic relationships with host organisms (see below). However, with no additional taxonomic or functional information, we can only conclude

that these species are abundant and may play a functional role associated with elevated H_2S, whether as producers, metabolizers, endosymbiotic mediators, or some combination of all three. At finer taxonomic scale, six OTUs were particularly enriched in H_2S streams: two *Acidithiobacillus* spp., *Sulfuricurvum kujiense*, *Sulfurimonas* sp., *Thiomonas* sp., and *Arctobacter* sp. All are candidate sulfur oxidizers in these streams [58–61]. *Arcobacter* have been found in sulfidic cave springs [62], but species in the genus are also associated with high levels of fecal pollution [63]. Elevated fecal pollution drives eutrophication and anoxic zones in streams, thereby favoring sulfur reducers which produce H_2S, making it difficult to disentangle the biotic underpinnings of *Arcobacter* enrichment in our study sites. It should be noted, however, that while we identified OTUs to the lowest taxonomic level available, our findings should be interpreted in the context of the limited resolution of 16S sequencing versus metagenomic data and therefore, the inherent challenge of identifying taxa below the family level [64].

Our efforts to clarify the sulfidic stream bacterial microbiome aligned well with the most similar effort by Headd and Engel [8]. In that study, the authors identified eight bacterial OTUs that made up the core sulfidic spring microbiome: one Epsilonproteobacteria OTU and seven *Gammaproteobacteria* [8]. We also recovered eight core OTUs from those classes, as well as one each from *Betaproteobactera* and *Alphaproteobactera*, two from *Clostridia*, and one each from *Actinobacteria* and *Bacteroidetes* (Table 5). Interestingly, both Headd and Engel [8] and others [62, 65] have reported abundant *Thiothrix* spp. sequences in sulfidic springs. While we did recover sequences associated with the genus, they were at low-frequency overall (~ 0.0015%) and equally common in non-sulfidic sites, indicating that at least in H_2S-rich streamwater of southern Mexico, *Thiothrix* spp. is not a member of the core microbiome.

Potential Links between Sulfur-Oxidizing Microbiota and Vertebrate Extremophiles

Of significant interest beyond microbial ecology are the physiological adaptations required for organisms, including vertebrates, to colonize extreme environments. Many poeciliid fishes can survive an array of extreme conditions making them interesting models for eco-evolutionary research [66, 67]. In southern Mexico, the colonization of sulfidic springs by poeciliids has been well-studied [26, 68–70]. Locally adapted fish populations primarily feed on microbial mats in sulfidic springs, and as such, sulfur-oxidizing microbes likely represent an important source of organic carbon and nitrogen [71, 72].

Trophic interactions aside, additional bacterial-vertebrate connections may be important. Symbiotic relationships between microbes and eukaryotic hosts have been described from H_2S-rich marine habitats [73–75] and range from surface-level to intracellular associations [74]. In gilled residents of H_2S-rich environments, endosymbiotic relationships typically involve sulfur-oxidizing *Epsilonproteobacteria* or *Gammapreoteobacteria* living within or on the surface of the host's gill epithelial tissue [52, 76, 77]. These bacterial symbionts are probably either sulfur detoxifiers, effectively limiting the host's exposure to the aerobic inhibition of H_2S, or nutritional symbionts, providing a food source in otherwise resource-limited habitats. While no H_2S-associated endosymbiotic relationships between bacteria and vertebrates have been described to date, it is unclear if this is because the relationships do not exist or that H_2S-tolerant vertebrates and their microbiota have been understudied [10]. While speculative, it is intriguing that the most abundant members of the core microbiome identified in this study belonged to the family *Helicobacteraceae*, a group known to contain marine gastropod endosymbionts living near H_2S-rich deep-sea vents [77]. Their abundance in our study streams raises the possibility that similar links may also be present in sulfidic streams. Of course, highly abundant taxa are not the only place to look for endosymbiotic relationships, as rare taxa can also be important in the right context (see [52]). Because we did not characterize bacterial communities of relevant vertebrate tissues (e.g., fish gill epithelia), it is also possible that species' abundances in streamwater do not align with abundances on or within potential hosts in the same habitats.

Conclusions and Future Directions

In this study, we identified clear differences in bacterial community composition between sulfidic and non-sulfidic streams of southern Mexico. The primary factor driving these differences was the presence or absence of elevated H_2S (> 20 μM), a finding that has also been observed for both macroinvertebrate and fish communities in the same sites. Taken together, our results support the notion that extreme conditions filter biological communities from micro- to macroscopic organismal scales [10, 25, 78, 79]. Future studies employing metagenomic tools to survey a greater diversity of microhabitats in these streams (e.g., sediments and biofilms and/or relevant vertebrate tissues), perhaps on temporal time scales or along H_2S concentration gradients, will no doubt yield a more refined perspective of microbial diversity and function in these ecosystems. The identification of several highly abundant taxa belonging to the family *Helicobactereracae* raises the intriguing possibility of prokaryote-vertebrate endosymbiotic connections remaining to be discovered. Finally, our results add another line of evidence to the broad theme in modern microbial ecology that the discovery of extensive undescribed diversity will continue as high-throughput sequencing methods and downstream classification pipelines are applied to an increasingly broad swath of habitats on Earth [80, 81].

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