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Microbial assemblages reflect environmental heterogeneity in alpine streams

Running Title: Microbial diversity in alpine streams

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Paper type: Primary Research Article

Abstract:

Alpine streams are dynamic habitats harboring substantial biodiversity across small spatial extents. The diversity of alpine stream biota is largely reflective of environmental heterogeneity stemming from varying hydrological sources. Globally, alpine stream diversity is under threat as meltwater sources recede and stream conditions become increasingly homogeneous. Much attention has been devoted to macroinvertebrate diversity in alpine headwaters, yet to fully understand the breadth of climate change threats, a more thorough accounting of microbial diversity is needed. We characterized microbial diversity (specifically Bacteria and Archaea) of 13 streams in two disjunct Rocky Mountain subranges through 16S rRNA gene sequencing. Our study encompassed the spectrum of alpine stream sources (glaciers, snowfields, subterranean ice, and groundwater) and three microhabitats (ice, biofilms, and streamwater). We observed no difference in regional (γ) diversity between subranges but substantial diversity differences among (β) stream types and microhabitats. Within stream (α) diversity was highest in groundwater-fed springs, lowest in glacier-fed streams, and positively correlated with water temperature for both streamwater and biofilm assemblages. We identified an underappreciated alpine stream type–the icy seep–that are fed by subterranean ice, exhibit cold temperatures (summer mean < 2°C), moderate bed stability, and relatively high conductivity. Icy seeps will likely be important for combatting biodiversity losses as they contain similar microbial assemblages to streams fed by surface ice yet may be buffered against climate change by insulating debris cover. Our results show that patterns of microbial diversity support an ominous trend for alpine stream biodiversity; as meltwater sources decline, stream communities will become more diverse locally but regional diversity will be lost. Icy seeps, however, represent a source of optimism for the future of biodiversity in these imperiled ecosystems.

Introduction:

Streams above the treeline in the alpine zone exhibit substantial environmental heterogeneity over small spatial scales, due in large part to variable hydrological sources (Brown *et al.*, 2007, Füreder, 2007, Hotaling *et al.*, 2017a, Ward, 1994). With such extensive habitat diversity, it is no surprise that alpine streams also support high among-stream (β) diversity across multiple taxonomic scales and classifications, including macroinvertebrate species diversity (Brown *et al.*, 2007, Finn *et al.*, 2011, Giersch *et al.*, 2016, Jacobsen *et al.*, 2012), macroinvertebrate genetic diversity (Finn *et al.*, 2013, Finn *et al.*, 2014, Hotaling *et al.*, 2019, Jordan *et al.*, 2016, Leys *et al.*, 2016), and microbial diversity (Fegel *et al.*, 2016, Freimann *et al.*, 2013a, Wilhelm *et al.*, 2013). The biodiversity of alpine streams is under threat, however, as rising ambient temperatures drive the ongoing decline of mountain glaciers and perennial snowfields worldwide, and with them, the loss of hydrological variation on local and regional scales (Hotaling *et al.*, 2017a).

A predicted rise in environmental homogeneity induced by climate change raises an important question: how will alpine stream biodiversity respond? For macroinvertebrate diversity, which includes the highest trophic levels in most alpine streams, the story is becoming increasingly clear: a reduction in meltwater will likely drive displacement of cold-tolerant species as less cryophilic taxa move uphill (Brown *et al.*, 2007, Hotaling *et al.*, 2017a, Lencioni, 2018). This community turnover is expected to yield higher within-stream (α) diversity but lower β diversity and, therefore, a decline in regional (γ) diversity as communities become more similar to one another (Jacobsen & Dangles, 2012, Jacobsen *et al.*, 2012). Microbial diversity plays an equally important role in alpine stream communities (Wilhelm *et al.*, 2014, Wilhelm *et al.*, 2013), yet its future is less clear. Of the previous studies most relevant to this question (i.e., Fegel *et al.*, 2016, Freimann *et al.*, 2013a, Wilhelm *et al.*, 2013), only Wilhelm *et al.* (2013) emphasized patterns of biodiversity, and their results revealed that for glacier-fed streams in the Austrian Alps, climate change impacts on microbial diversity will likely mirror macroinvertebrate predictions. Related perspectives have also shown that microbial α diversity is higher in rock glacier versus surface glacier-fed streams (Fegel *et al.*, 2016) as well as in groundwater versus glacier-fed streams (Freimann *et al.*, 2013a). Building upon these prior efforts, a

clear question emerges: across the full spectrum of hydrological variation in alpine ecosystems, how will climate change affect stream microbial diversity at regional scales?

The concept of what constitutes the full spectrum of hydrological variation in alpine ecosystems is also still being refined. Historically, alpine streams have been grouped into three main types based on their sources: groundwater-fed springs, snowmelt-fed streams, and glacier-fed streams (Ward, 1994), which reflect contrasting environmental conditions. Among these, glacier-fed streams are typically the coldest, and least physically stable, groundwater-fed springs the warmest and most stable, and snowmelt-fed streams intermediate between the two. Recently, a fourth unique alpine stream type, fed by subterranean ice-melt seeping from the subsurface, has been discussed (Hotaling *et al.*, 2017a). These "icy seeps" generally occur at geological transition zones, are cold like glacier-fed streams, but seasonally stable like groundwater-fed springs, and are most commonly fed by rock glaciers, masses of subterranean ice insulated by thick layers of inorganic debris (Janke, 2007, Janke, 2013, Millar *et al.*, 2013). Given their inorganic debris cover and/or subterranean nature, rock glaciers and other icy seep sources may be more buffered against warming atmospheric conditions than glaciers and perennial snowfields, making them less susceptible to climate change (Anderson *et al.*, 2018, Clark *et al.*, 1994, Knight *et al.*, 2019). In the contiguous United States alone, there may be more than 10,000 rock glacier features (Johnson, 2018) versus ~5,000 surface glaciers and perennial snowfields (Fountain *et al.*, 2017). Consequently, there is strong potential for icy seeps to serve as refugia for cold-adapted mountain stream species and unique ecological functions on a global scale.

The diverse microbial communities of glacier ice (Anesio & Laybourn-Parry, 2012, Anesio *et al.*, 2017), snow (Lutz *et al.*, 2016), and stream habitats (Zeglin, 2015) carry out important functional roles in terms of energy flow and nutrient transformation (Anesio *et al.*, 2010, Hotaling *et al.*, 2017b). As high-elevation headwaters, alpine streams are a key hydrological link between cryospheric processes and downstream habitats, both freshwater and marine (Hood *et al.*, 2015, Hotaling *et al.*, 2017b, O'Neel *et al.*, 2015). To date, most studies of microbial ecology in alpine headwaters have emphasized ice-fed streams, and particularly those fed by surface glaciers (Freimann *et al.*, 2013a, Ren *et al.*, 2017a, Ren *et al.*, 2017b, Wilhelm *et al.*, 2013) with rare rock glacier

examples (Fegel *et al.*, 2016). However, there has been considerably less focus on other stream types (but see Esposito *et al.*, 2016), and no comparisons across multiple streams types in the same geographic region have been made.

In this study, we investigated how microbial diversity (specifically Bacteria and Archaea) is structured in alpine streams across geographic, hydrological, and microhabitat space. We used sequence data from a variable region of the 16S rRNA gene collected from 13 streams and three microhabitats per stream in the Rocky Mountains. Our study included three levels of comparison: (1) between two disjunct regions of the Rocky Mountains in Wyoming and Montana, USA, (2) across four hydrologically defined stream types (glacier-fed, snowmelt-fed, groundwater-fed, and icy seeps), and (3) among three microhabitats (biofilms, streamwater, and source ice). Following a similar study from the Austrian Alps (Wilhelm *et al.*, 2013), we predicted that microhabitat would be the most important driver of community composition, with stream type acting secondarily. We also expected to observe some influence of mountain range on microbial diversity, presumably as a product of geological differences (as per Fegel *et al.*, 2016). To clarify how a warming climate may broadly affect microbial diversity in alpine streams, we also explored the relationship between within-stream α diversity and two in-stream environmental factors expected to shift with climate change: temperature and streambed stability. Taken together, our results expand general understanding of how environmental heterogeneity in alpine streams impacts microbial ecological pattern and process by adding a novel comparison across the full array of alpine stream types. Our identification and description of icy seeps as an ecologically important, but understudied, alpine stream type is particularly significant, as icy seeps may act as climate refugia for cold-adapted communities, microbial or otherwise.

Materials and methods:

Study sites and sample collection

In the summer of 2015, we collected microbial samples and environmental data from alpine streams in Grand Teton National Park and surrounding areas (GRTE) and Glacier National Park (GLAC; Figs. 1, S1; Table 1). All data, both microbial and environmental, were collected as close to the primary stream source as possible within a six-week interval from early August to mid-September. We designed our sampling scheme in this way to avoid the effects of any downstream hydrological inputs and because seasonal snowmelt is minimized late in the summer. Together, this approach ensures that stream hydrological conditions most closely reflected their primary source. We collected data from 13 streams: six in GRTE and seven in GLAC. GRTE and GLAC are technically subranges of the Rocky Mountains, but we refer to them as discrete mountain ranges here both for simplicity and because they are geographically and geologically disjunct from one another. We determined the primary source for each stream through satellite imagery, field confirmation, and the measurement of key environmental variables (see *Categorizing streams by hydrological source*).

For each stream, we sampled three microhabitats: streamwater, biofilm, and source ice or snow (when possible). For streamwater, we collected three replicate 1 L samples of flowing surface water into sterile Whirl-Pak bags (Nasco, Salida, CA). Each streamwater sample was a composite, with water collected from multiple sites along the stream surface. At one stream fed by subterranean ice emerging from a cave (Wind Cave in GRTE), we collected two sets of streamwater samples, one from the main stream channel and another from a seep emerging from high on the southern cave wall ~100 m inside of the entrance. We collected biofilm samples by scrubbing a ~10 x ~10 cm surface-facing section of three representative rocks from the stream bottom into an ethanol-sterilized plastic dish containing ~25 mL of sterile PCR water. Each rock represented a replicate of the biofilm community. Rocks were scrubbed with a wire-brush that was flame-sterilized before and after each sampling. When source ice was present and safely accessible, we sampled it from three locations typical of the focal glacier or snowfield surface. For each ice collection, we first removed the upper

~15 cm of snow and ice using a flame-sterilized ice axe adze. Next, we re-sterilized the adze and collected ~1 L of ice into sterile Whirl-Pak bags. We repeated this process for three independent ice collections and locations on each glacier or snowfield. Ice samples were transported in darkness and allowed to melt before filtering at a basecamp.

After collection, we immediately filtered biofilm and streamwater samples using sterile BD Luer-Lok syringes and filter holders (Becton, Dickinson and Company, Franklin Lakes, NJ) and 0.2 μm mixed cellulose ester filters (25 mm diameter; Millipore, Billerica, MA). Ice samples were filtered in the same way within six hours of collection. For streamwater and ice, we filtered ~0.5-1 L. For biofilm slurries, we filtered ~2-20 mL. All filters were immediately placed into sterilized sucrose lysis buffer (SLB; 20 mM EDTA, 400 mM NaCl, 0.7 M sucrose, 50 mM Tris, pH 9.0, Mitchell & Takacs-Vesbach, 2008) and stored in darkness in an insulated container until we returned from the field. Samples were then stored at -20°C until being processed for sequence data collection. At most sites, we also collected a "field negative" by using forceps to briefly expose a sterile filter to atmospheric conditions before preserving it in SLB and storing it in the same way as all other samples.

Categorizing streams by hydrological source

To assess the influence of hydrological source on patterns of microbial diversity, we categorized streams into one of four categories – groundwater-fed, snowmelt-fed, glacier-fed, or an icy seep – based upon spatial data and two environmental variables: a modified version of the Pfankuch Index (PI; a measure of streambed stability, Peckarsky *et al.*, 2014) and mean summer water temperature (summer solstice to autumn equinox, T_{SUMMER}). Water temperature was measured with *in-situ* dataloggers (HOBO, Onset Computer Corporation, Bourne, MA) which logged temperatures every hour for a calendar year in GRTE (2015-2016) and GLAC (2012-2013 or 2013-2014). Both streamwater and biofilm samples were collected in the same locations as one another and as close to the stream source as possible to control for spatial variation (e.g., other hydrological inputs downstream).

We categorized a stream as glacier-fed if satellite imagery (National Agricultural Imagery Program, 2005) revealed crevasses in the source ice, PI was > 30 (indicating low bed stability), and T_{SUMMER} was < 2°C. Thus, only traditional, surface glaciers were captured in our "glacier-fed" category. Any other streams fed by permanent surface snow were categorized as snowmelt-fed. We classified streams as groundwater-fed when the springhead could be identified, no permanent snowfield was present immediately above the catchment, T_{SUMMER} was > 3°C, and PI was < 15 (indicating a stable streambed). We categorized streams as icy seeps if we observed evidence of a subterranean ice source (e.g., clear lobes of a rock glacier) in satellite imagery on in the field, T_{SUMMER} was < 3°C, and the stream had a relatively stable streambed (PI = 15-24). We also georeferenced study streams with known glacier boundaries in publicly available GIS layers for GLAC (e.g., NPS Geospatial Dataset #1019881) and a rock glacier inventory database for the western United States (Johnson & Fountain, 2016), respectively.

To strengthen and confirm our hydrological classifications, we collected or calculated additional data for GRTE study streams, and compared sites according to the four variables commonly used in a stream "glaciality index" (Finn *et al.*, 2013, Ilg & Castella, 2006). These included: annual water temperature range (T_{RANGE}), specific conductivity (SPC; measured with a YSI Professional Plus Multiprobe, YSI Incorporated, Yellow Springs, OH), total suspended solids (TSS) following Hauer & Lamberti (2011), and PI (as above; Table S1). Using these data, we assessed the validity of our stream type classifications in GRTE with a principal components analysis (PCA) performed with PC-ORD (McCune & Mefford, 2006) following Finn *et al.* (2016). To assess the degree to which bed stability (PI) and mean temperature (T_{SUMMER}) varied across stream types, we compared means using one-way ANOVAs, followed by Tukey's HSD tests.

DNA extraction, library preparation, and amplicon sequencing

In the laboratory, we created composite samples by pooling replicate biofilm, streamwater, and source ice samples for the same site (e.g., all biofilm samples for the same site were combined into one tube). We vortexed composite samples briefly before extracting DNA from 100 μL of the well-mixed, SLB-preserved filter slurry following the direct-to-PCR method outlined by Flores *et al.* (2012) with two changes. First, we used Mllipore filters instead of swabs for biomass collection, and second, we exchanged the MoBio Powersoil DNA Isolation Kit for an Extract-N-Amp Ready Mix (Sigma-Aldrich, St. Louis, MO) kit. We used the 515f/806r PCR primer sets developed for the Earth Microbiome project (Gilbert *et al.*, 2014) and described in Bates *et al.* (2011) to amplify the V4 region of the 16S rRNA gene. PCRs were performed in 20 μL volumes containing: 10 μL of Extract-N-Amp Ready Mix, 1 μL mixtures of both forward and reverse indexed primers for each library at 5 μM concentrations, 4 μL of extracted DNA, and 5 μL of PCR-grade H_2O. Amplifications were performed with an initial denaturing step of 180 s at 95°C, followed by 35 cycles of: 30 s denaturation at 95°C, 60 s of primer annealing at 50°C, and 60 s of elongation at 72°C. All PCRs included negative controls and all negative controls used a single and unique indexed primer combination. PCR amplicons were gel-checked to ensure successful amplification and pooled in equimolar concentrations prior to sequencing. Sequencing was performed on an Illumina MiSeq at the University of Kentucky Advanced Genetic Technologies Center using 250 bp paired-end chemistry. In total, 48 samples (36 field samples, 11 field negatives, 1 PCR negative) were pooled and sequenced on a full MiSeq run followed by a Nano run that was dedicated to re-sequencing seven field samples for which the total reads recovered was less than 40,000 in the first run.

Raw sequence analyses and OTU calling

We demultiplexed our raw sequencing data with Illumina post-processing software and merged forward and reverse reads in FLASH v1.2.11 (Magoč & Salzberg, 2011) with a minimum and maximum of 20 and 250 bp of overlap, respectively. Non-merged reads were not included in

downstream analyses. Next, we filtered our reads for quality using the FASTX-Toolkit v0.0.13 (Gordon & Hannon, 2010) and only retained reads that had an average quality score of ≥ 24 across 80% of the read. All subsequent analytical steps were performed using the QIIME v1.8.0 pipeline unless otherwise noted (Caporaso *et al.*, 2010). We converted sequence files from FASTQ to FASTA (convert_fastaqual_fastq.py) then labeled and combined them for downstream processing (add_qiime_labels.py). Using the default 97% similarity threshold, we picked operational taxonomic units (OTUs) with a wrapper script (pick_de_novo_otus.py) that aligned sequences (align_seqs.py) and assigned taxonomy (assign_taxonomy.py) by comparing alignments to the Greengenes reference database (version 13_8; DeSantis *et al.*, 2006). As part of the OTU picking process, we filtered our database to remove OTUs for any of four reasons: (1) they were singletons (i.e., represented a by a single read), (2) assigned to eukaryotic chloroplasts, (3) enriched in our PCR and/or field negatives (>2% overall), or (4) literature searching revealed them to be likely contaminants (e.g., Salter *et al.*, 2014; see Table S2, Fig. S2).

Statistical analyses and visualization

To estimate biodiversity within a particular sample or group (e.g., site or stream type), we calculated α diversity using the Shannon diversity index (*H*). We defined regional γ diversity as the total *H* observed in each mountain range (GRTE and GLAC). We also calculated total α diversity within a group (e.g., stream type) by calculating *H* for the combined data from a focal group. We compared *H* for pairwise combinations among groups with two-sample, nonparametric t-tests and 1,000 permutations in QIIME (compare_alpha_diversity.py). We assessed possible associations between α diversity and water temperature (T_{RANGE}, T_{YEAR}, and T_{SUMMER}) or streambed stability using Pearson correlations in the R package 'Hmisc' (Harrell & Dupont, 2013).

We estimated β diversity using Bray-Curtis dissimilarity and visualized dissimilarities among samples through Principal Coordinate Analysis (PCoA; beta_diversity_through_plots.py). PCoA analyses provided a means for distilling dissimilarities among samples into principal coordinate axes

that each explain a portion of the variation. To test whether microbial β diversity differed among

groups (mountain range, stream type, or microhabitat), we performed adonis analyses (analogous to a

permutational multivariate analysis of variance or PERMANOVA) using the R package 'vegan'

(Oksanen *et al.*, 2007). Because our study design included inherently nested structure, we defined

strata (groups) to constrain permutations within focal groups (e.g., stream type) while controlling for

the influence of other groups (e.g., mountain range). For instance, using mountain range as an

example, we tested whether β diversity varied significantly between mountain ranges while

controlling for the effect of source and microhabitat with strata. For all adonis analyses, we used a

nonparametric approach to partition Bray-Curtis dissimilarity and 10,000 permutations to assess

significance. To quantify the degree to which microbial communities differed within each stream type

or microhabitat, we compared mean Bray-Curtis dissimilarity among samples for three groupings

(stream type, microhabitat, and a combination of stream type + microhabitat) using

make_distance_boxplots.py in QIIME. Specifically, we tested whether mean Bray-Curtis dissimilarity

within groups differed from one another with a two-sample t-test. For each comparison, we also tested

whether samples within groups were significantly more similar to one another than cross-group

comparisons with a two-sample t-test and Bonferroni correction for multiple comparisons.

We characterized the dominant taxa (phylum to genus) for each sample using the

summarize_diversity_through_plots.py script in QIIME. We also visualized how taxonomic

abundance and frequency corresponded with treatment groupings (whether source or microhabitat)

using ternary plots generated with the R package 'ggtern' (Hamilton, 2015). For our ternary plots, we

constructed separate plots for the 25 most abundant phyla and 50 most abundant families for samples

grouped by microhabitat or stream type. For three-way ternary comparisons among stream types, we

only included glacier-fed streams, icy seeps, and groundwater-fed springs as these categories best

covered the hydrological variation present in our sampling design and provide an additional means for

visualizing how icy seeps compare to the most harsh (glacier-fed streams) and most stable

(groundwater-fed springs) previously described alpine stream types (Hotaling *et al.*, 2017a, Ward,

1994). To incorporate snowmelt-fed streams, we conducted another set of two-group analyses, also in

'ggtern', to compare the 25 most abundant phyla and 50 most abundant families overall for icy seeps and snowmelt-fed streams. We visually compared the results of the three-way ternary and two-way plots to identify dominant taxonomic groups that may be indicative of icy seeps. We built on this analysis of dominant taxa by investigating associations between taxonomic groups and combinations of source and microhabitat in a categorical fashion with indicator species analyses using the R package 'indicspecies' (De Cáceres & Jansen, 2016). For this, we conducted 'indicspecies' analyses for all phyla, classes, orders, and families identified in this study. For each analysis, we used 1,000 permutations and a significance alpha of 0.05.

Finally, to identify which microbial taxa in streamwater and biofilms were closely associated with cold conditions, we categorized samples as being representative of colder ($T_{SUMMER} < 2°C$, $n = 10$) or warmer ($T_{SUMMER} > 2°C$, $n = 16$; Table S1) streams. We then mapped the overall 25 most abundant microbial families on a 2D line plot where position corresponded to relative frequency of each family in either category (colder or warmer) using 'ggtern' (Hamilton, 2015).

Results:

Sequencing and quality filtering

In total, we generated 13,093,344 sequence reads for 48 samples. We have archived the raw sequence data on the GenBank SRA (BioProject #PRJNA480048). After quality filtering, we retained an average of 56,328 reads per sample. For non-negative libraries, the minimum reads retained was 20,725 and maximum was 312,514. We determined that our sequencing depth was sufficient for resolving the amount of diversity present based upon rarefaction curves with little additional diversity discovered after 5,387 sequences per sample. For all biodiversity estimates, we rarefied samples to 20,725 reads, the lowest number recovered for any non-negative libraries after quality filtering and OTU removal. All results pertain to the 36-library treatment data set only (i.e., with no negative samples included).

Hydrological classifications

We identified four glacier-fed streams (two each in GRTE and GLAC), four snowmelt-fed streams (two each in GRTE and GLAC), three icy seeps (two in GRTE, one in GLAC), and two groundwater-fed springs (both in GLAC; Figs. 2, S1, Table S1). Stream beds were significantly less stable in glacier-fed streams versus all other stream types (Tukey's HSD, $P < 0.001$; Fig. S3a; Table S1). Icy seeps and glacier-fed streams were colder (T_{SUMMER}) than both groundwater-fed springs and snowmelt-fed streams (Tukey's HSD, $P < 0.05$; Figs. 2, S3b; Table S1). A PCA of the six GRTE streams, which included the four variables that make up the glaciality index, supported our broader stream type classification criteria (Fig. S4).

Community composition

In total, we identified 50,179 OTUs which were classified in 52 phyla, 187 classes, and 598 families (Table S3). Regardless of mountain range, stream type, or microhabitat, microbial diversity was dominated by OTUs belonging to two phyla: Proteobacteria and Bacteroidetes (Figs. 3a, 4). Within Proteobacteria, the majority of sequences were associated with α- and β-Proteobacteria, with α-Proteobacteria enriched in glacier-fed stream and icy seep biofilms (Fig. 4). Cyanobacteria-associated sequences were recovered from all samples and most abundant in biofilms but were present at conspicuously low levels in icy seep biofilms (Fig. 4). Across stream types, two phyla were primarily associated with glacier-fed streams (Firmicutes and Tenericutes), cyanobacteria were most common in groundwater-fed springs, and no dominant phyla were particularly associated with icy seeps versus other sources (Figs. S5a, S6a). At the family level, three common families were particularly abundant in icy seeps: Cellulomonadaceae, Micrococcaceae, and Pseudomonadaceae (Figs. S5b, S6b). For microhabitats, no phyla were closely tied to ice or biofilms (Fig. 3a), a number of families were associated with both microhabitats, and 23 families were largely shared by ice and streamwater only, suggesting a connection between the two (Fig. 3b). See additional discussion in this study's Supporting Information.

Through indicator species analyses of all, rather than the most abundant, taxonomic groups we identified one phylum, nine classes, 16 orders, and 28 families that were putative indicators of a specific stream type + microhabitat combination (Table S4). The bacterial phylum, NC10, was associated with icy seep streamwater ($P = 0.035$) and has been previously linked to anaerobic methane oxidation and nitrite reduction (Padilla *et al.*, 2016). All indicator classes were associated with streamwater in glacier-fed (two classes), groundwater-fed springs (seven classes), and snowmelt-fed streams (one class; Table S4). Similarly, 15 of 16 indicator orders were associated with streamwater with groundwater dominating (10 orders). One order linked to biofilms in snowmelt-fed streams was classified within the Synechococcophycideae ($P = 0.047$), a subclass of Cyanobacteria, members of which are common in stream biofilms, including meltwater streams of Antarctica (Van Horn *et al.*, 2016). At the family level, 24 of 29 indicator families were again linked to streamwater, mostly groundwater-fed springs (Table S4). Two families, Cellulomonadaceae ($P = 0.022$) and Sanguibacteraceae ($P = 0.014$), were associated with icy seep biofilms and are included in the "Polar and Alpine Microbial Collection" (Lee *et al.*, 2012), highlighting their commonality in cold habitats. Also, of note were two families of Archaea, Methanomassiliicoccaceae ($P = 0.028$) and Nitrososphaeraceae ($P = 0.039$), which were indicators of groundwater spring and icy seep streamwater, respectively (Table S4). A complete list of the 55 indicator groups identified in this study is provided in Table S4.

When streams were binned as colder ($T_{SUMMER} < 2°C$) or warmer ($T_{SUMMER} > 2°C$), four families (Exiguobacteriaceae, Carnobacteriaceae, Enterobacteriaceae, Pseudomonadaceae) were clearly enriched in biofilms of the coldest streams, with many more abundant in warmer stream biofilms (Fig. S7a). For streamwater, families were more evenly distributed between temperature categories, with a unique set of four putatively cold-associated families that were not detected in cold stream biofilms (Mycoplasmataceae, R4-41B, ACK-M1, Moraxellaceae; Fig. S7b).

Microbial diversity on regional and local scales

We observed no difference in regional γ diversity between mountain ranges (H_{GLAC} = 7.07 vs. H_{GRTE} = 6.87, P = 0.81) or total α diversity across stream types (pairwise P = 0.1-1; Table 2). For microhabitats, streamwater (mean H = 8.82) was more diverse than biofilm ($P < 0.01$) and ice ($P < 0.01$; Fig. 5a; Table 2) communities. Overall, the least diverse samples were biofilms in glacier-fed streams and icy seeps of GLAC (mean H = 2.39 and 2.52, respectively). Conversely, streamwater from groundwater-fed springs and snowmelt-fed streams, also from GLAC, were the most diverse (mean H = 10.04 and 9.93). Biofilms were consistently less diverse than corresponding streamwater samples across all stream types (Fig. 5b). Icy seeps had the largest difference in total α diversity between streamwater and biofilms with a mean difference of H = 5.97 (Fig. 5b). Both streamwater and biofilm α diversity were positively correlated with T_{YEAR} (Pearson's r = 0.72, $P < 0.01$ and r = 0.57, P = 0.05, respectively; Fig. 5c) and T_{SUMMER} (Pearson's r = 0.62, P = 0.02 and r = 0.70, P = 0.01, respectively; Fig. S8a). Biofilm diversity, but not streamwater diversity, was also positively correlated with T_{RANGE} (Pearson's r = 0.70, P = 0.01; Fig. S8b). Bed stability was negatively correlated with streamwater α diversity (Pearson's r = -0.70, $P < 0.01$), but had no significant relationship with biofilms (Pearson's r = -0.21, P = 0.52; Fig. S8c).

Mountain range had no effect on microbial community composition (P = 0.072). However, both stream type (P = 0.013, 22.59% of variance) and microhabitat (P = 0.038, 19.14% of variance) had strong effects on community assemblages. When visualized, these patterns were supported with samples generally grouping with those from the same stream type and microhabitat (Fig. 6). Across three focal groupings, samples within groups were more similar to one another versus the mean dissimilarity for all cross-group comparisons: stream type ($P < 0.001$), microhabitat ($P < 0.001$), and stream type + microhabitat ($P < 0.001$; Tables S5-7). Within stream types, mean Bray-Curtis dissimilarity among all samples within a given group was highest in glacier-fed streams (0.89) and lowest for groundwater-fed springs (0.74; Fig. 5d, Table S8) but no comparisons were significantly different from one another (Table S5). Microbial communities sampled directly from ice were the most similar to one another (mean Bray-Curtis dissimilarity = 0.63) and significantly more similar to one another than both streamwater (0.80) and biofilm samples (0.81; Fig. 6, Table S6). For stream

type + microhabitat groupings, only icy seep biofilms were significantly different from streamwater samples from the same habitat ($P < 0.001$; Fig. S5d, Table S7).

Discussion:

The global retreat of alpine glaciers and perennial snowfields is shifting biotic communities and altering ecosystems from headwaters to oceans (Hotaling *et al.*, 2017a, O'Neel *et al.*, 2015). The degree to which these effects might impact microbial diversity across the full spectrum of hydrological variation in alpine stream ecosystems, however, has remained largely unaddressed. In this study, we showed that alpine stream microbes exhibit largely similar patterns to those observed for their larger, well-studied macroinvertebrate counterparts (e.g., Cauvy-Fraunié *et al.*, 2015, Jacobsen & Dangles, 2012). Indeed, climate change will continue to drive rapid declines in permanent meltwater sources, namely glaciers and snowfields, which will lead to wide-scale loss of environmental heterogeneity in alpine headwaters. Tied to this heterogeneity is a wealth of microbial diversity that may decline on regional (e.g., mountain range) scales while increasing locally (e.g., within streams) as a more diverse, warmer water community shifts upward. Our results broaden the conclusions of Wilhelm *et al.* (2013) from a purely glacier-fed stream prediction to a more general prediction of climate change effects on the full suite of alpine stream hydrological variation.

Our findings also reveal the existence of an understudied alpine stream type–icy seeps–which emanate from subterranean ice sources and have been largely overlooked in the alpine stream biology literature (but see Fegel *et al.*, 2016, Hotaling *et al.*, 2017a). Icy seeps are cold like glacier-fed streams ($T_{SUMMER} < 2°C$), exhibit relatively high specific conductivity ($> 50\ \mu S\ cm^{-1}$) like groundwater-fed streams, and have moderately stable stream channels (PI ~20-25; Fig. S4, Table S1). Icy seeps also harbor unique microbial taxa that are at low abundance or absent in other alpine streams, but also contain many overlapping taxa with snowmelt- and glacier-fed streams. Beyond taxonomic overlap with other ice-fed stream types, icy seeps may be particularly important as climate change proceeds because they often stem from subterranean ice covered by thick layers of inorganic debris (e.g., rock

glaciers), and are predicted to be more resistant to warming (Anderson *et al.*, 2018, Fegel *et al.*, 2016, Millar *et al.*, 2013, Millar *et al.*, 2015). Furthermore, since rock glaciers may outnumber glaciers and perennial snowfields by a margin of 2:1 in North America (Fountain *et al.*, 2017, Johnson, 2018), with the potential for a similar story elsewhere in the world (Scotti *et al.*, 2013), icy seeps may be crucial climate change refugia for cold-adapted organisms worldwide (Hotaling *et al.*, 2017a).

Microbial diversity in alpine streams

Habitat conditions associated with stream type and microhabitat drive strong differences in total microbial diversity and community composition in alpine headwaters and these patterns are consistent across subranges of the Rocky Mountains. Different hydrological sources may carry different types of microbial cells into streamwater and biofilm microhabitats, while also dictating stream physicochemical characteristics that yield differential survival of narrowly adapted taxa. Microhabitat has been previously identified as an important predictor of microbial diversity in glacier-fed streams (Wilhelm *et al.*, 2013), and our results support and extend this finding to include all four common alpine stream types. The consistently lower diversity in biofilm communities relative to streamwater communities suggests that relatively few taxa derived from the surrounding water column are successful biofilm colonizers. Higher diversity in streamwater likely also reflects the high influx and low residence time of meltwater-associated microbial cells in alpine streams, particularly during the summer melt season (Wilhelm *et al.*, 2013). Though untested in this study, it is also possible that streamwater communities are more temporally dynamic than biofilm communities. However, microbial communities in streamwater outflows of the Lemon Creek Glacier in southern Alaska were relatively stable throughout a melt season, suggesting that the potential for dynamism in streamwater communities may not be as great as expected, particularly when streams are fed by a somewhat constant hydrological source (Sheik *et al.*, 2015). Collectively, our results echo a general premise: the flowing water of headwater streams represents a vital link between microbiota either

colonizing or otherwise associated with source ice and downstream communities developing in biofilms, sediments, and other specialized stream microhabitats (Hotaling *et al.*, 2017b).

Mountain range, however, had little to no influence on both total diversity and microbial community assemblage patterns. Fegel *et al.* (2016) identified clear differences in microbial diversity and community composition between the Rocky Mountains, Cascades, and Sierra Nevada. Our results contrast this, most likely due to geographic scope, as our comparison was made across disjunct subranges of the Rocky Mountains. Given the ~600 km of geographic separation and geological differences between GLAC and GRTE (see Love *et al.*, 1992, Ross, 1959, Smith & Siegel, 2000), a lack of subrange influence is still surprising and suggests geology may be, at least slightly, less important to microbial community structure than the results of Fegel *et al.* (2016) would suggest. However, one factor that likely contributed to our lack of mountain range-specific signal was our focus on a wide range of hydrological sources. Fegel *et al.* (2016) emphasized surface and rock glaciers, which both, to varying degrees, engage in active glacial comminution (i.e., grinding of bedrock into particles), a process known to substantially influence stream biogeochemistry (Telling *et al.*, 2015). By focusing on streams so inherently linked to active geological processes, their experimental design may have elevated signals of geological differences. Indeed, our study also included snowmelt- and groundwater-fed streams, stream types that are expected to be less influenced by bedrock than their glacier-associated counterparts. Regardless, both studies indicate that microbial community structure and diversity in alpine streams is strongly influenced by local factors, such as water temperature, conductivity, turbidity, or microhabitat, observations that align with more general studies of microbial biogeography (Fierer *et al.*, 2007).

The most common phyla observed in this study (e.g., Proteobacteria, Bacteroidetes) were not surprising as they have also been observed in many other studies of alpine and/or glacier-fed stream microbial ecology (Fegel *et al.*, 2016, Sheik *et al.*, 2015, Wilhelm *et al.*, 2013) and are typical components of freshwater stream microbial communities (Zeglin, 2015). Moreover, many other abundant phyla (e.g., Actinobateria, Firmicutes), classes (e.g., α- and β-Proteobacteria), and families (e.g., Exiguobacteraceae) have also been identified in various cryosphere-focused studies, leaving

little room for debate regarding their representation as common members of ice-associated habitats worldwide (Anesio & Laybourn-Parry, 2012, Anesio *et al.*, 2017, Chaturvedi & Shivaji, 2006, Hotaling *et al.*, 2017b). Given the role of cyanobacteria in primary production within streams, it was also unsurprising that they were most abundant in biofilms (Battin *et al.*, 2016), however the near absence of cyanobacteria in icy seep biofilm samples is difficult to explain. All three icy seeps had relatively stable beds unlike the only other streams with rare cyanobacteria-associated sequences (glacier-fed streams in GLAC). Moreover, all study streams were relatively shallow (i.e., < 45 cm in depth) and total suspended solids, a proxy for stream turbidity, was much lower in icy seeps versus glacier-fed streams (Table S1). Despite the limited presence of cyanobacteria, icy seep biofilms still support complex food webs, including a diversity of macroinvertebrates (Tronstad *et al.*, In review), so it is possible that algal primary producers may be more characteristic of icy seep biofilms or that icy seep food webs are supported by other basal energy sources.

Correlations between microhabitat diversity and environmental characteristics raise new questions about patterns of microbial community assemblage and sorting in alpine streams. For instance, both streamwater and biofilm diversity were positively correlated with mean stream temperature for the full year (T_{YEAR}) and summer (T_{SUMMER}), yet this relationship was decoupled when temperature range (T_{RANGE}) was considered (Figs. 5C, S8). Indeed, T_{RANGE} remained positively correlated with biofilm diversity yet had no significant relationship with streamwater diversity. This disconnect is likely due to the residency time of different microbial communities in the stream and the degree to which they reflect active colonization of the environment (Battin *et al.*, 2016) versus passive dispersal from upstream. Stream temperature range is also disproportionately influenced by seasonally reduced flow and high solar radiation and thus, is less reflective of the influence of the primary source on downstream conditions. Because biofilm communities are more permanent members of the stream ecosystem than microbes in flowing water, they are more likely to be constrained by high temperatures than streamwater communities. Finally, our data provide no insight into the metabolic status of the cells sampled in this study, and thus we cannot differentiate between cells that were alive or dead (Wilhelm *et al.*, 2014). It is, however, more likely that DNA recovered from a biofilm reflects

cells that can reproduce in their local microhabitat versus DNA recovered from streamwater which almost certainly originated elsewhere and thus are less indicative of sorting driven by local conditions.

The future of microbial diversity in alpine headwaters

As climate warming proceeds and permanent meltwater sources are lost, streams fed by glaciers and permanent snowfields will undergo substantial environmental changes, including warming temperatures, reduced flow and variability, and increasingly stable stream beds (Freimann *et al.*, 2014, Freimann *et al.*, 2013a, Jacobsen *et al.*, 2014). In some cases, streams will cease to flow or transition to intermittency (Haldorsen & Heim, 1999). Such dramatic habitat alterations are expected to shift the microbial community, with glacier-fed stream specialists (e.g., family Exiguobacteraceae) being lost as more generalist or groundwater-associated taxa become more prevalent (Freimann *et al.*, 2013a). However, the degree to which environmental shifts will translate to altered ecosystem functioning remains largely unknown. Reciprocal transplants of hyporheic sediments from a Swiss alpine floodplain revealed only fine scale effects of water source ecosystem function (Freimann *et al.*, 2013b), suggesting that microbial communities in alpine streams may be able to buffer the effects of changing hydrological regimes. Different biogeochemical cycles (e.g., certain nitrogen versus sulfur cycling functions) may also vary in their sensitivity to climate change (Ren *et al.*, 2017b) since pathways with low functional redundancy will be at an elevated risk of alteration with community turnover. From a broad perspective, it is important that future studies are mindful that the collective focus of the field on community-level changes may overlook the outsized impacts that rare but active taxa can play in ecosystem function (Wilhelm *et al.*, 2014).

Going forward, temporal monitoring of multiple alpine stream types, including icy seeps, will lend important clarity to the rate of environmental and biotic change in these imperiled ecosystems. To better understand ecosystem function, future efforts should take cues from the plethora of recent studies focused on microbial function in cryosphere-associated habitats (e.g., Edwards *et al.*, 2013, Ren *et al.*, 2017b, Wilhelm *et al.*, 2014) to better resolve connections between alpine stream microbial

diversity and ecosystem services, including the role biofilms play in supporting organisms at higher trophic levels, an important biotic coupling that stands to greatly shift in the decades to come. In the same vein, identifying and refining our collective understanding of stream types that will be most resilient to climate-induced habitat alteration (e.g., icy seeps), including the effects of climate change on biotic communities, is an important avenue for future research as these streams may represent the last stronghold of meltwater-associated biota in a landscape without glaciers and perennial snowfields.

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Tables:

Table 1. Sampling information for all streams included in this study. Mountain ranges are either Glacier National Park (GLAC) or Grand Teton National Park and the surrounding areas (GRTE). Sample abbreviations include: biofilm (B), streamwater (S), snow or ice (I), and field negative (N). Coordinates are in decimal degrees, elevations are in meters.

Stream	Range	Stream type	Latitude, longitude	Elev.	Samples
Clements Ck.	GLAC	Snowmelt	48.6899, -113.7335	2170	B, S, I
Oberlin Spring	GLAC	Groundwater	48.6983, -113.7305	2113	B, S, N
Reynolds Spring	GLAC	Groundwater	48.6823, -113.7311	2162	B, S, N
Lunch Ck.	GLAC	Snowmelt	48.7068, -113.7043	2189	B, S, I, N
Piegan Spring	GLAC	Icy seep	48.7031, -113.6941	2370	B, S, N
Grinnell Glacier	GLAC	Glacier-fed	48.7603, -113.7249	1917	B, S, I, N
Sperry Glacier	GLAC	Glacier-fed	48.6259, -113.7634	2318	B, S, I, N
Petersen Glacier	GRTE	Glacier-fed	43.7818, -110.8463	2922	B, S, I
South Cascade Ck.	GRTE	Icy seep	43.7217, -110.8377	3152	B, S, I[a], N
S. Fork Teton Ck.	GRTE	Snowmelt	43.6908, -110.8434	2987	B, S, I
N. Fork Teton Ck.	GRTE	Snowmelt	43.7774, -110.8595	2955	B, S, I, N
Wind Cave	GRTE	Icy seep	43.6661, -110.956	2692	B, S[b], N
Middle Teton	GRTE	Glacier-fed	43.7277, -110.7954	2955	B, S, I, N

[a] Ice samples were collected but the stream is primarily fed by a subterranean rock glacier.

[b] Two streamwater samples were collected, one in the main channel and one from a seep inside the cave entrance.

Table 2. Pairwise comparisons of overall α diversity (as measured by Shannon diversity index, *H*) among samples grouped by mountain range, microhabitat, and stream type using a two-sample, nonparametric t-test. Standard errors are in parentheses. Comparisons that are significantly different ($P < 0.05$) are in bold.

Comparison	Group, 1	Mean, 1	Group, 2	Mean, 2	*P*-value
Mountain range	GRTE	7.07 (2.26)	GLAC	6.87 (2.40)	0.81
Microhabitat	Ice	6.71 (0.59)	Biofilm	5.00 (1.95)	0.15
	Streamwater	**8.82 (1.66)**	**Biofilm**	**5.00 (1.95)**	**< 0.01**
	Streamwater	**8.82 (1.66)**	**Ice**	**6.71 (0.59)**	**< 0.01**
Stream type	Glacier-fed	5.35 (2.23)	Snowmelt-fed	8.45 (1.59)	0.1
	Groundwater	8.13 (1.97)	Snowmelt-fed	8.45 (1.59)	1
	Glacier-fed	5.35 (2.23)	Groundwater	8.13 (1.97)	0.88
	Icy seep	6.80 (3.03)	Snowmelt-fed	8.45 (1.59)	1
	Icy seep	6.80 (3.03)	Groundwater	8.13 (1.97)	1
	Glacier-fed	5.35 (2.23)	Icy seep	6.80 (3.03)	1

Figure captions:

Figure 1. Sample sites and stream types included in this study: a) Glacier National Park, b) Grand Teton National Park and surrounding mountains, c) geographic location of focal ranges in the Rocky Mountains, and d) microhabitats sampled for each stream. Acronyms: Grinnell Glacier (GR), Lunch Creek (LC), Piegan Spring (PS), Oberlin Spring (OS), Clements Creek (CC), Reynolds Spring (RS), Sperry Glacier (SG), Petersen Glacier (PG), Middle Teton Glacier (MT), North Fork Teton Creek (NFT), South Fork Teton Creek (SFT), South Cascade Creek (SC), and Wind Cave (WC).

Figure 2. Thermographs of mean daily temperatures for study streams in a) Grand Teton National Park and the surrounding area (GRTE) and b) Glacier National Park (GLAC) over a calendar year. T_{SUMMER} = mean water temperature between the summer solstice and autumn equinox (21 June – 22 September). Streams are color-coded by type. All GRTE profiles are from 2015-2016. All GLAC profiles are from 2012-2013 except for Grinnell Glacier, which was recorded from 2013-2014. Not shown: Sperry Glacier.

Figure 3. Distribution of taxonomic groups in streamwater, ice, and biofilms by percentage of reads associated with a) the 25 most abundant phyla and b) the 50 most abundant families. Circle position indicates the percentage of the associated taxon with each microhabitat and its size reflects the relative abundance of the associated taxon in the data set overall.

Figure 4. A bar graph of the most common phyla identified within sites, stream types and microhabitats included in this study. Each vertical bar represents one sample with each phylum identified by color. Only phyla present at > 1% frequency are included; therefore, bars vary in total height depending upon how many sub-1% phyla were identified. Abbreviations: GF = glacier-fed streams, IS = icy seeps, SM = snowmelt-fed streams, and GW = groundwater-fed springs.

Figure 5. Total α diversity by a) microhabitat + mountain range and b) microhabitat + stream type. Upper and lower lines for each group indicate the highest and lowest estimates (excluding outliers). Heavier lines are median values. Outliers are denoted with crosses. c) α diversity is positively correlated (Pearson's *r*) with mean annual stream temperature for both streamwater and biofilms. Temperature profiles for each point reflect the same thermographs in Fig. 2 and additional correlations are shown in Fig. S8. Each circle represents one composite sample. d) Average dissimilarity among samples when grouped by a combination of stream type + microhabitat with ice samples broken out separately. Higher values indicate greater dissimilarity within groupings. Abbreviations: GLAC = Glacier National Park; GRTE = Grand Teton National Park and surrounding mountains, BF = biofilm, SW = streamwater.

Figure 6. Bray-Curtis dissimilarity for all non-negative samples visualized as principal coordinate analysis plots: a) PCoA 1 and PCoA 2 and b) PCoA 2 and PCoA 3. Samples are color-coded by stream type and shapes correspond with microhabitat. Mountain range is not coded as it did not significantly affect microbial community structure ($P = 0.072$). The top 10 principal coordinate axes explained 70.5% of the variation in the data.

(a) GRTE

SNOWMELT	ICY SEEP	GLACIER-FED
S. Fork Teton Creek	S. Cascade Creek	Middle Teton
N. Fork Teton Creek	Wind Cave	Petersen Glacier
$T_{SUMMER} = 6.9°C$	$T_{SUMMER} = 2.2°C$	$T_{SUMMER} = 1.6°C$

(b) GLAC

GROUNDWATER	SNOWMELT	ICY SEEP	GLACIER-FED
Reynolds Spring	Lunch Creek	Piegan Spring	Grinnell Glacier
Oberlin Spring	Clements Creek		
$T_{SUMMER} = 4.1°C$	$T_{SUMMER} = 3.9°C$	$T_{SUMMER} = 0.3°C$	$T_{SUMMER} = 1.1°C$

(a) Top 25 phyla

Streamwater
- Nitrospirae
- OP3
- Fibrobacteres
- TM6
- Elusimicrobia
- Chlamydiae
- WS3
- Planctomycetes
- Chloroflexi
- GN02
- Chlorobi
- Armatimonadetes
- OD1

Ice & Streamwater
- Spirochaetes
- Gemmatimonadetes
- Acidobacteria
- Verrucomicrobia
- Tenericutes
- TM7

Shared
- Bacteroidetes
- Proteobacteria
- Firmicutes
- Actinobacteria

Biofilm
- Cyanobacteria
- Thermi

(b) Top 50 families

Ice & Streamwater
- Bacteriovoracaceae
- Rhodospirillaceae
- Moraxellaceae
- Bdellovibrionaceae
- Opitutaceae
- ACK-M1
- Rhodocyclaceae
- Aerococcaceae
- Mycoplasmataceae
- Succinivibrionaceae
- Ruminococcaceae
- Clostridiaceae
- Paraprevotellaceae
- Hyphomicrobiaceae
- Methylophilaceae
- Sphingobacteriaceae
- Verrucomicrobiaceae
- Prevotellaceae
- Oxalobacteraceae
- Sinobacteraceae
- auto67_4W
- Comamonadaceae
- Acetobacteraceae

Ice
- Microbacteriaceae
- Chitinophagaceae
- Sporichthyaceae

Streamwater
- R4-41B
- Coxiellaceae
- Cryomorphaceae
- Alteromonadaceae
- Chthoniobacteraceae
- Gemmataceae

Streamwater & Biofilm
- Neisseriaceae

Shared
- Caulobacteraceae
- Flavobacteriaceae
- Saprospiraceae
- Pseudanabaenaceae
- Rhodobacteraceae
- Xanthomonadaceae
- Sphingomonadaceae
- Cytophagaceae

Biofilm
- Phormidiaceae
- Micrococcaceae
- Chamaesiphonaceae
- Deinococcaceae
- Pseudomonadaceae
- Carnobacteriaceae
- Enterobacteriaceae
- Cellulomonadaceae
- Exiguobacteraceae