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RESEARCH ARTICLE

Influence of water source on alpine stream community structure: linking morphological and metabarcoding approaches

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Abstract

Alpine streams support unique biodiversity because of their isolation in the river network and harsh environmental conditions. Variation in water sources to alpine streams are an important driver of benthic community structure; however, studies with long-term data on stream abiotic characteristics and biological communities remain relatively rare. This is especially true for very high elevations where effects of climate change will be most pronounced. We used a combination of morphological and metabarcoding approaches to characterize benthic communities at nine locations within a high elevation catchment (~ 3200 to 3700 m) in the Southern Rocky Mountains, USA. Survey sites included ephemeral and permanent streams dominated by glacier meltwater, snowmelt, lake outflows, and mixed-source waters. Discharge, water temperature, conductivity, pH, total dissolved nitrogen, and total dissolved phosphorus differed between water source types. Environmental differences between source water types reflected their position in the catchment, the influence of subsurface ice meltwater on nitrogen and ions, and the biological activity and stabilizing effects of lakes. In total, we detected 130 macroinvertebrate taxa (52 via morphology and 99 via metabarcoding). Macroinvertebrate composition was more strongly correlated with stream permanence and conductivity than with water temperature or nutrient availability. Glacial and snowmelt-fed streams were dominated by Chironomidae (especially Diamesinae) and Oligochaeta, with increasing richness of Plecoptera, Ephemeroptera, and Trichoptera at the lake-outlet and mixed-source sites. Lake outlets showed the highest beta diversity, with unique communities dominated by various filter feeders (Simuliidae and Sphaeriidae). Alpha diversity was similar at the glacial, snowmelt, and lake outlet sites, but increased at the downstream mixed-source sites which had proportionally more non-dipteran taxa. Our results support the idea that distinct source waters have unique environmental conditions, leading to variation in their community structure. These results provide motivation for conservation efforts that protect a diversity of alpine stream types within and between catchments.

Keywords Alpine stream · Macroinvertebrate · Periphyton · Metabarcoding · Rock glacier · Cryosphere

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Introduction

The ecology of alpine streams is distinct from lower elevation streams for several reasons. Alpine streams have relatively low inputs of terrestrial organic matter, productivity may be limited by temperature, and dynamics of snow and ice drive key hydrological and geomorphic characteristics (Ward 1994). For these reasons, community structure and ecosystem function of alpine streams diverge from expectations developed for streams with forested headwaters, for which much of the early conceptual framework for stream ecology was based (Vannote et al. 1980; Doretto et al. 2020). Due to their steep elevational gradients, alpine streams also experience abrupt changes in geomorphology, temperature, hydrology, and water chemistry over much shorter longitudinal distances than lower elevation streams (Milner and Petts 1994; Maiolini and Lencioni 2001). As a result, ecological studies of alpine streams are especially important for developing a broader understanding of the linkages between physical and biological characteristics of lotic ecosystems.

Biological features of alpine streams are also influenced by their headwater position in the river network, sometimes resulting in unique contributions to regional stream biodiversity. In general, mountain headwater streams are thought to have relatively low site-level richness (i.e., low alpha diversity) due to their isolation and challenging environmental conditions (Clarke et al. 2008; Jacobsen and Dangles 2012). Early conceptual models for river ecosystems, such as the river continuum concept, suggested that richness should increase from headwater to mid-order streams (Vannote et al. 1980; Minshall et al. 1985). Yet simultaneously, alpine streams may have high turnover in species composition between sites (i.e., high beta diversity), and they can also contribute uniquely to regional diversity in a watershed (i.e., gamma diversity) (Finn et al. 2011). Headwater streams in mountainous regions are more isolated than downstream reaches, and this can influence community structure via constraints on dispersal, particularly for taxa that disperse within the aquatic environment (Tonkin et al. 2018). The presence of unique species in alpine streams is particularly important given their potential susceptibility to climate change effects, such as warming temperatures and shifts in hydrology that are prominent at high elevations (Hotaling et al. 2017; Brighenti et al. 2019). Climate change may result in losses of uniquely adapted alpine headwater taxa (Giersch et al. 2017). Shifts in source waters of streams from glacial and snow dominated to rainwater may also lead to homogenization of ecosystem functioning in the stream network, with potential consequences for food web stability (Bellmore et al. 2022). At the same time, certain alpine streams have the potential to act as climate refugia, especially those fed by subsurface ice (e.g., rock glaciers; Tronstad et al. 2020; Muhlfeld et al. 2020; Brighenti et al. 2021).

Differences in water sources to alpine streams are an important factor regulating environmental characteristics, with significant consequences for alpine stream community structure. Source waters to alpine streams typically include varying contributions from surface and subsurface ice, snow, and rainwater (Ward 1994; Sertić Perić et al. 2015; Brighenti et al. 2019). In general, streams fed by subterranean ice or groundwater tend to have greater stability in temperature, flow regime, substrate dynamics, and dissolved solutes than those fed by surface ice, snow, and rain, which have more extreme swings in environmental characteristics driven by seasonal pulses in snowmelt (Milner and Petts 1994; Ward 1994; Füreder 1999; Brown et al. 2009). Furthermore, many high-elevation streams are influenced by their position relative to alpine lakes, which can have a moderating effect on temperatures and can be hotspots for productivity and nutrient cycling (Hieber et al. 2003). These differences between source waters, in turn, have been correlated with unique communities of benthic macroinvertebrates (Hieber et al. 2005; Tronstad et al. 2020) and of microorganisms within periphyton (Wilhelm et al. 2013; Hotaling et al. 2019). For instance, glacially fed streams often have reduced alpha diversity of macroinvertebrates and high relative abundance of Chironomidae (Maiolini and Lencioni 2001; Milner 2016). Lake outlets tend to be dominated by non-insect taxa, often with high relative abundance of filter-feeders (Richardson and Mackay 1991). Groundwater-fed streams sometimes have the highest taxonomic richness compared to other stream types and higher proportions of Ephemeroptera, Plecoptera, and Trichoptera (Hieber et al. 2005). Similarly, for periphyton communities—which form the basis of the food web in most alpine streams—alpha diversity may be highest in groundwater-fed streams and lowest in glacial streams (Hotaling et al. 2019). Ecological functioning (e.g., metabolic pathways, primary productivity, decomposition) of the microbial community within periphyton of alpine streams can be strongly influenced by alpine source waters and is being altered by ongoing climate change (Kohler et al. 2022, 2024). Due to the variation in community structure associated with alpine stream source waters, conservation planning and watershed management that incorporates heterogeneity in alpine stream types may be useful for conserving the greatest diversity of unique stream taxa (Brown et al. 2009). Although the ecological importance of distinct alpine water sources is increasingly recognized (Füreder et al. 2001; Tronstad et al. 2020; Brighenti et al. 2021; Bellmore et al. 2022; Dunkle et al. 2024), studies that link long-term data on abiotic characteristics with variation in stream community structure of distinct source water types remain relatively rare, especially for high elevations (> 3000 m a.s.l.) where effects of climate change will be most significant (Pepin et al. 2015).

The unique species in alpine streams (Alther et al. 2019) underscore the importance of employing both morphological and molecular analyses to accurately detect and document species diversity, as each method can reveal different aspects of community structure. Improvements in molecular sequencing approaches have potential to improve detection of rare or cryptic taxa (Jackson et al. 2014; Serrana et al. 2019; Turunen et al. 2021), although traditional morphological identifications also have strengths in certain situations (e.g., when reference sequences are unavailable or sequencing results in taxonomic biases; Leese et al. 2021). The combination of morphological and metabarcoding approaches might result in a more thorough characterization of stream community structure than either method alone (Turunen et al. 2021). Relatively few studies have used both approaches together in alpine streams. A previous study used both approaches together in high elevation streams in the Colorado Front Range up to ~ 3500 m a.s.l. (Gill et al. 2014), providing relevant sequencing data on stream macroinvertebrate diversity within the study area.

In this study, we investigated the role of abiotic characteristics in shaping stream community structure of macroinvertebrates and periphyton within a high-elevation catchment in the southern Rocky Mountains of Colorado, USA. We specifically assessed variation in alpha and beta diversity across stream water source types and used long-term (> 30 years) datasets to characterize aspects of the abiotic environment. The research was conducted within the Niwot Ridge Long-Term Ecological Research site, where stream water sources have been extensively characterized based on hydrological and geochemistry studies (Hamann 2002; Liu et al. 2004; King 2012; Fields and Dethier 2019). We categorized the study sites as glacial meltwater, surface snowmelt, lake-outflows, or mixed water sources lower in the watershed. To better characterize potential cryptic diversity, we used a combination of morphological and DNA metabarcoding approaches to identify organisms. We expected the glacial and snowmelt-fed sites to have higher environmental variation over the year in temperature and hydrological variables, potentially limiting the establishment of some taxa. We predicted that alpha diversity would increase in the more stable environment of the lake outflows and mixed-source downstream sites, relative to the more variable glacial and snowmelt-fed sites. For beta diversity, we predicted that the ephemeral streams would have unique taxa and high turnover between sites, potentially contributing distinctly to overall diversity in the catchment.

Methods

Study area: We surveyed nine stream sites between 3221 and 3719 m a.s.l. within the Niwot Ridge Long Term Ecological Research (LTER) site, west of Boulder, Colorado, USA (Fig. 1, Fig. S1). This area forms the headwaters of North Boulder Creek and includes a series of six lakes within the Green Lakes Valley. Research has occurred at the LTER site since the 1950s, with a significant focus on hydrology, biogeochemistry, and snow/ice dynamics of the watershed

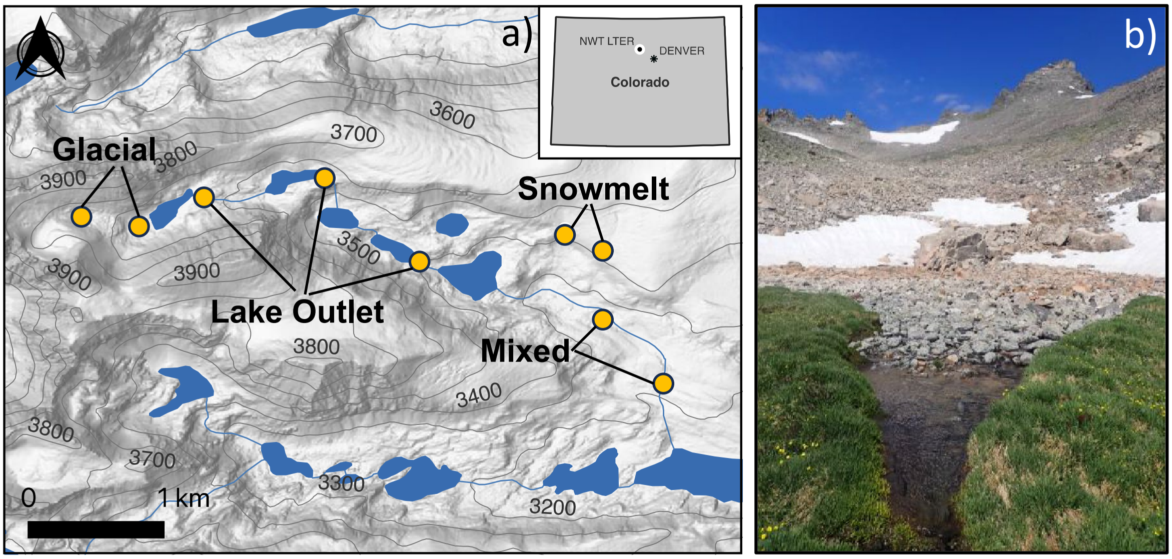

Fig. 1 Site map showing the nine sampling locations with stream types indicated with labels (**a**). The inset shows the location of the Niwot Ridge Long-Term Ecological Research Site relative to Denver, Colorado. The image at the right shows the highest elevation site (3719 m) at the top of the catchment, which is fed by glacial meltwater and permafrost (**b**)

(Bowman and Seastedt 2001; Bjarke et al. 2021). Ecological studies have mostly focused on terrestrial ecology and limnology, with less research focused on lotic ecology (Williams et al. 2015; Bjarke et al. 2021). A previous study developed a species list of invertebrates from sampling the littoral zones of lakes and the intervening stream segments (Bushnell et al. 1987). More recently, research has occurred on the lake macroinvertebrate communities in relation to environmental characteristics and seasonal changes (Yevak 2019). Several studies at the Niwot Ridge LTER have demonstrated shifts in terrestrial and aquatic ecosystems due to warming temperatures over recent decades (Leopold et al. 2015; Preston et al. 2016; Seastedt and Oldfather 2021).

We chose our study sites to span a diversity of environmental characteristics, including variation in water sources, as well as sites with long records of abiotic data collection. The two highest elevation sites were situated below the Arikaree Glacier, which lies between Arikaree and Navajo Peaks on the Continental Divide. Stream water at these locations is primarily composed of meltwater from the Arikaree Glacier and surrounding rock glacier ice within the talus fields at the head of the catchment (Liu et al. 2004). In contrast, two other ephemeral streams in the survey have sources dominated by snowmelt (Hamann 2002; Liu et al. 2004; King 2012; Fields and Dethier 2019). Stream water at these locations comes from surface run-off as well as snowmelt that has infiltrated the shallow subsurface with a short residence time. Three other stream sites were situated below lakes in the Green Lakes Valley. Water chemistry, temperature, and hydrology at these locations are mediated by in-lake processes, including ice phenology and productivity (Miller and McKnight 2012). Lastly, two sites were located just below the Green Lakes Valley, downstream of all other sites in the subalpine zone and below the tree line. Water sources at these locations therefore integrate all the upstream hydrological processes, incorporating surface and subsurface meltwater and precipitation (Liu et al. 2004). Hereafter, we refer to these four groups of sites as having water sources that are "glacial," "snowmelt," "lake outlet," and "mixed" (Fig. 1).

Environmental data: To characterize differences in the stream environment across sites, we used data on stream discharge, water chemistry, summer water temperature, stream width/depth, and substrate stability. The data on discharge and water chemistry are based on long-term datasets, while all other data were collected in summer 2021. Stream discharge and water chemistry data have been measured at six of the nine survey locations, with records dating back to the early 1980s or 1990s in most cases (Table S1). These six locations span all four types of water sources and include the highest elevation site and the second lowest elevation site (discharge datasets: Caine and Niwot Ridge 2021a, 2021b, Caine et al. 2023a, b, c; Caine et al. 2024). Water chemistry data, including pH, conductivity, total dissolved nitrogen (TDN), and total dissolved phosphorus (TDP), have been collected from the same six sites, with collections mainly occurring in the summer months when access to the sites is most feasible (chemistry datasets: Caine 2021a, b, c, d, e, f). Samples for TDN and TDP were digested and oxidized prior to spectrophotometric quantification using a Lachat QuikChem 8500 System Flow Injection Analyzer (APHA 2005; Solarzano & Sharp 1980). Additional details on discharge and water chemistry methods are available in the online datasets cited above. Stream temperature data were collected at all nine sites using either Onset HOBO temperature loggers (five sites) or Solinst Aquavent 3500 Pressure Transducers (four sites). For both types of loggers, data were continuously collected every 3 h in July and August of 2021, corresponding to when stream sampling of biological communities occurred. At the two ephemeral snowmelt-fed sites, temperature data were collected until the streams dried in mid-August. On the dates of stream surveys, we additionally characterized stream substrate stability using the Pfankuch index (Pfankuch 1975). The Pfankuch index uses qualitative measures of substrate brightness (due to presence/absence of biofilms), evidence of scour, substrate consolidation, and presence of aquatic vegetation to generate a relative measure of stability. We also measured stream width and maximum depth every 2 m along a 10-m reach spanning where macroinvertebrate samples were collected.

Macroinvertebrate sampling: Macroinvertebrate community structure was characterized using both morphological identifications and DNA metabarcoding from samples collected in summer 2021. Sampling occurred in mid- to late July for morphological samples and then again in early August for metabarcoding. Samples were collected with either a standard Surber sampler (930 cm^2 in area, 248 µm mesh) or a miniature Surber sampler (231 cm^2 in area, 248 µm mesh) if stream width was very narrow. We collected three replicate samples per site for morphological sample identification. When using the smaller Surber, we combined three to four collections into each sample to achieve a similar benthic area sampled as the larger Surber sampler. Samples were preserved in 80% ethanol and then identified in the laboratory at Colorado State University using Leica EZ4 microscopes (8 × to 35 × magnification). Samples were identified to genus for Ephemeroptera, Plecoptera, Trichoptera, Coleoptera, and some Diptera (Ward et al. 2002; Merritt et al. 2019). Chironomidae were identified to sub-family. Other taxa, including oligochaetes, molluscs, and crustaceans, were mostly identified to family or order (see Table S2 for a taxon list).

Macroinvertebrate metabarcoding: Metabarcoding samples were collected using the same Surber samplers described above. At one snowmelt-fed site, the stream had completely dried by the time we returned in August to collect metabarcoding samples; therefore, sequencing data were collected at eight of nine sites. Three replicate samples for metabarcoding were collected at each site. Not all samples were successfully sequenced, resulting in four replicate glacial site samples, seven lake outlet samples, two snowmelt samples, and four mixed-source samples (see Table S1 for sample sizes at each specific site).

Samples were preserved in 95% molecular-grade ethanol for DNA sequencing. We began cleaning each sample by removing pebbles but retaining fine sediment and organic matter (Pereira-da-Conceicoa et al. 2021). Additionally, we removed insects > 4 mm and returned a ~ 4 mm subsection of the abdomen to the sample, following Elbrecht et al. (2021). All cleaning was done using forceps sterilized with bleach and then ethanol and fire. We homogenized each cleaned Surber sample (together with preservation ethanol) with a Homgeek High Speed Professional Blender (model number NY-8608MCA) for 3 min at 21,000 rpm. The blender was sterilized between samples (see Supplemental Materials for details). Prior to DNA extraction, we homogenized samples by vortexing for 30 s, placing 2 ml of sample into a microcentrifuge tube, and then evaporating the ethanol using a ThermoSavant SpeedVac (Thermo Fisher Scientific, Waltham, MA, USA). We placed 50 mg dried sample directly into the Zymo BashingBead Lysis Tube. We carried out DNA extractions using the ZymoBIOMICS Miniprep Kit (Product number D4300) following a modified version of the manufacturer protocols (outlined in the Supplemental Materials). We quantified the DNA in each sample using a Qubit 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). We amplified the DNA in a two-step PCR amplification process using the freshwater macroinvertebrate degenerate primers developed by Leese et al. (2021). The full PCR program is provided in the Supplemental Materials. All PCR reactions were run on a Bio-Rad C1000 Touch Thermal Cycler (Hercules, CA, USA).

To normalize our samples, we used the Just-A-Plate Normalization Plate Kit (CharmBiotech) following the manufacturer's instructions but scaled down the initial volume to match the size of the final PCR product. We pooled 10 ul of each sample together by plate. Pooled libraries were evaluated on an Agilent FemtoPulse to estimate fragment size distributions. We then conducted size selection on the libraries on a Sage Science Blue Pippin, selecting for 250–500 bps. We estimated the molarity of the final sample with qPCR on a Thermo Scientific ViiA 7. The fragment analysis, size selection, and qPCR were all performed by the Brigham Young University DNA Sequencing Center.

Samples were sequenced on the Illumina MiSeq (Illumina, San Diego, CA, USA) using 500 cycle v2 chemistry. We sequenced the macroinvertebrate DNA libraries over three sequencing cartridges (two nano cartridges and one full cartridges) to ensure sufficient sequencing depth and combined all reads (see Fig. S2 for rarefaction curves indicating adequate sequencing depth). All initial processing was conducted using Qiime2 2022.2 (Bolyen et al. 2019). We paired and demultiplexed the forward and reverse sequences using the paired-end-demux commands and trimmed by aligning reads with target amplicons to identify primer boundaries. We trimmed at base pairs 25 and 167 to remove all primers and indexes from the raw reads. We denoised the sequences using DADA2 and then used a 98% similarity threshold to cluster.

To assign taxonomic identifications, we used blastn v.2.13.0 to query our OTUs against the NCBI non-redundant nucleotide database. We then used the following approach for assigning taxonomic resolution: taxa with < 90% similarity to existing sequences were not considered to be positive identifications and were omitted (~ 7% of sequences); taxa with matches between 90 and 95% were considered identified to the family level; taxa with 95% to 97% similarity were considered identified to the genus level; those > 97% were considered identified as species. In cases where an identification could potentially be redundant with another taxon (e.g., one organism matched only to the genus level but another was identified to the species level within that genus), the better resolved identification was kept and the lower resolution identification was dropped. Sequences from taxa that were not affiliated with the stream benthos, including taxa that are considered to have only terrestrial life stages and lake zooplankton, were removed from analyses. Taxa with semi-aquatic life stages were kept for analyses (see Table S3 for taxon list from metabarcoding).

Periphyton: Periphyton samples were collected from three randomly collected replicate rocks per site. Periphyton was scraped from the rocks into a collection dish using a toothbrush, and rocks were then rinsed with stream water from a sterile 50-ml syringe. Approximately 7 ml of the resulting slurry was transferred into a 15-ml conical tube containing 7 ml RNAlater. For each site, a new toothbrush and collection dish were used. The samples were kept on ice until processing in the laboratory. In the laboratory, samples were rinsed with nuclease-free water to remove RNAlater. After rinsing, DNA was extracted from ~ 250 mg of periphyton using a Powersoil DNeasy kit (Qiagen, Carlsbad, CA, USA) and quantified using a dsDNA HS Assay Kit and a Qubit Fluorometer (Life Technologies, Carlsbad, CA). DNA was submitted to the University of Minnesota Genomics Center for amplicon sequencing of the V4 region of the 16S SSU rRNA gene in bacteria and archaea and the V9 region of the 18S SSU rRNA gene in eukaryotes using the Earth Microbiome primer sets (Thompson et al. 2017) and ITS1 in fungi (Tedersoo et al. 2015). Dual indexed Nextera XT DNA libraries were prepared following Gohl et al. 2016, and amplicons were sequenced using MiSeq Illumina 2 × 300 bp chemistry. Only samples > 1 ng/µl DNA were submitted for sequencing. Due to low amounts of DNA, this resulted in only four of nine sites being successfully sequenced. These sites included two glacial sites, one lake outlet, and one mixed-source site. Post-sequence processing was carried out in mothur (v. 1.44.0; Kozich et al. 2013). For 16S and 18S, forward and reverse read pairs were joined as contigs prior to analysis. For ITS, only forward reads were used. Chimeric sequences were removed with the "uchime" step, and non-overlapping regions were trimmed in mothur (Kozich et al. 2013). Operational taxonomic units (OTUs) were clustered at 97% sequence identity, and taxonomy was assigned using the SILVA (V138) or UNITE (V8) databases.

Statistical analyses: We first assessed how the stream types varied in environmental variables. We analyzed differences in yearly peak discharge, summer temperatures, pH, conductivity, TDN, and TDP using either linear or linear mixed effects models. When data from multiple sites within all stream types were available, we included a random intercept for site identity (using the 'lme4' package in R). We used Tukey post hoc pairwise comparison to analyze which stream types were different from one another in these six environmental variables.

We next analyzed differences in benthic macroinvertebrate composition and beta diversity across the four stream types. We visualized community composition using non-metric multidimensional scaling (NMDS) and then tested for significant differences in composition between stream types with permutational multivariate analysis of variance (PERMANOVA) using Bray-Curtis dissimilarity matrices. For these analyses, we only used the morphological data because this allowed us to also include density in the dissimilarity matrices (i.e., for metabarcoding, we can only determine presence or absence, not counts). In addition to stream type ("glacial," "snowmelt," "lake outlet," or "mixed"), we included sampling site as a second predictor in the PERMANOVA to test for the relative importance of site-level versus stream group-level variation. To help interpret possible mechanisms underlying changes in community structure, we fitted vectors (or factors for categorical variables) of environmental data to the ordination. Environmental data included mean peak discharge, stream permanence (permanent or ephemeral), summer water temperature, pH, TDP, TDN, conductivity, channel stability (Pfankuch index), stream width, and stream depth. Mean values were used for all continuous variables. Because water chemistry and discharge were not available for three sites, we used data from the nearest connected upstream site. All other environmental variables were collected at all sites.

As a measure of beta diversity, we calculated mutivariate disperson for communities within each stream group (i.e., distances to the group centroid for each of the four stream types) (Anderson et al. 2006). We tested for overall and pairwise differences in dispersion across groups using a permutation test (Anderson 2006). Community analyses and visualization were conducted with the 'metaMS,' 'adonis2,', 'envfit,' and 'betadisper' functions in the R package 'vegan' (Oksanen et al. 2013).

Our next objective was to analyze changes in taxonomic richness and macroinvertebrate density. First, we summarized the aforementioned environmental variables (excluding stream permanence) using a principal components analysis (PCA). We then used a Poisson regression to test the relationship between the first principal component and sample-level taxonomic richness, including both the morphological and metabarcoding samples. We incorporated a fixed effect of sampling type in the model (morphological or molecular) with individual samples as replicates. We also included a random intercept for site identity. Additionally, we ran a second model with the same structure described above, including a categorical variable for stream type instead of the first principal component of the environmental variables. We analyzed log-transformed densities of macroinvertebrates using the same model structure, with stream type as a categorical predictor but with a Gaussian distribution. PCA was conducted with the 'prcomp' function in the R package 'stats' (R Core Team 2021), and regressions utilized the 'lme4' and 'emmeans' packages (Bates et al. 2015). In addition to including an effect of sampling type (morphological vs. molecular) in analyses of richness, we also made qualitative comparisons in the number of taxa detected with each method in the overall catchment, combining all sites together.

Our final objective was to explore how periphyton microbial diversity and composition changed across different stream types. All microbial diversity analyses were carried out using the R package 'phyloseq' (McMurdie and Holmes 2013). For each microbial group, we calculated Shannon diversity using the 'estimate_richness' function. To visualize compositional differences, we used a principal coordinates analysis of Bray-Curtis Dissimilarity indices.

Results

Environmental variables: Environmental variables generally differed across stream types in most cases (see Table S4 for pairwise comparisons from linear models). Peak discharge was highest for mixed-source downstream sites, followed by lake outlet, glacial, and snowmelt sites (Fig. 2a; $p < 0.05$ for all pairwise comparisons other than glacial vs. snowmelt). Summer water temperatures were lowest for the glacial sites, followed by the snowmelt sites, which had the highest coefficient of variation (Fig. 2b, Tables S4, S5). All pairwise comparisons were significant for summer temperatures (all $p < 0.04$; Table S4), other than the relatively stable lake outlet temperatures versus the mixed-source sites

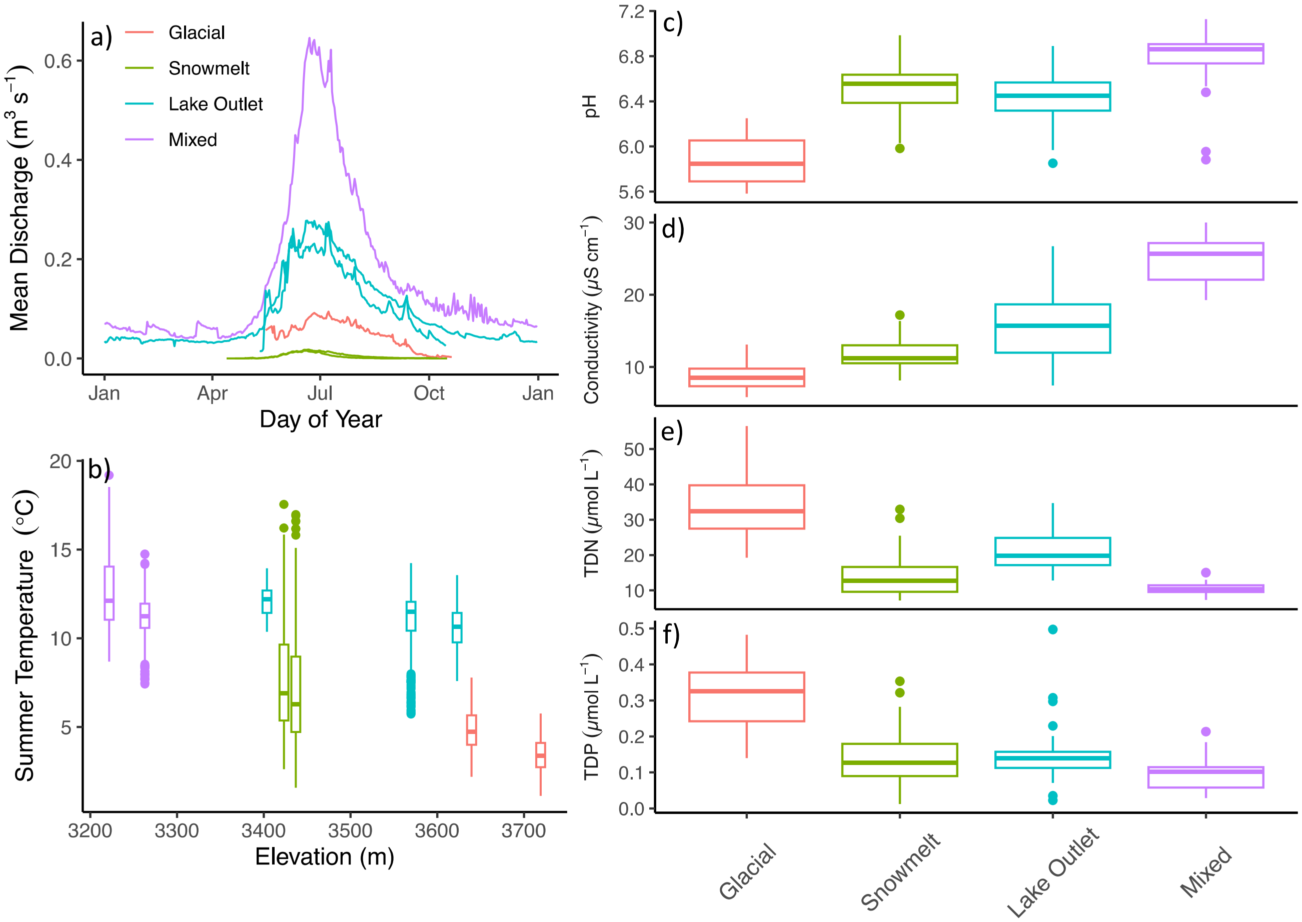

Fig. 2 Environmental data showing differences between stream groups. Upper left shows average daily discharge data for six stream sites where long-term flow measurements have been collected since the 1980s (**a**). The bottom left panel shows stream temperature data collected every 3 h over July and August 2021 from all nine sites. The x-axis shows elevation of the sites, and the colors indicate water sources, corresponding to the legend in the hydrograph panel at top left (**b**). The right panels show water chemistry data, including pH (**c**), conductivity (**d**), total dissolved nitrogen (**e**), and total dissolved phosphorus (**f**). All boxplots show the median value (horizontal line), the interquartile range (upper and lower ends of boxes), values within 1.5 times the interquartile range (whiskers), and outliers (points)

at lower elevation (Fig. 2b; $p > 0.05$). pH and conductivity generally were highest at the mixed-source sites and lowest at the glacial sites at the top of the watershed (Fig. 2c, d; $p < 0.05$ for 11 of 12 pairwise comparisons). TDN and TDP decreased from the top of the drainage to the downstream mixed-source sites, with intermediate values at the snowmelt and lake outlet sites (Fig. 2e, 2f, Table S4). Values for the Pfankuch index averaged 17 for the glacial sites, 30 for the lake outlets, 21 for the snowmelt sites, and 18 for the mixed-source sites. Lower Pfankuch index values indicate higher substrate stability.

Macroinvertebrates: Benthic macroinvertebrate composition differed in relation to stream type (PERMANOVA, $r^2 = 0.40$, $p < 0.001$). Glacial and snowmelt-fed sites were relatively similar to one another, as were mixed-source sites and lake outlets (based on location of their centroids and overlap in multivariate space, Fig. 3). Site identity also had a significant effect on community structure but explained lower variance than water source (PERMANOVA, $r^2 = 0.28$, $p < 0.001$). Communities from distinct water sources showed significant differences in beta diversity, as measured by multivariate dispersion (permutation test, $F = 17.1$, $p = 0.01$). Multivariate dispersion did not differ among glacial, snowmelt, and mixed-source sites (all $p > 0.05$) but did differ for all pairwise combinations involving lake outlet sites, which had the highest dispersion (all $p < 0.05$). Differences in the centroids between communities from different water sources were correlated with water depth ($r^2 = 0.31$), conductivity ($r^2 = 0.11$), peak discharge ($r^2 = 0.06$), water temperature ($r^2 = 0.06$), and stream width ($r^2 = 0.06$) (Fig. 3b). In general, the differences between glacial/snowmelt-fed sites relative to lake outlet/mixed sites were correlated with hydrological variables more so than water chemistry variables (Fig. 3b). Densities of macroinvertebrates were not significantly

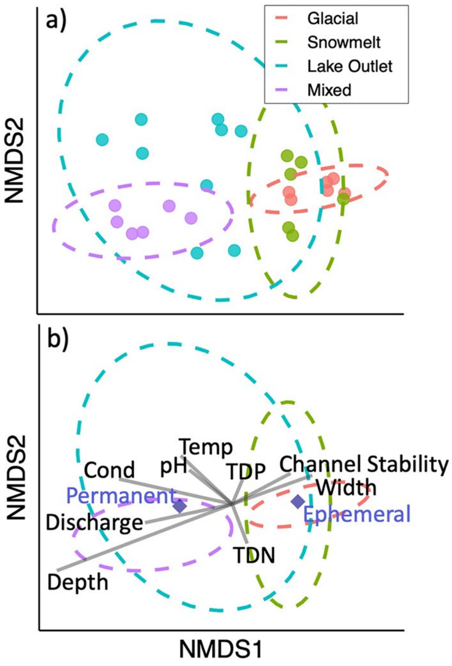

Fig. 3 Nonmetric multidimensional scaling plots, showing groupings of macroinvertebrate communities in relation to stream types (**a**) and with environmental vectors overlaid on the group ellipses (**b**). In both panels, the colors correspond to the stream groups shown in the legend at top right. Data are from Surber samples identified using morphological methods. Abbreviations for environmental variables are as follows: *cond* = conductivity, *temp* = temperature, *TDN* = total dissolved nitrogen, and *TDP* = total dissolved phosphorus

different between stream types (GLMM, all $p > 0.1$) although the lake outlet sites had much higher variation in invertebrate abundance than the other stream types (Fig. 4a).

Taxonomic richness of benthic macroinvertebrates also differed in relation to environmental variables. Taxonomic richness was positively correlated with the first principal component (GLMM, $z = 4.84$, $p < 0.001$), which explained 60% of the variation in the environmental data and was associated with increased discharge, conductivity, pH, temperature, and decreased TDN and TDP (Fig. S3). The mixed-source downstream sites had the highest richness (Fig. 4b). In the model including stream type, all pairwise comparisons involving the mixed-source downstream sites were significantly different in richness (GLMM, all $p < 0.001$). None of the other three stream types differed significantly in richness. When total unique richness per stream type was summarized from all sample types, the lake outlet and downstream mixed-source sites had proportionally more non-dipteran taxa than glacial and snowmelt sites (Fig. 4c).

Metabarcoding samples had higher richness on average than the morphological samples (GLMM, $z = 4.24$, $p < 0.001$, Fig. 4b). Much of the difference was caused by the higher taxonomic resolution of Diptera—especially Chironomidae—in the molecular samples, which accounted for 60% of the total taxa detected with all sampling methods. Both methods detected similar numbers of taxa for Plecoptera and Ephemeroptera, although some taxa were detected with only one of the methods (e.g., cryptic diversity within species groups detected by sequencing). Notably, the morphological sample processing included higher richness of Trichoptera than the molecular sampling (9 vs. 3 taxa, respectively) and slightly higher numbers of non-insect groups (Table S6). The combination of molecular and morphological analyses (after removing possibly redundant identifications) resulted in 130 taxa compared to 99 taxa in the molecular data and 52 taxa in the morphological data (Table S6). The metabarcoding of macroinvertebrates resulted in a total of 242,068 DNA

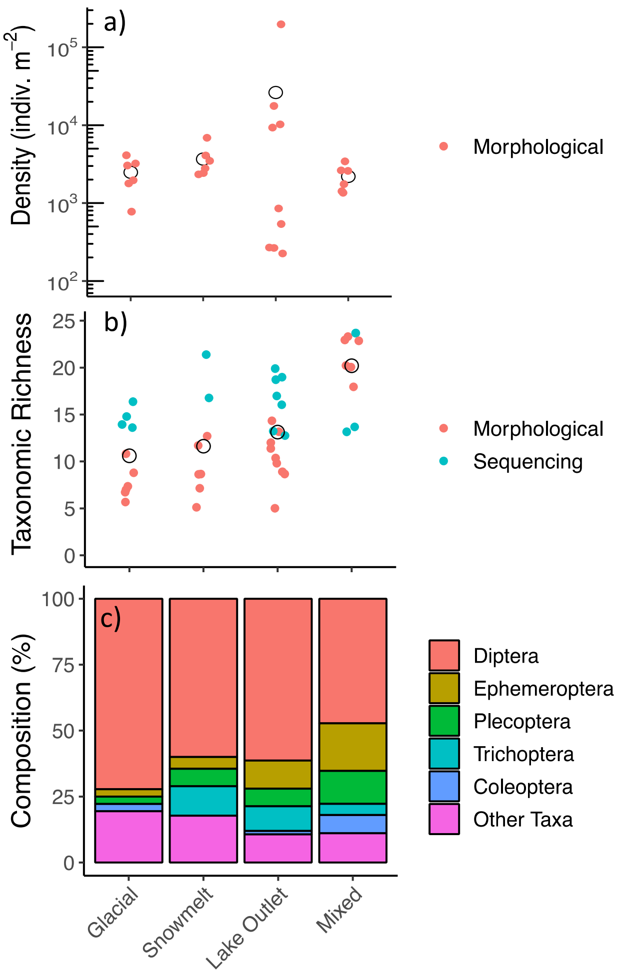

Fig. 4 Top panel shows macroinvertebrate densities from Surber samples across stream type. The hollow points are the mean values, and solid points are individual samples (**a**). The middle panel shows taxonomic richness detected in morphological and metabarcoding samples from the four stream groups (**b**). In the bottom panel, the proportional composition is shown by macroinvertebrate orders (**c**). Composition data in the lower panel incorporate both the morphological and metabarcoding data. The "Other Taxa" group includes hemipterans, crustaceans, bivalves, annelids, and nematodes

sequencing reads across 17 samples. After quality filtering, we retained 220,578 reads, ranging from 5876 to 19,990 per sample. When clustered at a 98% threshold, we detected 197 OTUs. Our taxonomic assignment protocols resulted in assignments of 21 unique families and 28 genera. Although many unique species were detected using the metabarcoding methods, only 15 OTUs were identified to species level to named taxa.

Periphyton: The microbial communities from glacial, lake outlet, and mixed water source stream types were generally distinct based on PCA plots (Fig. S4). For periphyton bacterial and fungal communities, glacial source periphyton samples clustered together, while mixed-source and lake outlets were more distinct. In contrast, the eukaryotic communities in glacial water source streams did not cluster together (Fig. S4). For both bacteria and eukaryotes, Shannon diversity was slightly higher in the downstream mixed-source site (Fig. S5) compared to the glacial or lake outlet sites. The proportional richness of Cyanobacteria taxa ranged from around 22–25% across sites (Fig. S6), while eukaryotic algae Diatomea ranged from ~ 2–25% (Fig. S6). The fungal phylum Ascomycota was particularly abundant, accounting for > 50% of the fungal OTUs in three of the four sites, including ~ 80% in the lake outlet (Fig. S6).

Discussion

Alpine streams form the headwaters of watersheds in mountainous regions globally and serve important roles in regulating downstream ecosystem services and supporting biodiversity (Hotaling et al. 2017; Brosse et al. 2022). Despite their importance, community characteristics of alpine streams are not well studied in many regions. We used a combination of morphological and molecular approaches to examine how environmental characteristics affect benthic community structure of alpine streams within a catchment in the Southern Rocky Mountains, USA. In some ways, our results supported our hypotheses regarding how alpha and beta diversity would change in relation to source waters. For instance, downstream mixed-source sites had the highest alpha diversity, but contrary to our predictions, we found the highest beta diversity among lake outlet sites. In the sections that follow, we discuss our findings for environmental characteristics, benthic macroinvertebrate communities, and microorganisms in relation to prior studies and expectations from conceptual models for alpine streams (Ward 1994; Milner 2016).

Environmental variables: Differences in channel stability and temperatures between stream types aligned with expectations in some but not all regards. Channel stability reflected several unique aspects of the study sites. For instance, geomorphology at the highest glacial site was characterized by an unconfined bench (i.e., meadow), resulting in relatively high channel stability, unlike most studies that typically find low stability below glaciers (Milner 2016). In contrast, channel stability scores were lower at some of the downstream lake outlet sites because of the steeper gradients at these locations. Temperature differences generally followed expectations and reflected both position in the catchment and source water types. For instance, a snowmelt-fed site was ~ 5 °C colder and had sevenfold more variable summer water temperatures relative to a lake outlet site at nearly the same elevation. This reflects the warming and variation-dampening effect of lake water residence times relative to the highly dynamic seasonal and diurnal patterns in discharge of snowmelt-fed streams (Ward 1994; Hieber et al. 2003).

Variation in water chemistry between stream types, in general, followed expectations for glacially fed versus streams with other source types. Meltwater from rock glaciers and permafrost has high concentrations of nitrogen, which was reflected in the highest TDN at the glacial sites (Williams et al. 2006; Barnes et al. 2014) and is in part due to a long history of atmospheric nitrogen deposition in the study area (Williams and Tonnessen 2000). Both TDN and TDP decreased in the downstream direction from the glacial to the lake outlet and mixed-source sites, which likely reflects the increasing demand of biological processes such as assimilation by primary producers in the lakes and riparian areas (Gardner et al. 2008; Miller and McKnight 2012). Glacial sites also had the lowest pH, reflecting the transport of acid anions with spring meltwater from surface and subsurface ice (Lepori et al. 2003; Williams et al. 2009). Glacial sites had lower conductivity than the snowmelt, lake outlet, and downstream mixed sites, consistent with prior studies finding low conductivity of glacial meltwater (Ilg and Castella 2006; Brown et al. 2010; Tronstad et al. 2020). This pattern may reflect the widespread contributions of permafrost and rock glacier ice in vectoring rock-weathering products throughout the upper Green Lakes Valley (Williams et al. 2006) as well as the contributions of shallow groundwater rich in cations at the two snowmelt-fed sites (Fields and Dethier 2019). It may also reflect the minimal biological uptake of these ions relative to dissolved nutrients, which showed the inverse pattern.

Macroinvertebrate community structure: Despite the clear differences in water chemistry between stream types, we found that other variables—especially those related to hydrology and stream size—seemed to have stronger correlations with invertebrate community structure. The glacial and snowmelt sites had relatively similar macroinvertebrate community structure, likely due in large part to their ephemeral hydrology. The glacial sites are thought to freeze entirely in winter while the snowmelt sites become intermittent in late summer most years, drying out completely by early fall. As a result, hydrological factors (i.e., permanence, stream depth, peak discharge) were most strongly correlated with differences in the centroids between stream source types in our multivariate analysis. Flow intermittency is a strong driver of macroinvertebrate composition in many types of lotic ecosystems (e.g., Bogan et al. 2013). In general, the communities at the lake outlet and mixed-source downstream sites overlapped in multivariate space but were distinct from the glacial and snowmelt sites and tended to have higher alpha diversity. These results are probably due to hydrological factors contributing to environmental stability at the lake outlet/mixed sites, which allow for colonization and persistence of taxa that require longer developmental periods and more consistent abiotic conditions (Hieber et al. 2003). Specially, the lake outlets have more stable water temperatures, and both the lake outlets and mixed-sources sites have a permanent hydroperiod.

Previous studies have found that glacial-fed streams are sometimes dominated by Chironomidae and non-insects (e.g., Oligochaeta, Nematoda), with increasing relative abundance of Plecoptera, Ephemeroptera, and Trichoptera as distance downstream from the glacier increases (Maiolini and Lencioni 2001, Milner 2016; but see Giersch et al. 2017, who found high densities of Plecoptera immediaetely below glaciers). Patterns from our study support this generalization overall, although the glacial sites were not as depauperate as many earlier studies have found. Channel stability and water temperature have been advanced as among the most important variables in shaping benthic communities in glacially fed streams (Milner and Petts 1994; Milner 2016; Hotaling et al. 2019). We found limited support for the key roles of these two factors, but this may have reflected somewhat unique local characteristics of our study sites. Maximum water temperatures at the glacial sites were above certain thresholds thought to limit taxonomic richness of glacially fed streams. When maximum water temperatures are < 2 °C and substrate stability is low, insect communities are expected to include only specific Chironomidae (i.e., Diamesinae) (Milner and Petts 1994; Milner 2016). We found relatively high alpha diversity at the glacial sites (comparable to the lake outlets and snowmelt-fed sites), probably because summer maximum temperatures were around 4 °C to 6 °C and channel stability was high compared to other sites in the survey. Glacially fed streams can also show distinct temporal patterns of macroinvertebrate drift relative to other types of streams (Fellman et al. 2023), and this represents an interesting future research direction in our study system.

Although differing in elevation by only about ~ 200 m, the three lake outlet sites had highly distinct macroinvertebrate communities, both from one another and from the other stream types (i.e., high beta diversity). The highest lake outlet (3623 m) was dominated by a large colony of blackflies (*Metacnephia coloradensis*) that have only been found in four other alpine lake outlets in Colorado (Finn and Adler 2006). These colonial blackflies averaged densities of 70,000 per m^2 in our samples from this location and have been reported at densities of 350,000 m^2 (Adler et al. 2004). Our sampling and previous studies (Bushnell et al. 1987; Finn and Adler 2006) suggest they are either absent or very rare in the other lake outlets of the Green Lakes Valley, and they only form a large colony at the Green Lake 5 outlet. A second lake outlet just downstream was dominated by fingernail clams (*Pisidium* sp.), which were rare at all other sites. Because alpine lakes are hotspots of aquatic productivity, they export particulate organic matter that can be utilized by filter-feeding taxa (Richardson and Mackay 1991; Green et al. 2022), such as blackflies and clams. Interestingly, filter-feeders were not found in abundance at high elevation lake outlets in the Swiss Alps (Hieber et al. 2005), but this may relate to differences in productivity. Most lower elevation lake outlets are found to support filter-feeders (Richardson and Mackay 1991). Given their proximity, we expected the lake outlet sites to be highly connected via dispersal and colonization, yet they had distinct communities. This may be related to the specific environmental niche of some of the filter feeders, such as *Pisidium* sp. clams, which tend to require soft sediments and moderate flow conditions (Cummings and Graf 2010). We did not observe large differences in summer water temperature or chemistry between the lake outlet sites, although they did vary in geomorphology (e.g., gradient, confinement, flow velocity), and one lake outlet was impacted by a previously constructed and since destroyed dam. Further research would be needed to thoroughly assess possible drivers of the lake outlet community differences in relation to microhabitat, resource availability, and species interactions.

Microorganisms: Periphyton amplicon sequencing, although based on a limited sample size, showed some differentiation among the glacial, lake outlet, and mixed-source sites. This is consistent with a previous study showing that periphyton communities in alpine streams differentiated based on source waters (Hotaling et al. 2019). We found slightly higher richness at the downstream mixed-source sites for bacteria and eukaryotes, which reflected the same pattern we observed for macroinvertebrates. This may be due to the warmer temperatures or more stable hydrological conditions at this downstream site and is consistent with previous observations that microbial diversity also tends to increase with decreasing elevation (Wilhelm et al. 2013). Other studies have shown that groundwater- and snowmelt-fed streams have high organic matter and relatively high microbial biomass (Battin et al. 2004). The higher richness of macroinvertebrate communities in downstream mixed-source sites may in part reflect the more diverse and higher biomass microbial community providing more abundant food resources to consumers at the base of the food web.

Morphology vs. metabarcoding: Metabarcoding detected more taxa than morphological identifications. Using morphological identifications, with chironomids identified only to sub-families, but many other taxa identified to genus, we detected 52 taxa (Table S3). Metabarcoding detected 99 unique taxa, with most of the added diversity being Diptera (especially Chironomidae-61 taxa). Resolving the midge taxa increased richness estimates for the glacial streams in particular, where midges dominated community composition (~75% of taxa). Morphological studies that do not resolve midges past sub-families, which is relatively common, probably underestimate richness of alpine headwater streams. Underestimation is likely not as severe in mid-order streams as they tend to have higher evenness with more easily identified taxa (e.g., Ephemeroptera, Plecoptera, Trichoptera, Coleoptera, etc.).

A high percentage of the macroinvertebrate sequences we obtained aligned to existing sequences reported from previous barcoding work carried out in adjacent watersheds (Gill et al. 2014). Sequences from only eight taxa (three insects, three worms, two crustaceans) matched with < 90% similarity to existing sequences. In the study by Gill et al. (2014), DNA barcoding revealed cryptic diversity of Ephemeroptera, Plecoptera, and Trichoptera (EPT) taxa that was not observed based on morphological identifications. They detected 49 EPT morphospecies and 69 distinct lineages using molecular methods. Similarly, based on morphology, we identified a single taxon each within several EPT genera (e.g., *Baetis*, *Cinygmula*, *Zapada*), where we found two to four distinct lineages per genera based on metabarcoding. As a result, metabarcoding helped detect a significant level of cryptic diversity, even among relatively well-studied genera.

The combination of morphological identifications with metabarcoding was more effective at characterizing macroinvertebrate communities than either method alone. Each technique has limitations. Specifically, the primers used in our study are known to exhibit taxonomic biases against Trichoptera and do not thoroughly detect non-insect taxa (worms, crustaceans, gastropods, etc.; Leese et al. 2021). When the data from the morphological identifications and metabarcoding were combined, we had a list of 130 taxa in total, indicating that both approaches detected unique taxa and contributed important information to the study results. Additionally, metabarcoding does not provide quantitative estimates of abundance or body size. This means that for many ecological questions, traditional sampling techniques that provide density or biomass will still be needed.

Conclusions: Using a combination of morphological and molecular barcoding approaches, our study found support for the hypothesis that distinct source waters would harbor unique benthic communities in alpine streams. Changes in community structure were linked to several abiotic factors, including stream permanence and conductivity. By examining linkages between environmental conditions and benthic community structure, this study adds to a growing body of knowledge that will be useful in predicting future shifts in alpine stream communities. Climate change is predicted to cause changes in stream flow patterns (e.g., more intermittent flow regimes, earlier peak discharge), losses of surface and subsurface ice, changes in streambed stability, and warming stream temperatures (Hotaling et al. 2017; Brighenti et al. 2019). These changes are likely to influence the abundance, size distribution, and local composition of benthic communities, with potential indirect effects on food web functioning, energy flow, and connectivity between aquatic and riparian environments (O'Gorman et al. 2021; Junker et al. 2024). Moving forward, experimental approaches that mechanistically manipulate environmental conditions (e.g., temperature, hydrochemistry) will be useful in understanding consequences of future environmental changes. Furthermore, comparative studies that evaluate links between alpine stream ecosystem function and community structure across distinct global regions will be useful in developing and testing further generalizations about the importance of alpine source waters. Lastly, our results provide support for conservation planning that incorporates distinct alpines stream types and recognizes their unique attributes. Because alpine streams are typically isolated and challenging to access, they have not received significant conservation attention, but their potential to support unique biodiversity and have strong impacts on downstream ecosystem services warrants our attention and their enhanced protection globally.

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Author contributions D. P., S. H., and K. A. developed the study design and coordinated research. D. P., K. A., S. Y., and J. M. conducted field research. I. E., P. F., T. P., and T. H. conducted molecular sequencing and associated analyses and laboratory work. D. P. wrote the first manuscript draft and all authors reviewed the manuscript and contributed feedback.

Data availability Amplicon data have been deposited in the National Center for Biotechnology Information Sequence Read Archive with the

BioProject Accession #PRJNA1124077. Other datasets will be made available from the Niwot Ridge LTER data catalog (https://nwt.lternet.edu/data-catalog) upon publication of the manuscript.

Declarations

Conflict of interest The authors declare no competing interests.

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