THESIS

SPATIAL AND ENVIRONMENTAL INFLUENCES ON MACROINVERTEBRATE COMMUNITY STRUCTURE THROUGH A STREAM NETWORK

Submitted By

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ABSTRACT

SPATIAL AND ENVIRONMENTAL INFLUENCES ON MACROINVERTEBRATE COMMUNITY STRUCTURE THROUGH A STREAM NETWORK

Stream ecosystems provide a unique framework for examining spatial variation in community structure. Streams generally have semi-predictable changes in abiotic characteristics from headwaters to mainstems, which leads to specific expectations for how longitudinal position along the stream gradient may shape benthic macroinvertebrate community structure. Streams also have a dendritic network configuration and directional connectivity, which may generate spatially-structured communities that are influenced by rates of dispersal between sites. In this study, we evaluated how benthic macroinvertebrate communities change along a longitudinal stream gradient, and we assessed the relative influence of local environmental variables versus dispersal-related mechanisms in shaping community structure. We hypothesized that (1) the position along the longitudinal stream gradient would be primary driver in shaping both alpha diversity (i.e., local taxonomic richness) and beta diversity (i.e., community variation between sites). Specifically, we expected that alpha diversity would increase and beta diversity decrease as stream order increased. Additionally, we expected that (2) pairwise differences in community structure between sites would increase with both greater geographic distances and greater environmental dissimilarity, reflecting the influence of both dispersal-related and local environmental filtering mechanisms. To assess the hypotheses, we collected macroinvertebrates and environmental data from 63 sites spanning 1st, 2nd, and 3rd order streams across three watersheds in the Sangre de Cristo Mountains of southern Colorado. Community structure was characterized using measures of density, biomass, alpha diversity, beta diversity, and pairwise community dissimilarity between sites (based on Bray-Curtis dissimilarity). Environmental variables were summarized using Principal Components Analysis, and pairwise environmental distances were used to evaluate the role of local environmental conditions relative to geographic distances between sites in shaping community structure. Geographic distances were quantified as both instream and overland distances, which were expected to affect aquatic and terrestrially dispersing taxa. Our results showed that alpha and beta diversity remained relatively consistent across stream orders along the longitudinal gradient. Density and biomass peaked in 2nd order sites, potentially due to increasing anthropogenic influences in 3rd order sites and challenging natural environmental conditions in 1st order sites. Although measures of diversity did not shift, taxonomic composition differed significantly between stream orders, indicating species turnover was happening along the stream gradient likely due to a shift from more specialist taxa to more generalist taxa at higher stream orders. Environmental dissimilarity and instream geographic distances were both significantly correlated with community dissimilarity, suggesting that local environmental conditions and dispersal-related factors affected community structure. Importantly, however, environmental dissimilarity was more strongly correlated than measures of geographic distance, indicating that local conditions play a more important role than dispersal at the spatial scale of our surveys. These findings highlight the role of environmental heterogeneity in shaping macroinvertebrate metacommunities within headwater stream networks. Additionally, the turnover in composition along the stream gradient highlights the importance of conservation measures that consider biodiversity patterns across the whole stream network, rather than specific isolated reaches.

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DEDICATION

To my family –

Your unwavering support, profound belief, and endless encouragement

have allowed me to pursue my passions.

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Introduction

A long-standing objective in stream ecology has been to understand and predict the factors that shape community structure across spatial and environmental gradients (Poff 1997, Heino et al. 2003, Poff et al. 2006, Brown and Swan 2010, Heino et al. 2015a). In particular, understanding the relative roles of local ecological factors relative to larger-scale biogeographic drivers is important for conservation planning and for predicting how environmental change may affect stream ecosystem functioning (Bae et al. 2005, Magbanua et al. 2010, Myers et al. 2021). However, the structure of benthic communities varies greatly across spatial scales, which can be due to abiotic and biotic causes that are difficult to disentangle (Palmer and Poff 1997, Palmer et al. 2010, Heino et al. 2015b).

Community structure is predicted to change across longitudinal spatial positions in stream networks. The River Continuum Concept (RCC, Vannote et al. 1980), coupled with Strahler’s stream order (Strahler 1957), describe longitudinal changes in stream ecosystems by outlining systematic patterns in stream size and ecological function along the gradient. Together, they have served as a foundational framework for examining patterns in stream community ecology. The RCC links physical and biological characteristics of a stream to describe environmental parameters and energy inputs that vary along the longitudinal gradient. In turn, these spatial patterns have been used to predict and test how benthic macroinvertebrate community structure varies along stream gradients (Doretto et al. 2020). The RCC describes how niche selection influences macroinvertebrate community structure but did not fully account for the roles of dispersal, spatial connectivity, or ecological filtering processes that can affect community assembly and species persistence at local scales (Poff 1997, Green et al. 2022). Additionally, the RCC’s predictions were made under the assumption of natural systems and did not incorporate anthropogenic factors as potential influences on community structure (Vannote et al. 1980, Doretto et al. 2020). Alternatively, considering lotic ecosystems as a network with dendritic organization , rather than a linear system, can change predictions about community structure and stream functioning (Clarke et al. 2008, Altermatt 2013, Richardson 2019, Doretto et al. 2020). As a result, the original RCC has become more nuanced and has evolved over time (Doretto et al. 2020).

Headwater streams are important components of the of stream network because they are the most abundant by stream length and may contribute uniquely to stream functioning and biodiversity (Meyer et al. 2007, Downing 2012, Allen et al. 2018). Headwater streams (defined here as 1st order to 3rd order) have a number of characteristics that may cause ecological dynamics and macroinvertebrate assemblages to differ from higher order streams. Headwaters can exhibit greater among-stream differences in local habitat characteristics because of heterogeneity in environmental conditions across relatively small spatial scales (Heino et al. 2004, Finn et al. 2011, Hotaling et al. 2017, Green et al. 2022). Headwaters also differ in discharge, light, substrate, and resource availability relative to higher order reaches (Palmer and Poff 1997). For instance, some montane headwater streams lack canopy cover leading to increased light availability and reduced inputs of allochthonous material that may be a main energy source in downstream reaches. Additionally, environmental conditions are typically most challenging in headwaters, with lower temperatures, shorter growing seasons, and sometimes volatile flow regimes (Hotaling et al. 2017, Green et al. 2022). In addition to their unique environments, the spatial isolation of headwater streams relative to more connected mainstem reaches can result in the development of distinct community assemblages (Sarremejane et al. 2017).

Metacommunity theory provides a conceptual basis to describe spatially heterogeneous landscapes and processes that affect diversity and organismal distributions across the stream network (Altermatt 2013, Tonkin et al. 2018a, 2018b). This framework considers the structure and dynamics of multiple local communities that are linked to varying degrees via dispersal across the stream network (Leibold et al. 2004, Heino 2013). The metacommunity concept allows for exploration of how abiotic and biotic processes shape community structure via “ecological filters”. In particular, it considers the roles of dispersal-related mechanisms (“dispersal filters”), and local environmental variation (“environmental filters”) as drivers in shaping diversity and composition. This provides a useful conceptual framework for understanding drivers of community structure in stream ecosystems.

Common ways to measure community composition within a metacommunity structure framework are through alpha and beta diversity. Alpha diversity describes local site-level community richness. Local alpha diversity is commonly perceived to be hierarchically regulated by the longitudinal stream gradient. For instance, as streams progress from 1st order to larger mid-order sites, increases in habitat area and resource availability are expected to provide a greater array of niches that can support more diverse macroinvertebrate assemblages (Vannote et al. 1980, Heino and Paasivirta 2008, Sarremejane et al. 2017). Beta diversity was not considered in the early conceptual frameworks of stream community structure (e.g., the RCC), but is used to describe variation in community composition between different locations (Whittaker 1960, Tonkin et al. 2018a). Using pairwise comparisons of community dissimilarity (e.g., Bray-Curtis dissimilarity) between sites as a measure of beta diversity can highlight spatial turnover along the stream gradient and identify community variation across stream orders (Anderson et al. 2006, 2011, Baselga 2010). Research suggests that low order headwater streams may have unique communities and relatively high beta diversity, due to heterogeneous stream characteristics across headwater sites (Palmer and Poff 1997, Finn et al. 2011).

One factor that can strongly shape local stream community structure is geographic distance between communities. Distance decay relationships typically describe an increase in community dissimilarity due to increased distance between localities, although this pattern may vary with the dispersal abilities of the local communities (Heino and Mykrä 2008, Morlon et al. 2008, Brown and Swan 2010, Heino et al. 2015b). Distance decay relationships are sensitive to spatial processes, particularly dispersal barriers that may limit the movement of organisms across landscapes, making them a valuable tool for testing mechanisms underlying community composition (Morlon et al. 2008). When examining the possible role of dispersal throughout a stream network, it is important to consider the dispersal traits of organisms, such as the potential to move long distances in aquatic or terrestrial environments (Tonkin et al. 2018a). Assessing both overland and instream distances as distinct dispersal pathways is valuable in this regard, as different organisms within the stream community may rely on different modes of movement (Brown and Swan 2010). By characterizing dispersal pathways and considering stream network configuration, we can enhance the understanding of how spatial factors contribute to stream community organization through dispersal filtering (Heino et al. 2015b).

In addition to geographic distance, the local environmental conditions at a site are predicted to be a key factor in controlling community structure. Environmental filtering involves community sorting across environmentally heterogeneous habitats according to ecological requirements of community members

(Clarke et al. 2008, Stewart and Schriever 2023). Fluctuating environmental conditions – such as changes in temperature, flow, or substrate conditions – can exert strong filters on species composition, impacting local macroinvertebrate populations due to their specific habitat requirements. Anthropogenic influences, such as changes to flow regimes from dams and diversions or human land use in riparian corridors (e.g., agriculture) can modify habitat conditions resulting in shifts towards more generalist and tolerant taxa (Baker et al. 2011, Hotaling et al. 2017, Li et al. 2018). However, local environmental conditions may also exhibit spatially structured variation (Mykrä et al. 2007) which can make it hard to differentiate between the roles of geographic distance relative to environmental mechanisms in shaping community structure. Studies that assess both factors – geographic distances and local environmental influences – can help to disentangle the relative importance of each mechanism (Brown and Swan 2010, Schmera et al. 2018, Green et al. 2022).

In this study we examined benthic stream macroinvertebrate community structure from both a longitudinal-linear stream order perspective and a more network-based stream distance perspective. Our first objective was to identify how stream invertebrate communities changed over the longitudinal gradient. We hypothesized that position along the longitudinal stream gradient would be a primary driver in shaping both alpha and beta diversity. We specifically predicted that alpha diversity would increase as stream order increased, following predictions of the RCC. In contrast, we predicted that beta diversity would decrease as stream order increased, with communities becoming more similar to one another at downstream locations relative to headwaters. Next, we examined the relative roles of ecological filters in shaping community structure across the steam network, with a focus on dispersal-related mechanisms and local environmental variation as drivers of community dissimilarity. We expected that greater geographic distances between sites and increased environmental dissimilarity would both lead to increased community dissimilarity across the headwater stream network.

Methods

Study location and site selection – Field sampling occurred in the Sangre de Cristo Mountains of southern Colorado, USA and included three watersheds – Sangre de Cristo Creek, Ute Creek, and Trinchera Creek (Fig. 1). All sites were on the Trinchera-Blanca Ranch, which is managed for a variety of uses including agriculture, tourism, and the conservation of native cutthroat trout populations. A total of 63 headwater sites were sampled during the summer months of 2022 to 2024. Survey sites were between 2400 m to 2700 m elevation and included 23 sites on 1st order streams, 18 sites on 2nd order streams, and 22 sites on 3rd orders streams. Stream orders were determined in the field and confirmed using satellite imagery. A reach of 60 m was established at each site. The reach was selected as a representative section of the stream incorporating dominant habitat, in terms of riffle-run, vegetative cover, streambed substate, and stream gradient (see Fig. S1 for example site photos). Total reach length and top and bottom UTM coordinates of the reach were recorded in the field.

Macroinvertebrate collection and identification – Macroinvertebrate collection methods were modified from procedures outlined in the Colorado Department of Public Health and Environment's Aquatic Life Use Attainment, Appendix B, Benthic Macroinvertebrate Sampling Standard Operating Procedure (Colorado Department of Public Health and Environment and Water Quality Control Commission 2020). All samples were collected from riffle habitats within the reach. Macroinvertebrates were collected from the stream benthos using a kicknet (600 μm mesh, 1045 cm^2 opening) from an area of approximately 0.5 m^2. The sampling area was estimated using the kicknet dimensions as a reference. To collect the sample, we disturbed substrate within the sampling area for one minute. Large rocks within the 0.5 m^2 sampling area were scrubbed with a nylon bristle brush to release macroinvertebrates. The kicknet sample was then transferred into a dissecting tray, and all macroinvertebrates within the kicknet bag were collected. The sample was then transferred to a collection jar with a site label and preserved in 70% ethanol. In the laboratory, macroinvertebrate samples were sorted and identified primarily to family level (see Table S1 for taxonomic resolution) using a Leica EZ4 dissecting microscope (Voshell 2002, Ward and Kondratieff 2002, Merritt et al. 2019). Macroinvertebrates were categorized as larva, pupa, or adult to discern between aquatic and terrestrial life stages. Within each sample, total counts were recorded for each taxonomic group and a subset ($n = 10$) of each taxon per sample were measured for body length to the nearest 0.5 mm. Body length measurements were converted to estimated dry biomass using existing length-to-dry mass regressions (Culver et al. 1985, Ganihar 1997, Benke et al. 1999, Miserendino 2001, Sabo et al. 2002, Baumgärtner and Rothhaupt 2003, Cummins et al. 2022).

Environmental variables – At each site, the reach was subdivided into six cross sections where wetted width, stream depth, canopy openness, stream slope, and streambed substrate measurements were recorded. Wetted width (cm) was measured as the bank-to-bank width at current summer discharge. Depth (cm) was taken in the thalweg of each cross section using a meter stick. Canopy openness was measured using a Model-A spherical densiometer at the center of each cross section. Canopy values were expressed as percent canopy openness based on the number of open quadrants from the densiometer. Stream slope was calculated as rise over run, using the author's height (rise) and a Halo XL450 Rangefinder to measure the furthest point upstream at 0° angle (run). A total of 30 streambed substrate particles were measured at each site with five measurements taken per cross section using a Wildco 14-D40 gravelometer. Streambed substrate size was determined as the smallest opening a particle would fit through along its b-axis (Wolman 1954). Opening sizes were: 2, 2.8, 4, 5.6, 8, 11, 16, 22.6, 32, 45, 64, 90, 128, 180, >180 mm.

Elevation, grazing intensity, and presence of water diversions were also recorded for each site. A single measurement of site elevation (m) was collected at the macroinvertebrate sampling location and site UTMs were recorded with a GPS. Grazing intensity was estimated by the author as the observed riparian vegetation damage and the presence of hoofprints and scat of wild and domestic ungulates. Grazing intensity was quantified on a relative scale of 0 to 3 (0 = none, 1 = light, 2 = moderate, and 3 = heavy) within a 3-meter buffer on each bank of the stream reach. Based on field observations and aerial imagery, the presence or absence of upstream water diversion structures relative to the sampling reach were recorded. This measure was considered an indirect assessment of whether stream flows were likely to be natural or altered by water withdrawal.

Remote sensing data – Anthropogenic landcover, upstream watershed area, and geographic distances between sites were quantified with aerial imagery and ArcGIS Pro version 3.3.2. Anthropogenic landcover area (m^2) was quantified with GIS using a 100 m buffer around the sampling site. GIS layers obtained from Trinchera-Blanca ranch managers included landcover of agricultural use (center pivots, irrigated acres), timber harvest, and ranch roads. Railroad and county road GIS layers were obtained from the Colorado Information Marketplace within the Data Catalogue from the Transportation category using datasets: Railroads in Colorado, Major Roads in Colorado, and Local Roads in Colorado (CDOT - Colorado Department of Transportation 2025). Railroad and road data were transformed from line features to polygon features to obtain area measurements. Upstream watershed area (km^2) was calculated for each site using GIS Ready-to-Use watershed tools.

Geographic distances between sites were used as a proxy to test the role of dispersal-related mechanisms in shaping benthic macroinvertebrate community structure. Distances were measured in two ways – overland distance and instream distance. Overland distance (km) was measured as the Euclidean distance between sites and was used as a representative measure of terrestrial dispersal distances. Instream distance (km) was measured as the distance between sites along the stream network and was used as a representative measure of in-channel dispersal throughout the aquatic environment. We calculated overland distances between sites using the *sf* package (Pebesma and Bivand 2023) and instream distances using the *sfnetworks* package (Meer et al. 2024) in the R programming language (R Core Team 2023).

Analyses – Our first objective was to test for differences in macroinvertebrate community structure in relation to position in the stream network. Site-level macroinvertebrate density (individuals per m^2), biomass (mg per m^2), and alpha diversity (richness, measured as the number of unique taxa) were analyzed across stream orders using non-parametric Kruskal-Wallis test, followed by Dunn's post hoc. These analyses were also conducted on a subset of taxa consisting of Ephemeroptera, Plecoptera, Trichoptera, which are generally more sensitive to environmental changes than many other taxa.

Using the *vegan* package in R (Oksanen et al. 2024), we created non-metric multi-dimensional scaling (NMDS) plots based on Bray-Curtis dissimilarities. NMDS plots were used to visualize differences in macroinvertebrate community composition between stream order groups and the three focal drainages. To test for differences in community composition across stream orders, we conducted a permutational multivariate analysis of variance (PERMANOVA) utilizing the 'adonis2' function in R (Anderson 2017). P-values and r^2 values were obtained from 999 permutations. This analysis tested for significant differences in NMDS stream order group centroid positions across multivariate space. Group centroid position represents potential variation in community composition across stream order groups. To further explore differences in community composition, we fitted taxon vectors to the NMDS plot using the 'envfit' function in R (Oksanen et al. 2024). Within the NMDS ordination, beta diversity was measured as the multivariate dispersion within each stream order group (Anderson et al. 2006). We used the ‘betadisper’ function (Oksanen et al. 2024) to calculate the distance from each site to its corresponding stream order group centroid. Dispersion within a stream order group represents the variability of community composition for that stream order. For example, larger within-group dispersion (i.e., greater point-to centroid distances) is equivalent to increased beta diversity (i.e., greater differences in community composition) for that stream order group. Differences in within-group dispersion (e.g., 1st vs 2nd vs 3rd order dispersion) were analyzed across stream order groups using ANOVA, followed by Tukey’s post hoc tests for pairwise comparisons.

Our second objective was to test the relative influence of ecological filtering processes – dispersal-related mechanisms versus environmental variables – in shaping community structure. To accomplish this, we examined the relationships between community dissimilarity and three hypothesized predictors: the overland distance between sites, the instream distance between sites, and the environmental dissimilarity between sites. To quantify community dissimilarity, we calculated pairwise Bray-Curtis dissimilarity values based on abundance data using the ‘vegdist’ function in R (Oksanen et al. 2024). Overland and instream distances were calculated as described above. Environmental dissimilarity was based on a Principal Components Analyses (PCA). The PCA used site-level environmental variables summarized with the *factoextra* package (Kassambara and Mundt 2020). The PCA included elevation (m), average wetted width (cm), average depth (cm), average percent canopy openness, average slope, average grazing intensity, median streambed particle size (d50), upstream watershed area (km^2), anthropogenic landcover area (m^2), and a categorical diversion variable (presence/absence). Fitted vectors representing the direction and magnitude of environmental variables were visualized on the PCA biplot. Environmental dissimilarity was calculated as Euclidean distance between site pairs across multivariate space within the PCA (Brown and Swan 2010). We used Mantel’s test to analyze relationships between each predictor variable (overland distance, instream distance, or environmental dissimilarity) and the response variable (community dissimilarity based on Bray-Curtis values). P-values and r-values were obtained using 999 permutations. Environmental variables were also analyzed individually across stream orders using non-parametric Kruskal-Wallis test, followed by Dunn’s post hoc tests to help interpret changes along the stream gradient. Additionally, we analyzed relationships between geographic distances (instream and overland) with specific subsets of the community that were expected to be primarily aquatic dispersers (lacking a terrestrial life stage) or taxa that could disperse overland (e.g., winged life stage [see Table S1 for dispersal association]). We expected that the aquatic dispersers would be influenced most by instream distances while the terrestrial dispersers would be influenced most by overland distances. These analyses were conducted using Mantel's tests in the same manner as described above. All analyses were preformed using R Statistical Software (R Core Team 2023) in RStudio (Posit Team 2023).

Results

Density, biomass, and alpha diversity – Density and biomass, but not alpha diversity, differed across stream orders (Fig. 2 and Table S2). Macroinvertebrate density was highest for 2nd order streams (Kruskal-Wallis: p = 0.0027), with significant differences between 1st and 2nd order and between 2nd and 3rd order streams. Density increased by 55% between 1st and 2nd order and then decreased by 48% between 2nd and 3rd order streams (Dunn's: p = 0.005 and p = 0.0004; Fig. 2a). Biomass also significantly differed across stream orders and followed the same relative pattern as density (Kruskal-Wallis: p = 0.018). Biomass increased by 35% between 1st and 2nd order and then decreased by 26% between 2nd and 3rd order streams (Dunn's: p = 0.005 and p = 0.007; Fig. 2b). No significant pairwise differences were found between 1st and 3rd order streams for density nor biomass. In contrast, alpha diversity (i.e. local taxonomic richness) remained relatively consistent across stream orders (Kruskal-Wallis: p = 0.88; Fig. 2c). Ephemeroptera, Plecoptera, and Trichoptera (EPT) followed similar patterns as the overall community, with the highest density and biomass in 2nd order streams (Fig. S2, Table S2). EPT biomass significantly differed across stream orders (Kruskal-Wallis: p = 0.0049), with differences observed between 1st and 2nd and 2nd and 3rd order sites (Dunn's: p = 0.0012 and p = 0.0038).

Community composition – Across the 63 sampling sites, a total of 92139 macroinvertebrates were identified, representing 65 unique families and 18 orders (Table S1). Based on relative abundance, five orders accounted for 91.5% of all individuals across sites: Diptera (33% relative abundance, 18 families), Ephemeroptera (30%, 7), Coleoptera (15%, 4), Trichoptera (8%, 13), and Plecoptera (6%, 7). Seven families accounted for 78.4% of all individuals: Diptera Chironomidae (28% relative abundance), Coleoptera Elmidae (14%), Ephemeroptera Baetidae (13%), Ephemeroptera Heptageniidae (12%), Ephemeroptera Ephemerellidae (5%), Diptera Simuliidae (3%), and Plecoptera Nemouridae (3%). Notably, the orders Diptera and

Ephemeroptera together comprised 63% of the total abundance and were the most prevalent taxa in each stream order group, accounting for more than 50% of the individuals within each stream order (Fig. S3).

Community composition differed significantly across stream order groups (PERMANOVA: $p = 0.001$, $r^2 = 0.105$; Fig. 3). The greatest separation in community composition was observed between 1st and 3rd order streams. First order sites were generally associated with negative NMDS1 values while 3rd order sites clustered toward positive NMDS1 values, and 2nd order sites were intermediate between 1st and 3rd order communities (Fig. 3). Nine taxa were significantly associated ($p < 0.05$) with 1st order streams and 14 were significantly associated with 3rd order streams (see Table S3 for all statistically significant associations). For instance, taxa associated with 1st order streams included Plecoptera Nemouridae, Trichoptera Rhyacophilidae, and Arthropoda Ostracoda. In contrast, taxa including Ephemeroptera Leptophlebiidae, Ephemeroptera Leptohyphidae, and Odonata Gomphidae were associated with 3rd order streams.

Differences in beta diversity were not statistically significant across stream orders (ANOVA: $p = 0.42$, $F = 0.87$; Fig. 2d). Multivariate dispersion was highest within 1st and 3rd order streams (mean dispersion = 0.44 and 0.41, respectively), indicating greater community dissimilarity within these groups. Second order stream sites had the lowest dispersion (mean dispersion = 0.39), suggesting lower community dissimilarity.

Local environment versus geographic distance – Within the PCA, 39.7% of environmental variation was explained by the first principial component (PC1 axis) and 19% of environmental variation was explained by the second principal component (PC2 axis). Stream orders occupied distinct regions with the PCA indicating that environmental variables differed consistently along the stream gradient (Fig. 4). Elevation, wetted width, depth, slope, grazing intensity, anthropogenic landcover area, and upstream watershed area all differed with stream order (Kruskal-Wallis: all $p < 0.05$), but no significant differences were observed for percent canopy openness, and d50 particle size (Fig. 5, see Table S4 for medians and additional statistical results). Notably, diversions were observed exclusively in 3rd order streams affecting 50% of 3rd order sites (Fig. 5j). Additionally, environmental variables differed less between the three focal waterheds than across stream orders (Fig. S4).

Community dissimilarity was more strongly correlated with environmental dissimilarity than geographic distances between sites (Fig. 6). As community composition became more dissimilar (i.e., higher

Bray-Curtis values), environmental variables also become more dissimilar, resulting in a significant positive relationship (Mantel: *p* = 0.001, *r* = 0.34; Fig. 6a). Instream distances were also positively correlated with community dissimilarity (Mantel: *p* = 0.01, *r* = 0.11), suggesting a weak but significant relationship (Fig. 6c). However, instream distances explained less variation in community dissimilarity (11%) than did environmental dissimilarity (34%). Overland distances were not significantly correlated with community dissimilarity (Mantel: *p* = 0.16, *r* = 0.03; Fig. 6b). Additionally, overland distances, instream distance, and environmental dissimilarity were not significantly correlated with one another (Fig. S5), suggesting these drivers acted independently in shaping community dissimilarity in our dataset. Lastly, when we analyzed subsets of the community that were classified as either terrestrial or aquatic dispersers (Table S1), we did not find significant relationships between community dissimilarity and overland or instream distances (Fig. S6).

Discussion

Our primary objective was to examine shifts in benthic macroinvertebrate community structure from both a longitudinal-linear stream order perspective and a more network-based geographic stream distance perspective. We hypothesized that position in the stream network may influence macroinvertebrate composition through mechanisms related to both environmental changes and dispersal-related processes associated with geographic distance. We found some support for both hypotheses, although environmental changes seemed to explain observed changes in macroinvertebrate community more so than geographic distances. In the sections that follow we place our findings into the broader context of early conceptual foundations (i.e., the River Continuum Concept) and more recent metacommunity-based approaches.

We expected that position along the longitudinal gradient would be a primary influence in shaping diversity, where alpha diversity would increase, and beta diversity would decrease as stream order increased from 1st to 3rd order sites (Finn et al. 2011). Contrary to our expectations, neither alpha diversity nor beta diversity significantly differed across stream orders, indicating that these measures were not strongly influenced by position along the stream gradient, at least for the headwaters section of the network. Consistency in these diversity measures could indicate that species richness in local communities is constrained by the regional species pool (i.e., gamma diversity), resulting in similar richness throughout stream orders (Heino 2011, Al-Shami et al. 2013). Additionally, consistent local environmental conditions or consistent dispersal across sites may result in similarly diverse communities across the sampling gradient (Finn and Poff 2005, Poff et al. 2006, Heino and Tolonen 2017). However, fairly large differences in environmental conditions were observed across stream orders, creating unique niche spaces along the stream gradient (Fig. 4, Fig. 5, Table S4). But, unlike other studies, environmental differences were not followed by shifts in alpha or beta diversity (Brown and Swan 2010, Altermatt et al. 2013, Li et al. 2019, Green et al. 2022).

Prior studies have generally found an increase in alpha diversity, but variable patterns in beta diversity, across the longitudinal stream gradient. For instance, the increase in alpha diversity from headwaters to mid-order streams, as predicted by the RCC, has been supported by other studies in diverse systems (Melo and Froehlich 2001, Heino et al. 2005, Finn and Poff 2005, Altermatt et al. 2013, Stewart and Schriever 2023). However, unlike alpha diversity, patterns for beta diversity have varied considerably. Some studies found results confirming the hypothesis that as stream order increases, beta diversity decreases (Clarke et al. 2010, Finn et al. 2011, Green et al. 2022). These studies typically attribute relationships between diversity and stream order to changes in physical habitat characteristics. For example, variable conditions across high elevation headwater streams (water temperature, steep stream gradients, flashy hydrology, etc.) may limit the number of taxa that can establish in certain locations, reducing alpha diversity, while simultaneously creating highly unique communities across sites and thus increasing beta diversity (Finn et al. 2011, Hotaling et al. 2017, Green et al. 2022). Other studies, however have reported beta diversity increasing with stream size (Al-Shami et al. 2013) or that beta diversity had no response to spatial position, with similar variation in community structure across the longitudinal stream gradient (Heino et al. 2015a, Harrington et al. 2016). Two considerations are that we identified taxa primarily to the family level and were focused on headwater stream orders (1st through 3rd) rather than the entire downstream network. It is possible that species level identifications would have resulted in greater ability to detect subtle differences in alpha and beta diversity. However, Clarke et al. (2008) identified studies utilizing a wider range of stream orders (1st through 5th) that measured longitudinal changes in alpha diversity and reported total taxonomic richness values that encompassed ours, suggesting that our richness levels were likely high enough to detect differences across orders if they existed.

Although alpha and beta diversity remained consistent across stream orders, macroinvertebrate communities differed significantly in composition (i.e., NMDS group centroids differed), indicating that stream order influenced which taxa were present, rather than how diverse those communities were. Our findings suggest that compositional turnover occurred along the gradient, where changes in community composition reflect taxa replacement between stream order groups (Anderson et al. 2011, Heino and Tolonen 2017). This consistency in diversity, coupled with shifts in taxonomic composition, supports the concept of species turnover. We found more sensitive and specialist taxa were associated with 1st order streams (Trichoptera and Plecoptera) while more facultative and generalist taxa were associated with 3rd order streams (Odonota and Gastropoda) which is consistent with the expectations for habitat requirements of these different taxa (Voshell 2002).

In addition to changes in community composition, we found significant differences in macroinvertebrate density and biomass across stream orders, with higher values occurring in 2nd order streams compared to 1st and 3rd order sites. Elevated density and biomass in 2nd order streams may reflect an optimal balance of environmental conditions that provide refuge from difficult headwater conditions and/or sanctuary from impacts of anthropogenic stressors in 3rd order sites (Meyer et al. 2007, Tonkin et al. 2018a, 2018b, Richardson 2019). Across the survey gradient, 1st order sites were associated with narrow, shallow streams while 3rd order sites were associated with increased levels of grazing, presence of anthropogenic water diversions, as well as more human-impacted landcover in the riparian zone (roads, railroads, and agricultural landcover). These changes in environmental conditions could be partially responsible for the decreases in density and biomass observed in 1st and 3rd order sites. Together, these findings suggests that stream order not only structures which taxa are present but also influences the functional capacity and productivity of benthic communities.

For the second hypothesis, we tested the role of ecological processes – including dispersal-related mechanisms and local environmental differences – as potential filtering mechanisms in shaping community dissimilarity across the stream network. First we examined the role of distance decay relationships to evaluate how different geographic distances may act as dispersal filters through effects on species movement and subsequent community composition (Brown and Swan 2010). By partitioning geographic distances into overland and instream components, we attempted to test the relative roles of terrestrial and aquatic dispersal pathways in structuring communities. We observed that overland distances were not correlated with community dissimilarity, potentially indicating that terrestrial pathways are not acting as dispersal filters at the spatial scale of our survey design. This may suggest that macroinvertebrates have strong dispersal abilities, allowing them to move across the stream network without being constrained by terrestrial topographic features (e.g., taxa with a winged life stage [Coleoptera, Ephemeroptera, Plecoptera, Trichoptera])(Finn and Poff 2005, Poff et al. 2006). However, we detected a correlation, although weak, between instream distances and community dissimilarity, suggesting that aquatic pathways may be more strongly affecting community dissimilarity that overland distances. These findings are not unexpected due to the hierarchical spatial structure of dendritic networks and the limited swimming ability of some taxa which may disperse primarily via passive movement (Altermatt et al. 2013, 2013). Additionally, impediments to connectivity – such as instream diversions – may act as a dispersal barriers, restricting movement for some species and contributing to differences in community composition (Sarremejane et al. 2017, Schmera et al. 2018). As stream distances between sites increased, the likelihood of both natural and anthropogenic aquatic dispersal barriers (e.g., waterfalls and diversion structures) also likely increased in our study area. By defining subsets of communities based on dispersal abilities, we aimed to further evaluate whether organismal traits affected the expected role of dispersal filters in structuring communities. Specifically, we assessed aquatic dispersers in relation to instream distances and terrestrial dispersers in relation to overland distances. However, we found no indication that geographic distances were more strongly correlated with these subsets of the community, which may suggest that dispersal filtering may not be a crucial factor for structuring macroinvertebrate communities across the stream network. Alternatively, the relationships between geographic distance and community dissimilarity were highly variable, suggesting that local mechanisms other than dispersal filtering may more strongly affect community composition (Altermatt et al. 2013).

Environmental heterogeneity plays a fundamental role by creating a mosaic of conditions that may support distinct communities in streams. We found that environmental differences had the strongest correlation with community dissimilarity, suggesting that local environmental variables are key ecological drivers in structuring benthic communities throughout the stream network. From our PCA, environmental variables created clear separations across stream orders. As expected, stream characteristics such as steeper gradients and smaller stream size were associated with 1st order streams, while 3rd order streams had greater wetted width, depth, and all measurements of anthropogenic influences. These findings align with studies that found the geophysical characteristics of stream network structure to be associated with community variability (Altermatt et al. 2013, Leszczyńska et al. 2017, Schmera et al. 2018). Perhaps, some of the most influential environmental characteristics were stream size and anthropogenic variables (grazing, diversions, agriculture, timber harvest, roads), which were strongly associated with 3rd order streams. These variables have also been shown to alter community structure (Magbanua et al. 2010), and human impacts tend to shift communities toward more generalist taxa. Previous studies have demonstrated that environmental filtering often outweighs spatial processes, highlighting the importance of local environmental conditions in shaping community structure even in systems heavily influenced by spatial gradients (Brown and Swan 2010, Astorga et al. 2012, Heino and Tolonen 2017, Stewart and Schriever 2023). However, it is important to acknowledge that environmental conditions are also spatially structured in stream networks, which may lead to misinterpretations of the relative role of environmental variables (Heino 2011, Heino et al. 2015b, Schmera et al. 2018, Tonkin et al. 2018b). This can be especially true in studies within a single watershed, but was avoided in our study because we focused on three watersheds, helping remove the correlation between geographic distances and environmental conditions. Additionally, we found no evidence of distance between sites being correlated with differences in local environmental conditions, suggesting that these variables acted independently in shaping community structure.

Conclusion

In headwater streams macroinvertebrate community assemblages are influenced by the interplay of environmental and spatial processes throughout the stream network. Our results indicate that, while alpha and beta diversity remained relatively consistent, macroinvertebrate communities exhibited turnover in composition through the longitudinal stream gradient. We found that differences in community structure were driven largely by differences in environmental conditions across sites. Understanding the processes regulating the assembly of communities is fundamental to ecology and provides the basis for environmental management (Schmera et al. 2018). Our results help highlight how diversity is maintained and recognition of distinct communities along the gradient has important implications for stream biodiversity and entire stream network conservation. Understating how these communities vary and the ecological drivers shaping them can lead to more informed management decisions improving restoration efforts and conservation priorities moving forward.

Figures & Tables

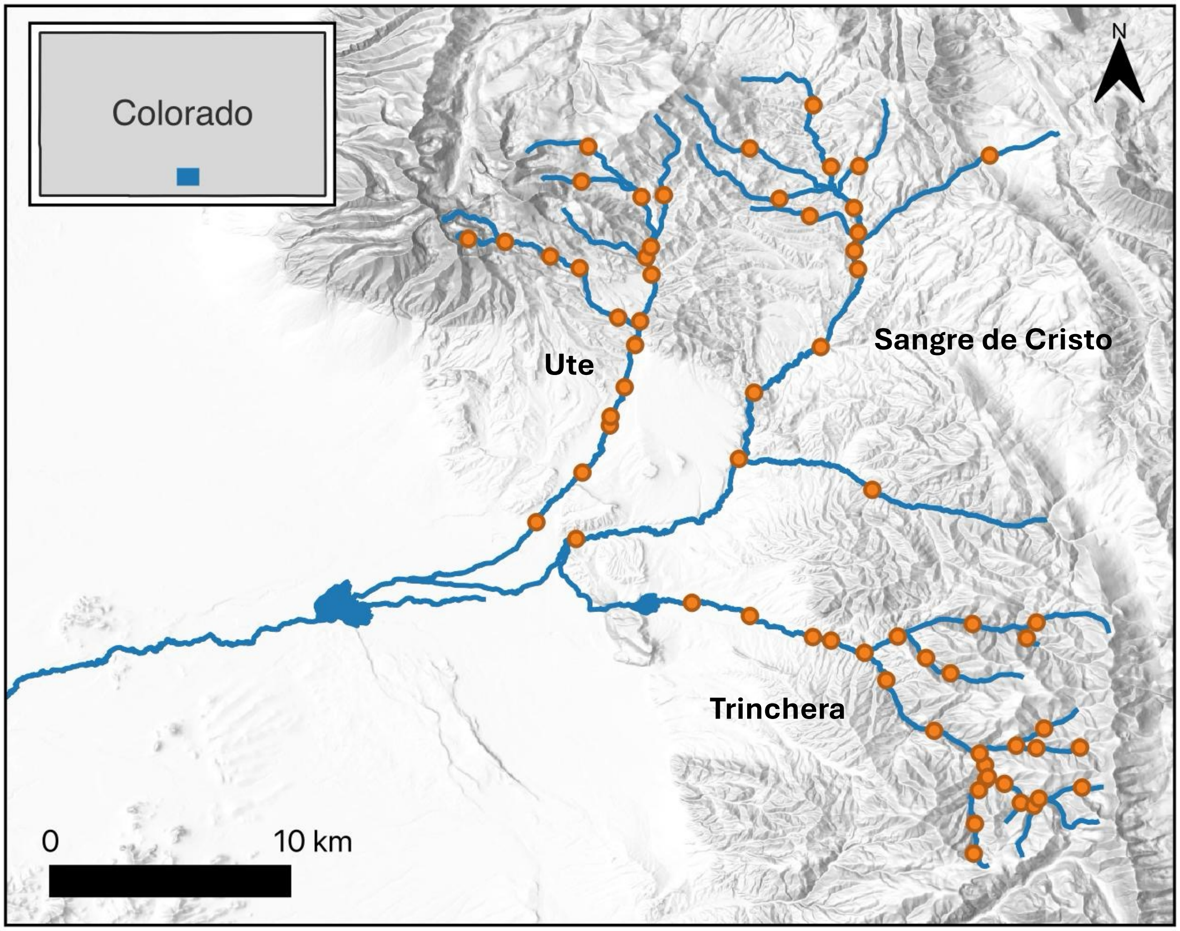

Figure 1. Map of study streams and sampling locations in southern Colorado, USA (inset). Orange points indicate 63 sampling sites across three watersheds: Ute Creek (easternmost), Sangre de Cristo Creek (central), and Trinchera Creek (southernmost). All watersheds drain into Smith Reservoir, ultimately flowing into the Rio Grande River.

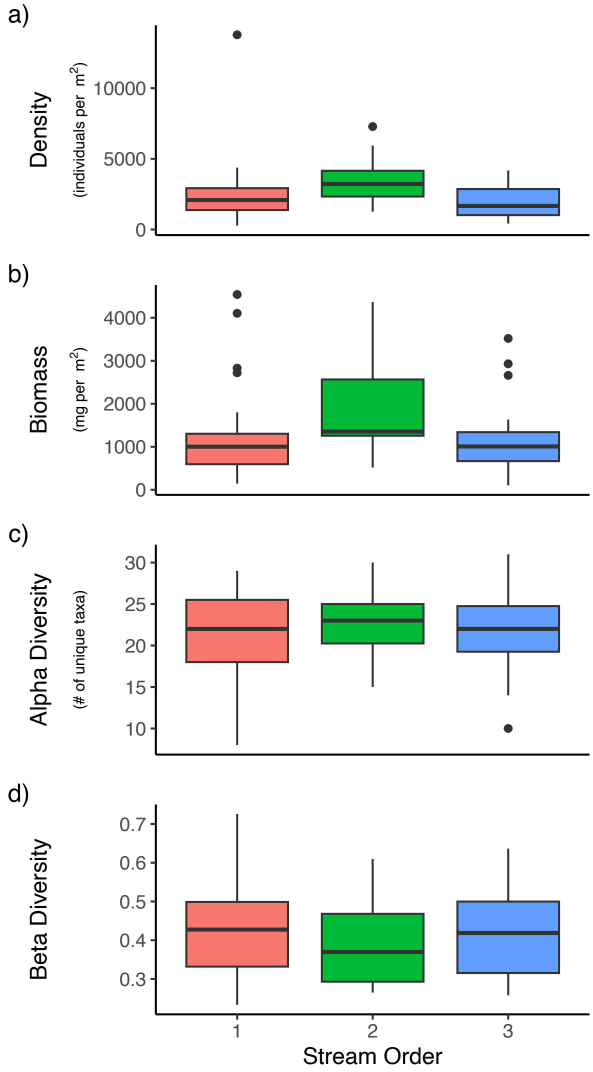

Figure 2. Relationships between stream order and (a) density, (b) biomass, (c) alpha diversity, and (d) beta diversity of benthic macroinvertebrates. Significant differences were observed for density (Kruskal-Wallis: p = 0.0027) and biomass (Kruskal-Wallis: p = 0.018). Alpha diversity is defined as local taxonomic richness at a site, while beta diversity values are based on multivariate dispersion from ordinations of community structure across stream orders.

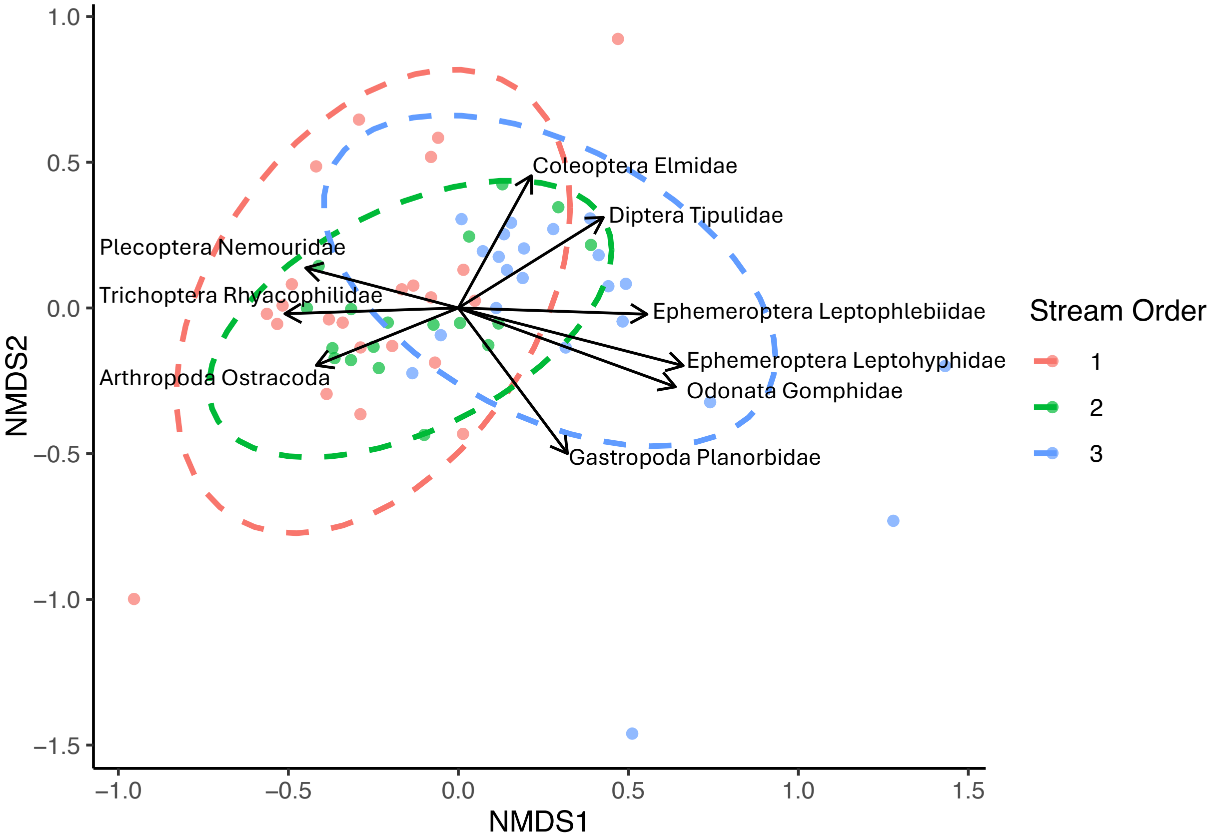

Figure 3. Non-metric multidimensional scaling (NMDS) plot of benthic macroinvertebrate community structure grouped by stream order. Beta diversity did not differ between groups (dispersion within stream order groups, ANOVA: $p = 0.42$) but we did find differences in community composition between groups (variation in group centroid position, PERMANOVA: $p = 0.001$, $r^2 = 0.105$). Taxon vectors are overlaid, indicating the direction and strength of taxon associations with stream order groups ($p < 0.01$). Stress value = 0.18.

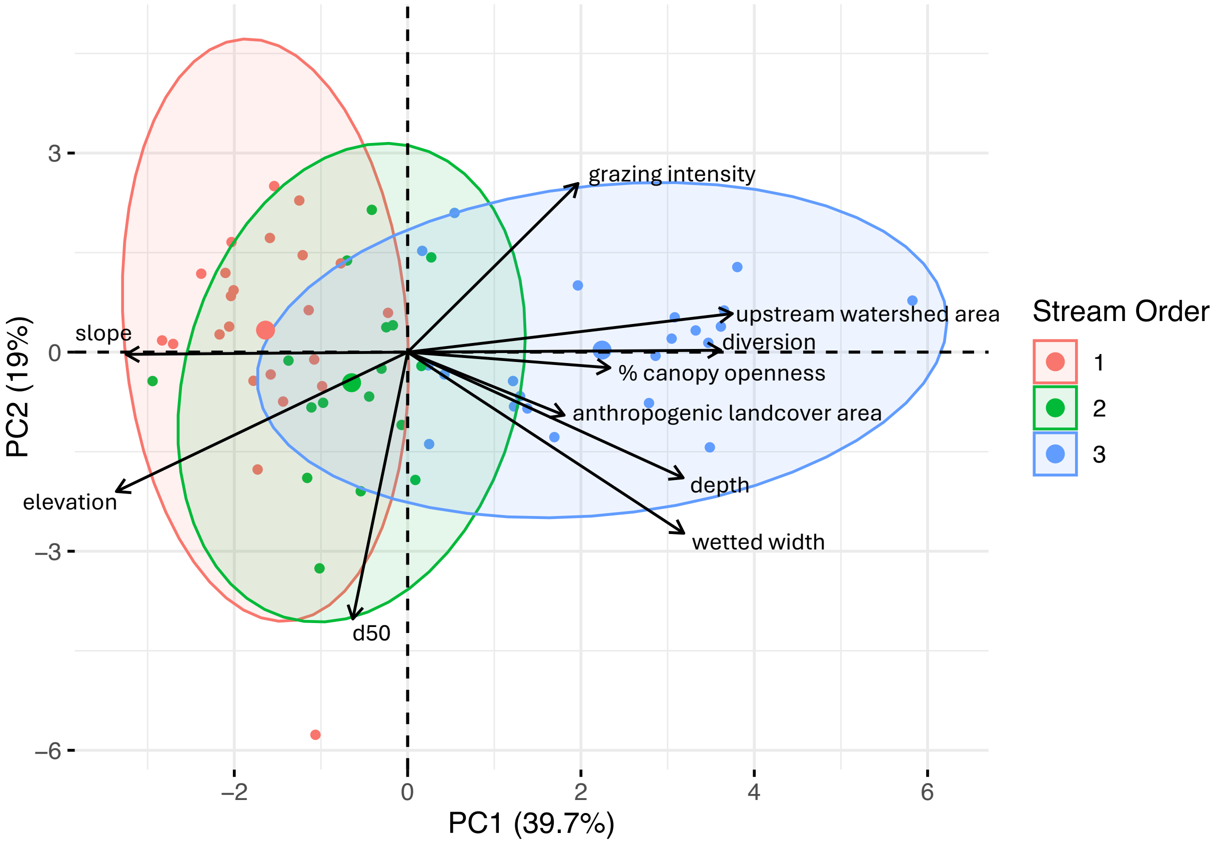

Figure 4. Principal Component Analysis (PCA) biplot of environmental variables with sites grouped by stream order. Vectors represent direction and magnitude of environmental effects. The PC1 axis explains 39.7% of environmental variation and PC2 explains 19%.

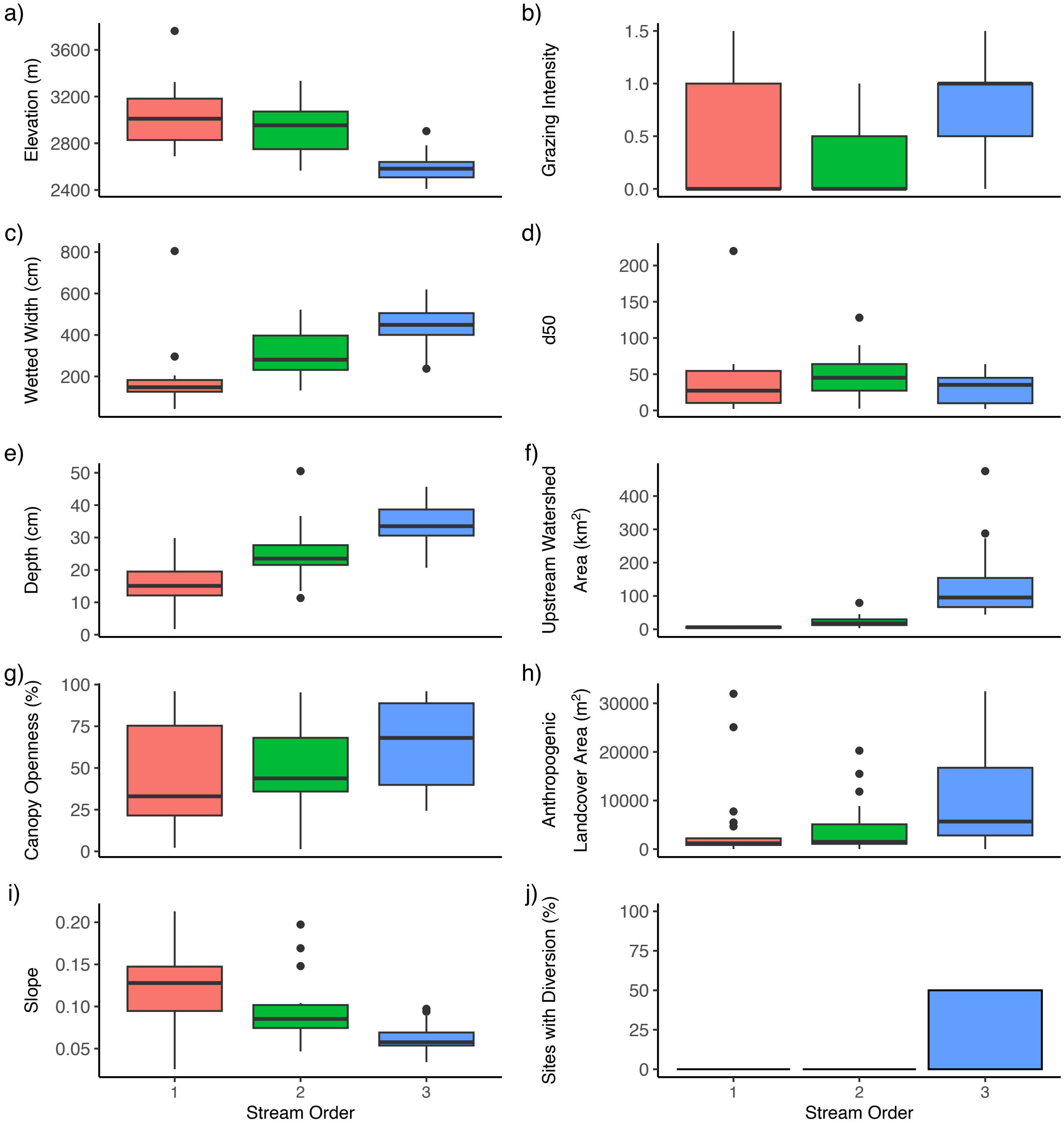

Figure 5. Relationships between stream order and environmental variables: (a) elevation, (b) grazing intensity, (c) wetted width, (d) d50 particle size, (e) depth, (f) upstream watershed area, (g) percent canopy openness, (h) anthropogenic landcover, (i) slope, and (j) percentage of stream order sites with diversion. See Table S4 for median values and statistical results.

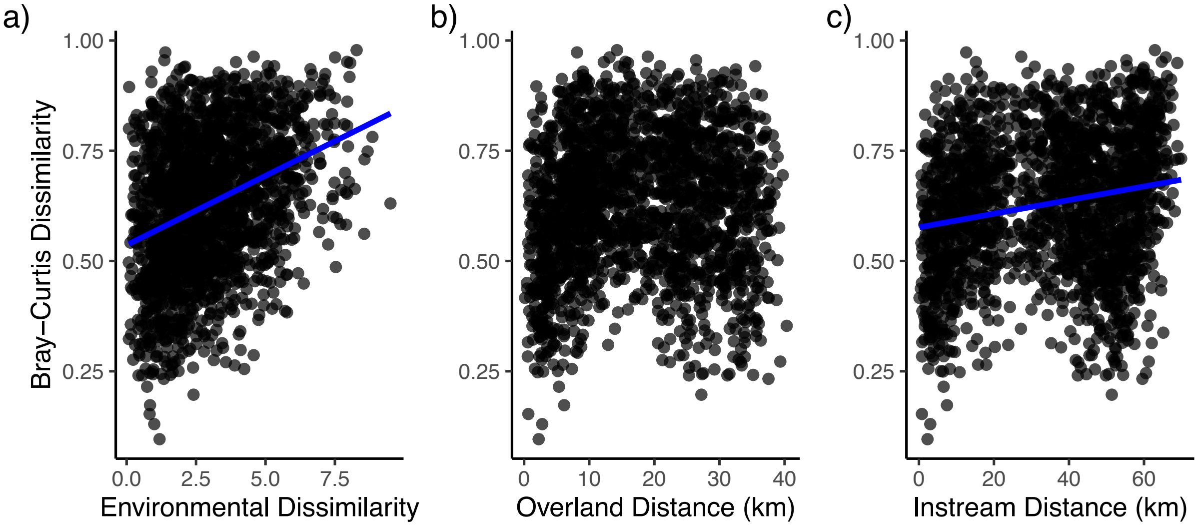

Figure 6. Pairwise relationships of community dissimilarity (Bray-Curtis) with (a) environmental dissimilarity (Mantel: $p = 0.001$, $r = 0.34$), (b) overland distance measured as Euclidean terrestrial distance (Mantel: $p = 0.16$, $r = 0.03$), and (c) instream distance measured as within river network distance (Mantel: $p = 0.01$, $r = 0.11$). Pairwise Bray-Curtis dissimilarities are the community response variable on the y-axes where values range from 0 (exactly similar) to 1 (completely dissimilar). Blue lines indicate significant relationships.

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Appendices

Supplemental Figures & Tables

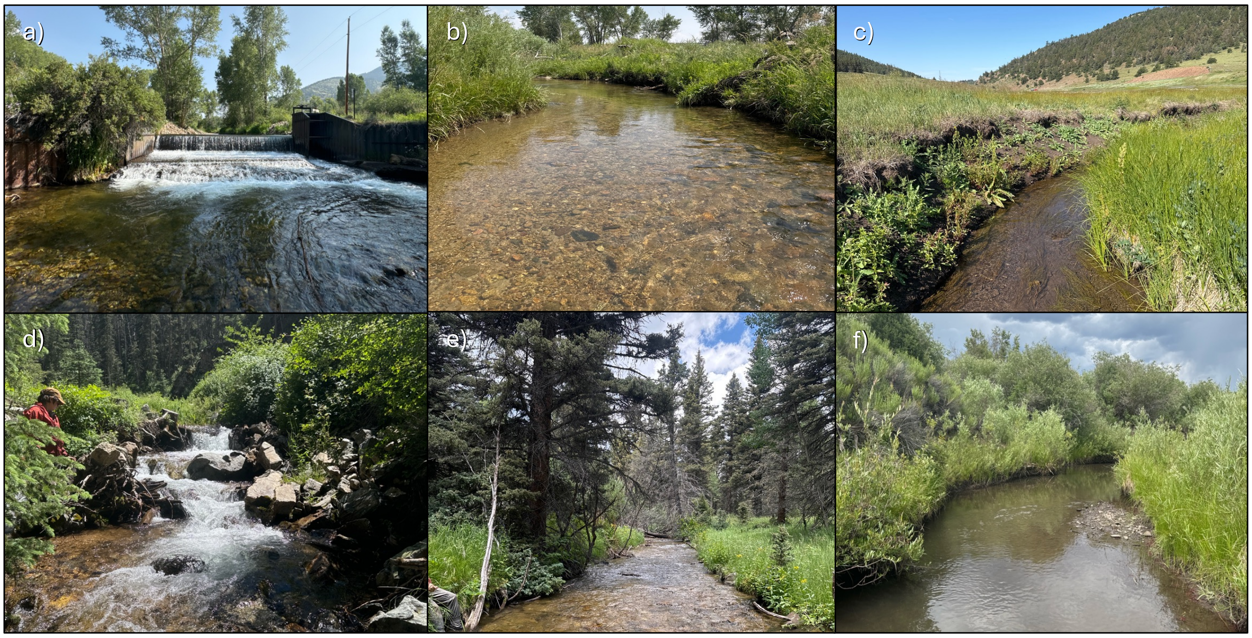

Figure S1. Habitat heterogeneity across sites: (a) anthropogenic infrastructure (water diversion), (b) site adjacent to agricultural land, (c) open canopy headwater, (d) step-pool, (e) coniferous dominated canopy, (f) site adjacent to railroad and highway system.

Table S1. All taxa identified across 63 sites – representing 18 orders and 65 families. Count and relative abundance are based on total macroinvertebrates (92139 individuals) sampled across all sites. Terrestrial dispersers are indicated with an asterisk (*).

Order	**Family**	**Count**	**Relative Abundance (%)**
Arthropoda	Amphipoda	1287	1.40
	Anomopoda	1	0.00
	Copepoda	478	0.52
	Ostracoda	468	0.51
Bivalvia	Sphaeriidae	508	0.55
Coleoptera *	Dryopidae	1	0.00
	Dytiscidae	87	0.09
	Elmidae	13331	14.47
	Hydrophilidae	20	0.02
Collembola	springtail	24	0.03
Decapoda	Cambaridae	2	0.00
Diptera *	Athericidae	1	0.00
	Blephariceridae	3	0.00
	Ceratopogonidae	233	0.25
	Chironomidae	25845	28.05
	Culicidae	5	0.01
	Dixidae	131	0.14
	Empididae	685	0.74
	Ephydridae	9	0.01
	instar	1	0.00
	Limoniidae	38	0.04
	Pediciidae	39	0.04
	Phoridae	6	0.01
	Psychodidae	58	0.06
	Ptychopteridae	31	0.03
	Simuliidae	2822	3.06
	Stratiomyidae	4	0.00
	Tabanidae	1	0.00
	Tipulidae	176	0.19
Ephemeroptera *	Ameletidae	82	0.09
	Baetidae	11526	12.51
	Ephemerellidae	4948	5.37
	Heptageniidae	11147	12.10
	instar	89	0.10
	Leptohyphidae	108	0.12
	Leptophlebiidae	107	0.12
Gastropoda	Ancylidae	9	0.01
	Lymnaeidae	11	0.01
	Physidae	14	0.02
	Planorbidae	10	0.01
	Valvatidae	5	0.01
Hemiptera *	Hebridae	3	0.00
	Macroveliidae	2	0.00
Hirudinea	leech	5	0.01
Hydrachnidia	water mite	1762	1.91
Lepidoptera *	Pyraloidea	2	0.00
Nematoda	nematode	139	0.15
Odonata *	Gomphidae	9	0.01
Oligochaeta	aquatic worm	2081	2.26
Platyhelminthes	flatworm	1038	1.13
Plecoptera *	Cap-Leuc	102	0.11
	Chloroperlidae	1256	1.36
	instar	29	0.03
	Nemouridae	2639	2.86
	Perlidae	159	0.17
	Perlodidae	709	0.77
	Pteronarcyidae	260	0.28
Trichoptera *	Brachycentridae	1594	1.73
	Glossosomatidae	1776	1.93
	Helicopsychidae	3	0.00
	Hydropsychidae	528	0.57
	Hydroptilidae	235	0.26
	instar	16	0.02
	Lepidostomatidae	280	0.30
	Limnephilidae	693	0.75
	Philopotamidae	10	0.01
	Polycentropodida	193	0.21
	pupae	475	0.52
	Rhyacophilidae	1180	1.28
	Uenoidae	610	0.66

Table S2. Median values and statistical results for density, biomass, and alpha diversity for all benthic taxa (Fig. 2) and EPT taxa (Fig. S2). Significant results are in bold. Median values are reported due to non-parametric analyses (Kruskal-Wallis and Dunn's post hoc).

	median values			*p* -value			
	1st	**2nd**	**3rd**	**Kruskal-Wallis**	**Dunn's 1v2**	**Dunn's 1v3**	**Dunn's 2v3**
density	2082.5	3216.9	1666.9	**0.0027**	**0.0052**	0.1991	**0.0004**
biomass	1001.7	1353.6	1006.9	**0.0182**	**0.0053**	0.4616	**0.0074**
alpha diversity	22.0	23.0	22.0	0.8763			
EPT density	977.1	1291.7	804.0	0.0967			
EPT biomass	671.2	1172.9	771.4	**0.0049**	**0.0012**	0.3594	**0.0038**
EPT alpha diversity	10.0	12.0	13.5	0.0829			

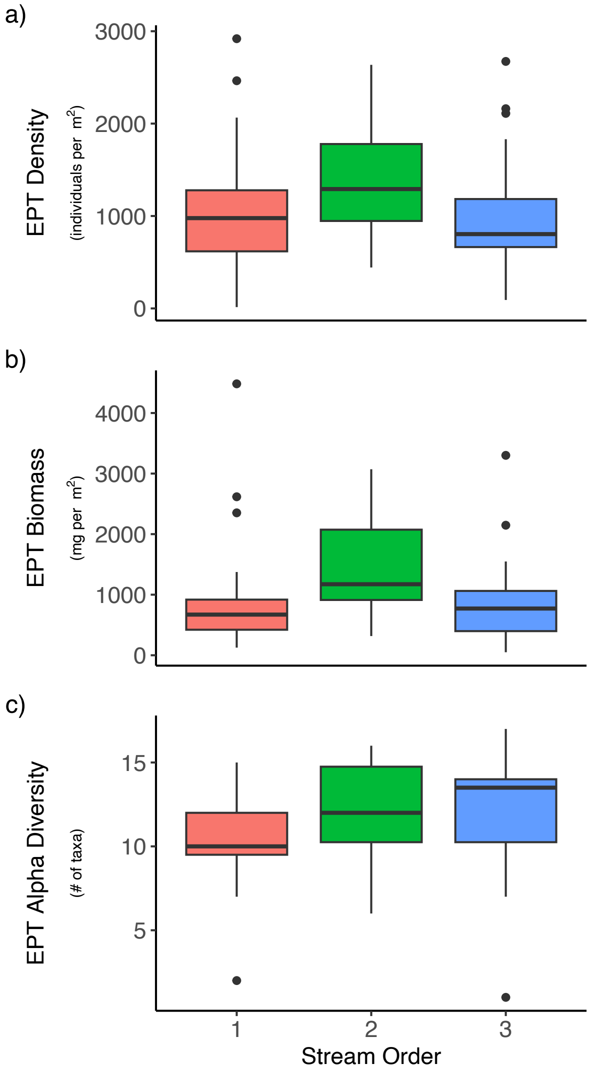

Figure S2. Relationships between stream order and (a) density, (b) biomass, and (c) alpha diversity of Ephemeroptera, Plecoptera, and Trichoptera (EPT) macroinvertebrates. Significant differences were observed for biomass (Kruskal-Wallis; p = 0.005) and pairwise differences were found between 1st and 2nd order and between 2nd and 3rd order streams (Dunn's; p = 0.0012 and p = 0.0038).

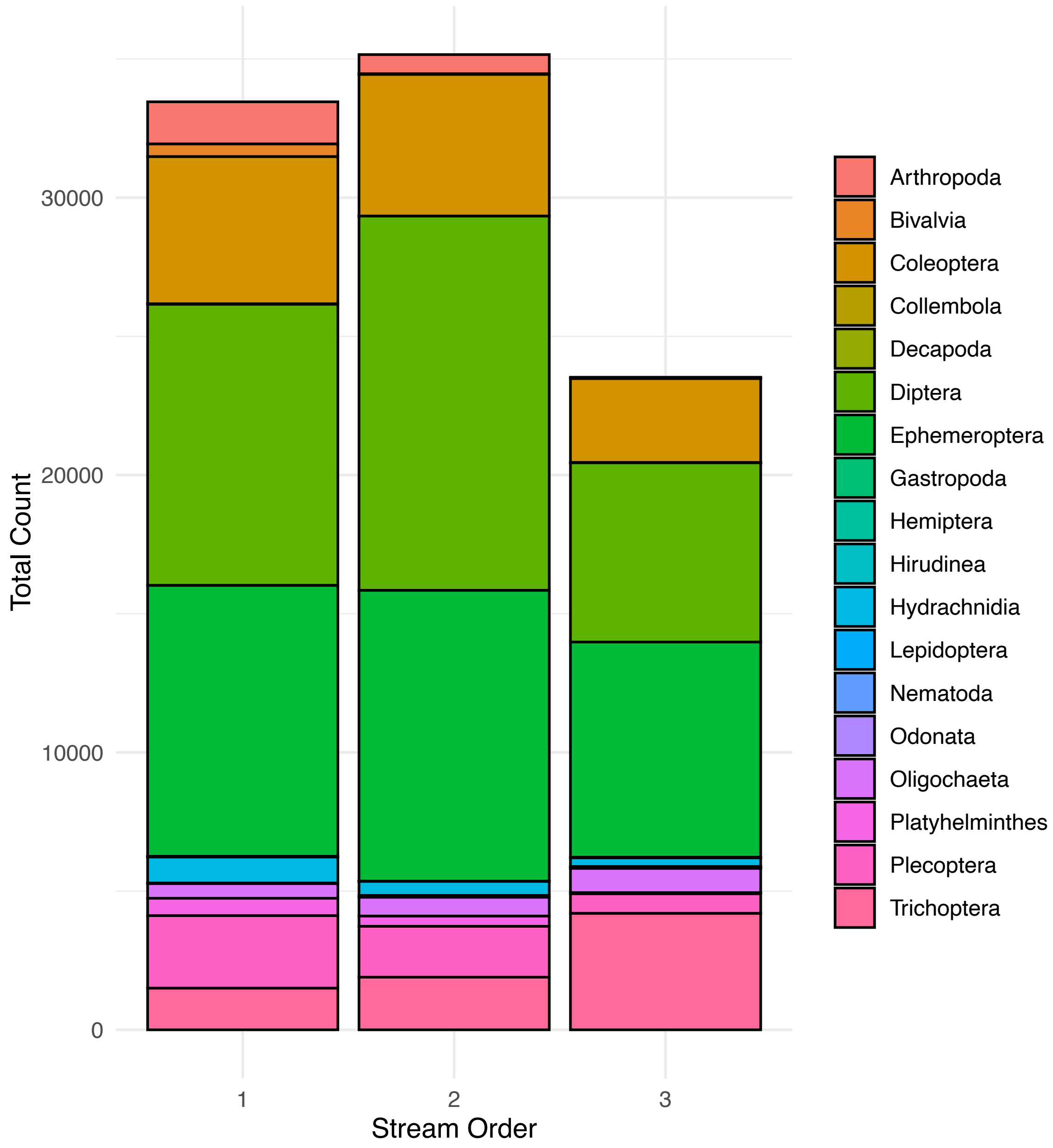

Figure S3. Abundance of taxa based on total counts across steam orders. Different colors represent different taxonomic orders**.**

Table S3. All taxa significantly associated with NMDS1 axis ($p < 0.05$). Negative NMDS1 values correspond to taxa associated with 1st order streams, while positive NMDS1 values correspond to taxa associated with 3rd order streams. Taxa included as vectors in Fig. 3 ($p < 0.01$) are indicated with an asterisk (*).

taxa	**NMDS1**	**NMDS2**	***p*-values**
Trichoptera_Rhyacophilidae *	-0.510	-0.020	0.001
Plecoptera_Nemouridae *	-0.449	0.138	0.005
Platyhelminthes_flatworm	-0.434	0.026	0.010
Arthropoda_Ostracoda *	-0.418	-0.197	0.005
Diptera_Empididae	-0.392	-0.039	0.015
Diptera_Dixidae	-0.373	0.072	0.016
Plecoptera_Cap-Leuc	-0.356	-0.046	0.041
Plecoptera_Perlodidae	-0.326	-0.184	0.034
Hydrachnidia_water mite	-0.264	0.289	0.011
Coleoptera_Hydrophilidae	0.093	0.388	0.038
Diptera_Stratiomyidae	0.101	0.396	0.042
Plecoptera_Pteronarcyidae	0.104	0.358	0.030
Diptera_Tabanidae	0.128	-0.538	0.019
Coleoptera_Elmidae *	0.215	0.454	0.006
Trichoptera_Helicopsychidae	0.216	0.292	0.030
Gastropoda_Planorbidae *	0.321	-0.500	0.003
Arthropoda_Anomopoda	0.385	-0.282	0.029
Lepidoptera_Pyraloidea	0.386	-0.205	0.024
Diptera_Tipulidae *	0.427	0.311	0.001
Gastropoda_Ancylidae	0.508	-0.199	0.011
Ephemeroptera_Leptophlebiidae *	0.555	-0.021	0.004
Odonata_Gomphidae *	0.639	-0.271	0.001
Ephemeroptera_Leptohyphidae *	0.662	-0.198	0.001

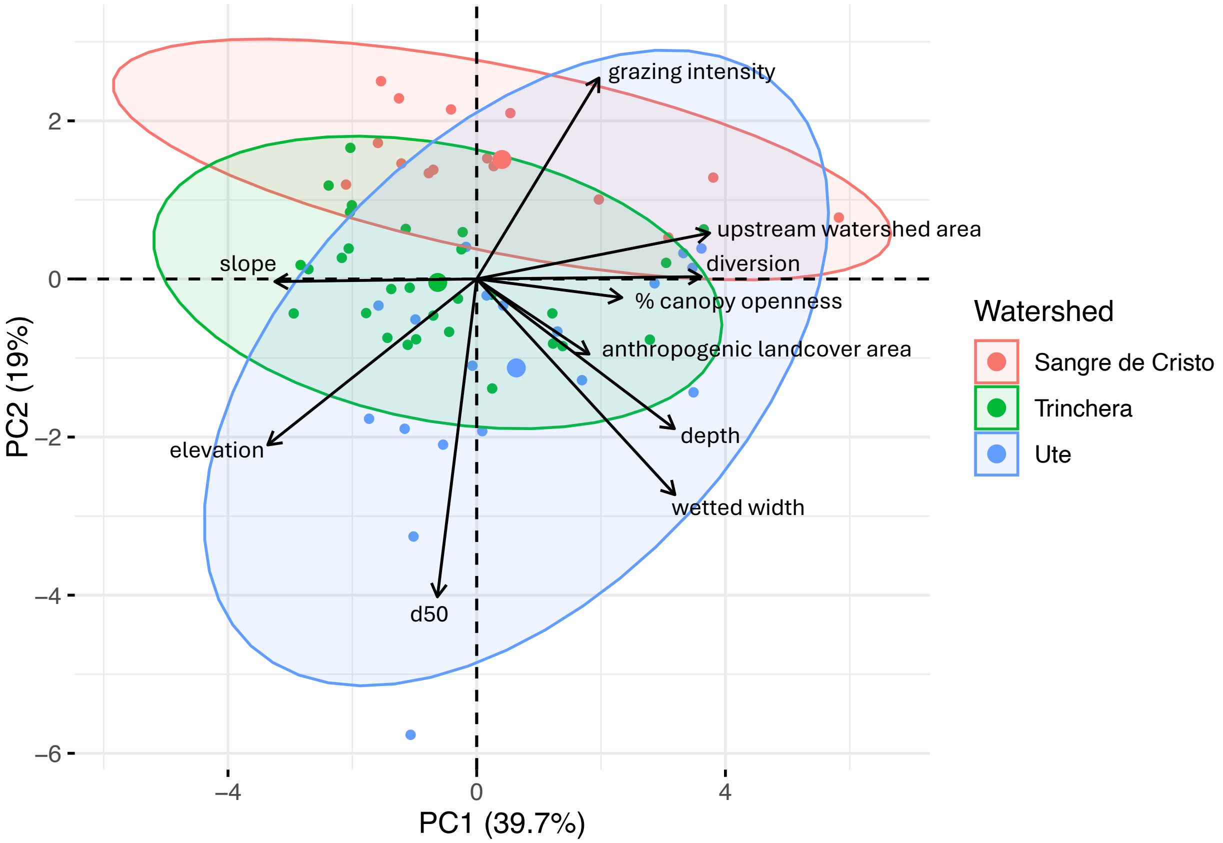

Figure S4. Principal Component Analysis (PCA) biplot of environmental variables with sites grouped by watershed. Vectors represent direction and magnitude of environmental associations. The PC1 axis explains 39.7% of environmental variation and PC2 explains 19%.

Table S4. Median values and statistical results of environmental variables across stream orders. Significant results are in bold. Median values are reported due to non-parametric analyses (Kruskal-Wallis and Dunn's post hoc).

	median values			*p*-value			
environmental variable	**1st**	**2nd**	**3rd**	**Kruskal-Wallis**	**Dunn's 1v2**	**Dunn's 1v3**	**Dunn's 2v3**
elevation (m)	3010.43	2954.15	2582.41	**4.0E-08**	1.3E-01	**0.0E+00**	**0.0E+00**
wetted width (cm)	147.74	280.67	448.73	**2.3E-08**	**1.3E-03**	**0.0E+00**	**4.8E-03**
depth (cm)	15.08	23.50	33.50	**1.3E-08**	**1.6E-03**	**0.0E+00**	**3.2E-03**
% canopy openness	34.32	45.50	70.72	9.0E-02			
d50	27.30	45.00	35.25	2.2E-01			
slope	0.13	0.09	0.06	**1.1E-05**	3.5E-02	**0.0E+00**	**3.8E-03**
grazing intensity	0.00	0.00	1.00	**3.0E-03**	1.2E-01	**1.1E-02**	**5.0E-04**
upstream watershed area (km^2)	6.05	17.80	95.35	**1.7E-11**	**1.5E-03**	**0.0E+00**	**1.0E-04**
anthropogenic land cover (m^2)	1194.41	1493.36	5684.09	**8.4E-04**	1.7E-01	**1.0E-04**	**6.5E-03**
diversion	0.00	0.00	0.50	binary variable - no tests conducted			

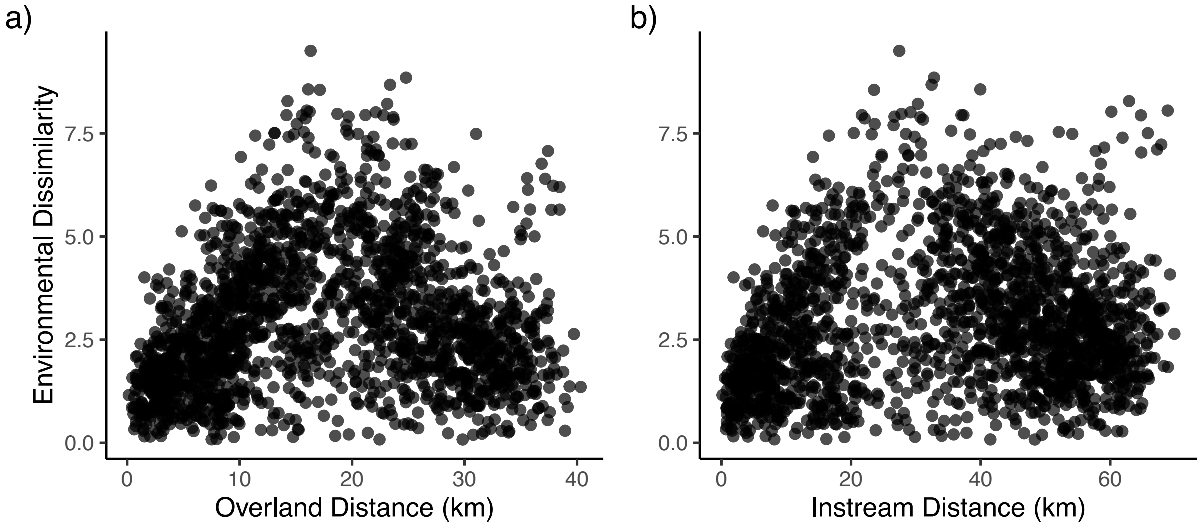

Figure S5. Pairwise (a) overland distances and (b) instream distances in relation to environmental dissimilarity. No significant relationships were found (Mantel's: (a) p = 0.232, r = 0.02 and (b) p = 0.213, r = 0.04), indicating independent interactions of geographic distances and environmental dissimilarity as predictors of community structure.

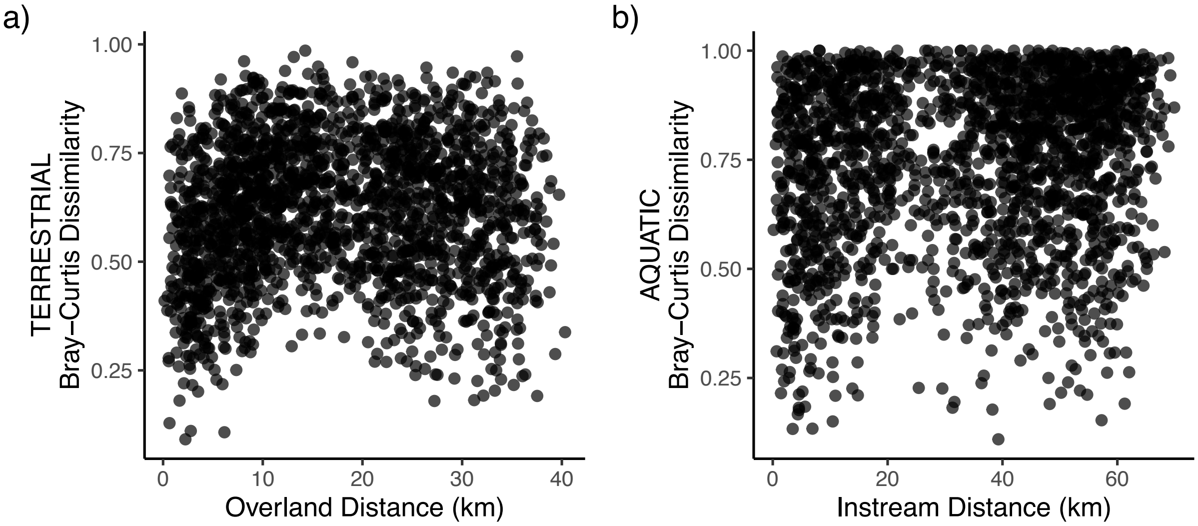

Figure S6. Pairwise geographic distances as predictors of community dissimilarity for subsets of the macroinvertebrate community based on dispersal traits. The left panel (a) evaluates the role of overland distances shaping communities with terrestrial dispersal capabilities, and the right panel (b) evaluates the role of instream distance shaping communities with aquatic dispersal capabilities. No significant correlations were found (Mantel's: (a) $p = 0.163$, r = 0.03 and (b) $p = 0.0567$, r = 0.07). Bray-Curtis dissimilarity values range from 0 (exactly similar) to 1 (completely dissimilar).