

SPATIAL VARIATION IN MACROINVERTEBRATES IN GROUNDWATER-
DOMINATED RIVERS: IS COMMUNITY COMPOSITION EXPLAINED
BY DISCRETE OR CONTINUOUS RIVERINE MODELS?

by

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	iv
LIST OF TABLES	vii
LIST OF FIGURES.....	viii
LIST OF ABBREVIATIONS.....	ix
ABSTRACT.....	x
CHAPTER	
I. INTRODUCTION.....	1
II. METHODS AND MATERIALS.....	5
III. RESULTS.....	14
IV. DISCUSSION.....	20
REFERENCES.....	43

LIST OF TABLES

Table	Page
1. Proportion of variation explained by reach and local predictor variable sets for benthic macroinvertebrate community composition for the upper San Marcos (SMR) and Comal (CR) rivers.....	40
2. Total β -diversity and the percent contribution of taxon replacement and richness difference to β -diversity in each mesohabitat across the three upstream-to-downstream study reaches in the San Marcos and Comal Rivers	41
3. The top three contributor taxa to species contributions to b-diversity (SCBD) for the San Marcos and Comal Rivers.....	42

LIST OF FIGURES

Figure	Page
1. Map of the upper San Marcos River with the three study reaches (R1, R2 and R3) indicated	29
2. Map of the upper Comal River with the three study reaches (R1, R2, and R3) indicated	30
3. Principal component analysis of mesohabitat and reach-level conditions in the upper San Marcos River.....	31
4. Principal component analysis of mesohabitat and reach-level conditions in the upper Comal River	32
5. Box and whisker plots of water physicochemistry	33
6. Partial and global redundancy analyses (RDA) plots of community composition for the upper San Marcos River.....	34
7. Partial and global redundancy analyses (RDA) plots of community composition for the upper Comal River	35
8. Box and whisker plots of taxonomic diversity for each mesohabitat type for the San Marcos River.....	36
9. Box and whisker plots of taxonomic diversity for each mesohabitat type for the Comal River.....	37
10. Areal biomass estimates for the top five contributing taxa in the San Marcos River for mesohabitat types across reaches	38
11. Areal biomass estimates for the top five contributing taxa in the Comal River for mesohabitat types across reaches	39

LIST OF ABBREVIATIONS

Abbreviation	Description
SMR	San Marcos River
CR	Comal River
R(1,2,3)	Reach 1, 2, 3
RCC	River Continuum Concept
PCA	Principal Components Analysis
RDA	Redundancy Analysis
SCBD	Species Contribution to β -Diversity
ANOVA	Analysis of Variance

ABSTRACT

Benthic macroinvertebrate communities in riverine ecosystems are shaped by both local and large-scale influences. Most models of riverine community structure are grouped into either discrete or continuum conceptual models. However, there is much uncertainty in how these two classes of conceptual models apply to macroinvertebrate communities in the physicochemically consistent headwaters of spring-influenced rivers. This thesis examined benthic macroinvertebrate community composition and biomass among local discrete geomorphic mesohabitats (riffles, runs, and pools) and along an upstream to downstream gradient in two spring-fed rivers in central Texas (San Marcos River and Comal River, Hays County). The goal of my thesis was to examine the relative importance of downstream distance from springhead versus smaller-scale habitat conditions (i.e., mesohabitat units) in explaining the variation in macroinvertebrate community structure in these two spring-based subtropical riverine systems. I hypothesized that, because I sample in the upper physicochemically consistent reaches of both rivers that local mesohabitat conditions would explain more variation in community composition than downstream distance from springhead. I expected this change in community composition would be driven by replacement of taxa rather than differences in taxonomic richness. I also predicted that non-native benthic species would be more evenly distributed throughout the study reaches due to the consistency of physicochemical conditions. Finally, I predicted that the distribution of taxa specific biomass contributions will follow patterns seen in community composition change and

will reflect taxonomic density estimates. To test these hypotheses, I sampled macroinvertebrates and local habitat parameters from mesohabitat types along three reaches in the first ~4 km of each river. I also determined biomass of each taxon and community-wide patterns of biomass distribution of benthic macroinvertebrates. I found that variation in taxonomic composition in both rivers was driven to a greater or equal amount by upstream to downstream distance from spring-influence, and by mesohabitat type. Taxonomic diversity did not vary with distance from springhead but was consistently lower in pool mesohabitats when compared to runs and riffles in both rivers. Compositional changes and patterns in b-diversity within mesohabitat types among study reaches indicated that replacement (as opposed to richness differences) was the main mechanism leading to patterns of b-diversity. I found that a non-native snail (*Tarebia granifera*) and an endemic spring snail (*Elimia comalensis*) appear to be spatially partitioning habitat in an upstream- to downstream manner. Finally, I found that *Tarebia granifera* constituted the largest proportion of benthic macroinvertebrate biomass in several study reaches downstream from springheads.

I. INTRODUCTION

Freshwater habitats, including groundwater and groundwater-influenced systems, despite constituting a small percent of the earth's total water, support disproportionate amount of global animal biodiversity (Balian, 2008; Dudgeon, 2006; Reid, 2019). Freshwater ecosystems and the communities that occupy them generally experience greater amounts of anthropogenic alteration and climate change pressure than many terrestrial or marine ecosystems (Sala, 2000; Dudgeon & Strayer, 2010). Spring openings and spring-influenced waterways comprise a small proportion of total freshwater, but represent unique physicochemical habitats which sustain endemic fauna, many of which are imperiled (Hubbs, 1995; Cantonanti, 2012; Nair, 2021). Although geographically limited, biodiversity at groundwater influenced systems and the surrounding headwaters can account for large proportions of stream-wide biodiversity and can also contribute to ecosystem functions like the transformation of groundwater quality (Meyer, 2007; Boulton, 2008; Cantonanti, 2012). Springs and associated headwater habitats are among the more at-risk freshwaters due to high levels of human interaction, flow alteration, and groundwater extraction (Hubbs, 1995; Cantonanti, 2012; Di Sabatino, 2020). Although spring ecosystems are biologically unique and represent important reservoirs for biological diversity within drainage networks, springs and their associated communities are largely underrepresented in conservation legislation and scientific research (Sala, 2000; Strayer, 2006; Dudgeon & Strayer 2010; Reid, 2019).

Previous Characterizations of Benthic Communities

Abiotic habitat characteristics such as flow velocity, substrate type, and water chemistry influence patterns of abundance and diversity in benthic freshwater

macroinvertebrate communities. In general, conceptual models that predict the abundance and distribution of macroinvertebrate communities fall into two main categories: so-called “discrete” models which posit that the composition and diversity of riverine systems is driven by variation in hydrogeomorphic characteristics at local (i.e., reach) and larger (e.g., river valley) scales (Poff and Ward 1990; Maasri et al. 2021). For example, macroinvertebrate community structure in rivers can be driven by invertebrate associations with specific habitat types which are arranged in a spatial mosaic across smaller reaches or larger hydrogeomorphic river sections. In contrast, continuum-based models predict changes in community structure with increasing stream size, from headwaters to larger order reaches. Among continuum models, the River Continuum Concept (RCC) is the most prominent and theorizes that continuous longitudinal variation in abiotic factors like canopy cover, flow velocity, and streambed structure affect resource availability and in-stream conditions and therefore influence benthic community composition (Vannote, 1980). While continuum models such as the RCC are applicable across large downstream spatial scales (i.e., from 1st to 12th order lotic systems), such as the basin or catchment levels, less is known about community patterns at smaller scales (<10 km) along hydrogeologic gradients (but see Maasri et al. 2019; Maasri et al. 2021). Although ecologists have long acknowledged that community structure in rivers is affected by processes occurring at multiple spatial and temporal scales, much of the previous work on invertebrates conducted at smaller spatial scales indicates that hydrogeomorphic factors often outweigh local physiochemical factors, and that such influences can be more influential at local levels (Doisy, 2001; Meißner, 2019). Furthermore, similar studies have found consistent differences in benthic

macroinvertebrate taxa at even smaller spatial scales, as predictable changes have been observed in riffle community composition along a hydrologic gradient within the same discrete habitat type (Boyero, 2001; Fenoglio, 2004).

Groundwater influence

Groundwater discharge (i.e., spring discharge) into stream systems can increase the effects of local discrete habitat types on benthic community structure, but the evidence for this is equivocal among studies. For example, Barquin and Death (2011) found that benthic taxonomic richness increased with distance from a springhead in New Zealand streams, but the opposite was found in a Florida karst spring system (Mattson, 1995). Larger-scale patterns of community composition are also inconsistent, with some regional spring studies finding similar benthic community structure at different springheads within the same region, and other studies finding distinct springhead communities which then homogenize further downstream (Koperski, 2011). Additionally, factors that shape spring influenced stream benthic community structure longitudinally can differ across systems, but include increased downstream temperature variability, changes in water chemistry and in organic matter concentrations, the amount of leaf litter input, and flow permanence (Glazier & Gooch, 1985; Smith et. al., 2003; Barquin & Death, 2004; Barquin & Death, 2011; Koperski et al, 2011). Indeed, influence of groundwater contributions on physicochemical consistency generally declines with distance from springhead, but the downstream influence of groundwater varies with a variety of factors, including the magnitude of groundwater contributions (Nichols et al. 2014). There is still a great deal of uncertainty in how macroinvertebrate communities respond in the context of distance from springhead, and a need to understand the relative

influence of discrete (i.e., hydrogeomorphic habitat units) and longitudinal (i.e., distance from springhead) drivers of benthic macroinvertebrate community structure in spring-fed systems, particularly in the light of their conservation value.

Goals and Predictions

The goal of my thesis was to examine the relative importance of downstream distance from springhead versus smaller-scale habitat conditions (i.e., mesohabitat units) in explaining the variation in macroinvertebrate community structure in two spring-fed subtropical riverine systems. I predicted that benthic community composition would change with increasing distance downstream from spring emergence points, with a compositional shift from spring-associated taxa to more cosmopolitan surface water taxa. However, I also predicted that because my study was limited to the upper spring-influenced and physicochemically consistent portion of each river that variation in benthic community composition and site specific taxa richness would be more strongly influenced by variation in environmental conditions across mesohabitat types (i.e., local mesohabitat type would explain more variation in community composition) when compared to distance from springhead. I expected this change in community composition to be driven by replacement of taxa rather than differences in taxon nestedness (subsets of the larger benthic assemblage) among habitat types and reaches. I further predicted that within groundwater influenced reaches non-native benthic species would be evenly distributed throughout my study reaches due to the consistency of physicochemical conditions in the upper reaches. Finally, I predicted that the distribution of taxon-specific biomass contributions will correlate with patterns seen in community composition and will reflect taxonomic density estimates.

II. METHODS AND MATERIALS

Study sites

This study examined these patterns in the first ~4 km of the Comal and San Marcos rivers, two strongly groundwater-influenced rivers in central Texas, USA. The Comal and San Marcos rivers are the first and second largest spring outflows in Texas, respectively (Brune, 1981). Both river headwaters are physiochemically stable, maintaining consistent temperature (~23°C) and dissolved ion composition. Both rivers discharge from the Edwards Aquifer, a karst carbonate aquifer which provides water for agricultural, industrial, and individual use (Charbenau, 2011). The “Rule of Capture” was in place for Edwards Aquifer water use until 1992, but protection of spring outflow at the Comal and San Marcos River headwaters was implemented by a legislative ruling in 1993 in favor of the Sierra Club (Charbenau, 2011). This ruling was imperative to these communities, because between the two headwaters and their associated springs, seven aquatic species are listed as federally endangered and threatened, including the Texas blind salamander (*Eurycea rathbuni*), the Comal Springs riffle beetle (*Heterelmis comalensis*), the Comal Springs dryopid beetle (*Stygoparnus comalensis*), the Fountain Darter (*Etheostoma fonticola*), the Peck’s Cave amphipod (*Stygobromus pecki*), the San Marcos salamander (*Eurycea nana*), and Texas wild rice (*Zizania texana*) (Fish and Wildlife Service, 2013; TPWD, 2020). Like many spring systems, these rivers are also invaded by several non-native species, including two snails, the red-rimmed melania (*Melanoides tuberculata*) and the quilted melania (*Tarebia granifera*). The San Marcos and Comal rivers are located within the larger Guadalupe drainage and groundwater discharge contribute substantially to the flows of the basin, particularly during low

precipitation years (Perkin et al. 2011). Long-term mean annual discharge for the upper Comal and San Marcos rivers is 8580 L/s and 4984 L/s, respectively (<https://waterdata.usgs.gov>). The Comal River headwaters (Comal Springs) flows from four major spring runs, which contains multiple spring openings into a small artificial lake (Landa Lake) and then an outflow to the upper Comal River. The San Marcos River headwaters issue from a series of six main artesian openings and hundreds of additional smaller orifices into Spring Lake (a small man-made impoundment), and these waters flow downstream to confluences with the Blanco and then the Guadalupe rivers. The land use surrounding both headwater sites is largely urban.

Field sampling

Three 100-150 m reaches each from the Comal and San Marcos Rivers were selected using a combination of in-person field surveys and satellite imagery (Fig.1 & Fig 2). The initial reaches of both rivers were selected as Reach 1 with the proceeding 2 reaches occurring within 5 river km of Reach 1 (R1). In the San Marcos River, the first reach (SMR1) began just below the Spring Lake dam and extended for 0.16 km downstream. SMR2 began 1.65 km from Spring Lake and was 0.12 km long, and SMR3 began 3.2 km from the outflow of Spring Lake and was 0.2 km long. The first reach of the Comal River (CR1) began immediately downstream from the major outflows of the first spring run (Spring Run 1) and extended downstream for 0.3 km. CR2 began 1.1 km from the spring run (designated as the old channel of the river) and was 0.15 km long. CR3 began 2.65 km from the spring run and was 0.13 km long.

To assess benthic macroinvertebrate community structure across discrete geomorphic habitat units, each reach was surveyed and segregated into three meso-

habitat types based on flow velocities, depths, and substrate types: runs, pools, and riffles. All sampling occurred within the spring of 2021, from April 6th to May 13th. Although I categorically defined mesohabitat types in each study reach during field sampling, I used sampling point conditions (e.g., depth, velocity, substrate composition) as continuous variables in data analysis (see below). Within each study reach, habitat characteristics and benthic macroinvertebrate samples were taken from five sampling points chosen haphazardly within each meso-habitat type, yielding a total of $n = 15$ points per reach (i.e., 5 riffle points, 5 pool points, and 5 run points) and a total of $n = 45$ sampling points per river. After data collection, satellite imagery of each of the study reaches was used to determine the spatial extent of each mesohabitat type and to generate the areal estimates of each mesohabitat type per-reach.

At each sampling point, prior to the collection of invertebrate samples, habitat characteristics were measured, including flow velocity, physicochemical conditions, canopy cover, macrophyte and algal cover, substrate type, and water depth. Flow velocity was measured using a Hach flow meter. Water physicochemical properties (temperature, specific conductivity, pH, and dissolved oxygen) were measured using a Eureka Manta +30 sonde. Percent substrate composition, macrophyte cover, and algae cover were estimated visually using a modified Wentworth method having five categories: fine silt, sand, gravel, cobble and boulder (Nielsen & Johnson, 1989; Diaz, 2015). A handheld densiometer or the Canopy Surveying App (Public Interest Enterprises) was used to determine percent canopy cover above each sampling point.

Benthic macroinvertebrates were quantitatively sampled at each sampling point using a 0.33 m diameter Hess sampler (500- μ m mesh; sample area = 0.086 m²) locations with

adequate flow and shallower depths. At lower velocity sites or sites that were deeper than the top of the Hess sampler, I used a 10 cm x 10 cm Lang benthic basket sampler (500- μm mesh; sampling area = 0.0242 m²); duplicate Lang samples were collected at each point and pooled in the field. Samples were examined in the field to remove endangered species and place them back into the river. Samples were then preserved with 75% ethanol until sorted in the lab.

Density and Biomass

In the lab, each sample was evenly dispersed into a flat-bottomed pan and separated into four equally sized quadrants. Invertebrates were enumerated and counted from a haphazardly selected quadrant and identified to the lowest feasible taxonomic level (typically genus) using appropriate keys (Merritt & Cummins, 1996; Diaz, 2017). Enumeration and identification were performed by quadrant until >200 individuals had been identified and a minimum of a single quadrant was processed. This subsampling approach was used because of the exceptionally high invertebrate densities found at some sampling points (Diaz et al. 2015). Population densities (number/m²) for each taxon were calculated at each sampling using count data, number of quadrants picked, and sampling device area.

To estimate areal biomass for macroinvertebrates in mesohabitat types for each reach and river, individuals from each taxon were measured using Zeiss Stemi 305 stereomicroscope interfaced with an Olympus Cellcens© camera system at a standard shutter speed of 3.395 milliseconds. Morphological characters were measured in the Cellcens© software system that makes measurements to the nearest 0.01 mm, dried, and then weighed on a Mettler Toledo MX5 microbalance. Individuals of a taxon of similar

body size were pooled in groups of $n = 5$ to 80 individuals for dry weight measurement (minimum of $n = 3$ individuals or groups of individuals per taxon were weighted); dry mass was expressed on a per capita basis. When numbers allowed, body sizes and dry masses were estimated for the Comal and San Marcos rivers separately; however, body sizes of taxa substantially overlapped between rivers; thus, for rare taxa I occasionally grouped San Marcos and Comal invertebrates to get adequate dry mass. Larval insect body sizes were measured using head capsule width, and adult invertebrates and nymphs were measured using total body length. Snails were measured by spire height, but soft tissues were removed prior to determining dry mass. Taxa which displayed pronounced ranges in body size in a river were separated into two to three size classes. Once measured, individuals or groups of individuals of a taxon were dried for 48 hours at 60°C and weighed. To calculate areal dry mass for each taxon at each sampling point, the median per capita dry mass of each taxon in each river was multiplied by the density of individuals at a sampling point, yielding areal dry biomass (g/m^2).

Data Analysis

Spatial variation in stream environmental conditions, both among mesohabitat types and longitudinal reaches for each river was initially assessed with principal component analysis (PCA). Prior to analysis, redundant variables were removed by performing a Pearson correlation matrix and variables were removed if $r \geq |0.7|$. The final PCA dataset included 14 variables, which included reach identity (R1, R2, or R3), water temperature, flow velocity, conductivity, water depth, percent substrate composition (sand, gravel, cobble, and boulder), percent algal cover, percent canopy cover, and percent macrophyte cover. Removed variables were flow velocity taken at the

benthos level, and depth in meters. Data were z -score transformed prior to analysis.

Examination of the data indicated that water temperature, DO, and conductivity exhibited some spatial variation among reaches, but the overall magnitude of those differences was small. Nevertheless, these differences in physicochemical conditions exerted a strong influence on the PCA (see Results). I additionally assessed longitudinal differences in temperature, DO, and conductivity among reaches through analysis of variance (ANOVA) in which the physicochemical parameter was the dependent variable and reach identity (R1, R2, and R3) were the independent variables. Prior to analysis, data were assessed for assumptions of homoskedasticity of variances and normality and if these assumptions were not met, data were \log_{10} transformed. If assumptions were still not met, a non-parametric Kruskal-Wallis test was used. If an overall significant effect of reach was detected, then differences among reaches were assessed with a Tukey's post-hoc test. Significance for all analyses was inferred at a ≤ 0.05 .

To assess if variation in benthic macroinvertebrate community composition was more influenced by related local mesohabitat conditions or spatial distance from springhead (the upstream, middle, or downstream reaches) in each river, I performed redundancy analysis (RDA). Environmental variables were separated into reach-level predictors (categorical variables of R1, R2 or R3) and local sampling site characteristics (e.g., water velocity, benthic substrate composition). Due to multicollinearity in the local environmental predictor data sets, I performed the 'ordistep' function in the 'vegan' package using a forward selection process and unrestrained permutations to reduce the predictor data sets and to accomplish some degree of parsimony (Borcard, 2018; Oksanen, 2020). This process resulted in the two most important local variables for each

river: flow velocity and percent macrophyte cover for the San Marcos River and percent silt cover and water depth for the Comal River. The species matrix data set used densities of each taxon at each sampling point. To reduce the influence of rare taxa, I reduced the number of taxa used in the analysis for each river so that each RDA included taxa which constituted $\geq 3\%$ of the total number of individuals (Zhao, 2017). Taxa density data were Hellinger transformed before analysis. I then performed a partial RDA analysis using predictor variable set: the first RDA only used the reach category (Reach 1, 2, or 3) and the second used the local variable predictor set. I then ran a ‘global’ RDA for each river, using both predictor sets. All RDA analyses were performed in the R platform using the ‘vegan’ package (Oksanen, 2020). Finally, I used the function ‘var.part’ function in the ‘vegan’ to determine the proportion of variation in community composition explained by reach identity versus mesohabitat predictors, as well as the proportion of variation in community composition that is shared between the two predictor sets.

To assess patterns in taxonomic diversity among reaches and mesohabitat types, I examined spatial differences in taxonomic richness (S) among mesohabitat units (i.e., riffles, runs, and pools) and among reaches (R1, R2, and R3) using a two-way ANOVA with mesohabitat unit and reach identity as the independent variable. Each sampling point was used as an independent observation. To meet assumptions of homogeneity of variances and normality, taxonomic richness was \log_{10} -transformed prior to analysis. The analysis yielded main effects of mesohabitat type and reach, as well as the interaction. If an overall significant effect was detected, then differences among reaches or mesohabitat types were assessed with Tukey’s post-hoc tests. Significance for all analyses was inferred at $\alpha \leq 0.05$.

To explore patterns turnover in benthic community composition longitudinally in both rivers, I conducted an analysis of b-diversity within mesohabitat types across the study reaches. Classically, b-diversity is defined as the difference in species composition among local habitat units (a-diversity); however, the amount of species difference is a consequence of either species replacement (i.e., simultaneous loss and gain of taxa between two sites) or richness difference (i.e., subsets of taxa found between sites, with one having more or less total unique taxa than the other) (Legendre, 2014; Borcard, 2018). To estimate b-diversity along an upstream to downstream gradient and the proportion of that diversity associated with replacement and richness difference, I used the functions ‘beta.div.comp’ and ‘beta.div’ in the R package ‘adespatial’(Dray, 2018). I used taxon incidence data (e.g., 0, 1) from within each mesohabitat type from each reach in the ‘beta.div.comp’ function, and used Podani’s Jaccard indices to decompose pairwise measurements of b-diversity into richness difference and taxon replacement (Legendre, 2017; Borcard et.al 2018). I additionally estimated the species contributions to b-diversity (SCBD) to determine the taxonomic components of b-diversity along a gradient from upstream to downstream within each mesohabitat type and to identify the changes in specific taxa which may be driving b-diversity patterns. For this analysis, I used the ‘beta.div’ function with Hellinger transformed abundance data within each mesohabitat type across study reaches in each river.

To examine community and taxon-specific patterns in biomass for each river and each reach, the median per-individual biomass of each taxon was multiplied by per-site population density estimates to yield per-site areal biomass estimates (mg dry mass/m²). Per-site biomass estimates were averaged across mesohabitat type (i.e., run, pool, and

rifle) to create per-habitat type biomass estimates for each reach. Finally, per-habitat type biomass estimates were multiplied against the total area of each meso-habitat type in each reach to create total biomass estimates for each reach.

III. RESULTS

Variation in reach and site conditions

Firstly, I analyzed the general limits in flow velocity and depth between my three mesohabitat types across both rivers. Run velocity water velocities ranged from 0.04 and 1.04 m/s with depths between 14 and 119 cm. Riffle velocity was between 0.15 and 0.9 m/s with depths ranging from 6 to 56 cm. Finally, pool velocity was between 0 and 0.18 m/s with depths from 12 to 125 cm.

The PCA for SMR site conditions described 37.21% of the variation in the first two components (Fig. 3A & B). The first principal component accounted for 21.33% of the variation and described a general downstream to upstream gradient of sites with lower temperature and conductivity in the most downstream reach (R3) to sites with higher temperature and conductivity in R1. PC2 described a gradient of conditions associated with flow velocity among sites. The gradient contrasted sites with lower flow velocities, greater depths and higher percent silt to sites (pool sites) with sites having higher velocities and greater percent cobble (riffle sites).

The first two axes for the CR described a total of 45.18% of the variation in the dataset (Fig. 4A and B). The first principal component accounted for 25.15% of the total variation explained, and showed an upstream to downstream gradient, like the SMR: lower DO and high conductivity in the initial reach (R1) to sites in the two lower reaches (R2 and R3) had higher DO and lower conductivity. PC 2 described a gradient of depth, temperature, and velocity across discrete meso-habitat types.

Because physicochemical gradients exerted a strong influence on the PCAs for each river, I further examined how water temperature, DO, and conductivity varied

across reaches and determined the magnitude of those differences. In the CR, temperature was similar in R1 and R2, but was $\sim 1^\circ\text{C}$ cooler in R3 (Fig. 5A; $H_{2,42} = 28.49$, $p < 0.001$). DO differed among all three CR reaches ($F_{2,42} = 81.639$, $p < 0.001$), with DO increasing by 2 to 3 mg/L from R1 to R2 (Fig. 5C). This variability could have been due to measurements taken at different times of day, or with greater or lesser cloud coverage. Conductivity in R1 differed from R2 and R3 but was only $\sim 20\ \mu\text{s}$ greater ($H_{2,42} = 27.521$, $p < 0.001$) Fig. 5E). In the SMR, both temperature ($F_{2,42} = 161.72$, $p < 0.001$) and conductivity ($F_{2,42} = 7.62$, $p = 0.002$) differed amongst reaches (Fig. 5B and F), with temperatures being $\sim 2^\circ\text{C}$ cooler in R3 than in R1. Conductivity in SMR differed in R1 in comparison to both R2 and R3, although this difference was very small ($\sim 4\ \mu\text{s/cm}$). In the SMR, DO did not significantly differ among reaches (Fig. 5D).

Invertebrate community responses to longitudinal and local habitat gradients

The RDA and variation partitioning analyses revealed that for the SMR, reach identity played a greater role than local variables in shaping community composition. The partial RDA examining the influence of reach identity found that the first two reach RDA axes accounted for 20.46% of the total variation in benthic macroinvertebrate community composition (Fig. 6, A & B). R1 was associated with the first RDA (14.23% of the variation) and R2 was largely associated with the second axis (7.23% of the variation). R1 was characterized by higher densities of the amphipod *H. azteca* and the endemic snail *E. comalensis*, whereas the non-native snail *T. granifera* associated with R2, and several genera of elmids were associated with R3. The partial RDA that assessed the influence of local environmental conditions (Fig. 6 C & D) explained 16.13% of the variation in community composition, with the first axis portraying a gradient of low to

high flow velocities (9.15%) and the second axis (6.98%) describing a gradient of high macrophyte cover to low macrophyte cover. Sites were largely grouped by mesohabitat type with annelids and *T. granifera* associated with lower velocities limited macrophyte cover (i.e., pool sites), whereas *H. azteca*, baetid mayflies, and *E. comalensis* were found mainly in higher macrophyte coverage sites (runs and riffles). Finally, elmids larvae and caddisflies were found mainly in higher flow velocity sites (i.e., riffles). The global RDA for the SMR explained 26.91% of the variation in community composition (Fig. 6, E & F) and portrayed taxa associations demonstrated in both partial RDAs. Variation partitioning showed that reach identity explained more (14.30%) of the variation in community composition than local predictors (8.70%), with shared variation of the two predictor sets explaining a smaller amount (3.12%; Table 1).

The RDA and variation partitioning analyses for the CR revealed that reach identity and local variables played an equal role in shaping community composition, and that reach identity only separated the lower reaches (R2 and R3) from the most spring associated reach. the partial RDA examining the influence of reach identity explained 16.93% of the variation in community composition (Fig. 7, A & B), with R1 and R2 separating across the first axis (13.2% variation explained) and the second axis explaining a substantially lower amount (3.73%). Like the SMR, the amphipod *Hyaella* was associated with R1, but the elmids *Microcyloepus* was also found predominantly in the upper study reach. The non-native snail *T. granifera* was associated with R2, while R3 sites had higher densities of the caddisfly *Helicopsyche* and the endemic snail *E. comalensis*. The partial RDA examining local site-level conditions (Fig. 7, C & D) explained 17.38% of the variation in composition, with the first axis (16.49%) showing a

gradient of sites with greater water depth to sites with higher % silt, and the second RDA axis accounting for a much lower amount of variation (0.89%). The native snail *E. comalensis* and *Helicopsyche* were associated with the shallower sites with the low percent silt (i.e., riffles), while *T. granifera* was associated with the deeper slower velocity sites (i.e., pools). Interestingly, the larvae of the riffle beetle *Microcyllloepus* and *H. azteca* were associated with siltier sites. The global RDA (Fig. 7, E & F) explained 26.79% of the variation and the first axis explained a clear majority of the variation (22.77%) and separating siltier R1 sites from less silty and downstream R2 and R3 sites. Variation partitioning indicated that both reach identity and local variables explained approximately equal amounts of variation in community composition (~7%), with a similar percentage (6%) explained by the shared of the two sets of predictors (Table 1).

Spatial variation in taxon richness and β -diversity

Overall patterns in richness upheld my prediction in both rivers, as richness did not differ greatly by reach, but did differ between mesohabitat types. In the SMR, taxon richness did not vary with reach identity ($F_{2, 42} = 2.87, p = 0.071$; Fig 8), but richness differed among mesohabitat types ($F_{2, 42} = 10.449, p < 0.001$) and the reach \times mesohabitat type interaction was not significant ($F_{4, 42} = 1.91, p = 0.132$). Pools in the SMR had lower richness than other mesohabitat types. In the CR, richness varied with both reach identity ($F_{2, 42} = 3.466, p = 0.043$) and mesohabitat type ($F_{2, 42} = 16.973, p < 0.001$) and that there was a significant interaction term ($F_{2, 42} = 3.853, p = 0.011$). However, post-hoc Tukey's HSD tests failed to reveal differences in richness by reach. As observed in the SMR, pools had lower in richness than either riffles or runs in the CR.

β -diversity analyses for the SMR was consistent with my predictions, and showed

that between mesohabitat types in an upstream to downstream direction, replacement rather than richness difference contributed the highest amount of β -diversity for runs, riffles, and pools. β -diversity was highest within pools among reaches (87.36 %) and the lowest in runs (58.27%) (Table 2). The SCBD estimates for the top five taxon-specific contributors to β -diversity the SMR indicate that replacement contributions to β -diversity along an upstream to downstream gradient are likely being driven by replacement of *H. azteca* and *E. comalensis* with *T. granifera* and *Helicopsyche* (Table 3).

In the CR, replacement was again the primary contributor to β -diversity in runs (73.24%) and riffles (55.24%), but pool β -diversity was primarily driven by richness differences (55.38%; Table 2). SCBD analysis in the CR showed that β -diversity along the upstream to downstream gradient was likely due to changes in abundance of *T. granifera* (much higher abundance in R2 and R3), and the replacement dynamics of *Helicopsyche*, *H. azteca*, and the elmid *Macrelmis* (Table 3).

Benthic Macroinvertebrate Biomass

In the San Marcos River, the top biomass contributor in R1 were annelids, with their greatest biomass contribution in deeper runs and pools (Fig. 9A – C). In R2 and R3, *T. granifera* was the largest contributor to community biomass, again occurring mostly in runs and pools (Fig. 9D – J). Within-reach community biomass composition was most similar between runs and pools. Indeed, among the top five biomass contributors, runs and pools from each reach had at least three shared taxa that were in the top five biomass contributors. Riffle composition was more unique across all three reaches and also consistently contributed the lowest amount of reach-wide biomass.

In the Comal River, R1 biomass was dominated by larval *Psephenus*, and the

biomass composition of communities was similar across all three mesohabitat types (Fig. 10A – C). However, in R2 *T. granifera* was the largest biomass contributor, and the biomass composition riffle mesohabitats was distinct from those in runs and pools. In R2 reach, *T. granifera*, *E. comalensis*, leeches, and annelids were dominant biomass contributors in pools and runs, while *Psephenus*, simuliid larvae, and the elmids *Microcyloopus* and *Macrelmis* were the largest biomass contributors in the riffle community (Fig. 10D – F). In CR, R3 had similar community biomass composition across all three habitat types, but the naucorid *Ambrysus* was the largest biomass contributor in runs, *T. granifera* was the largest biomass contributor in pools, and annelids were the largest biomass contributor in riffles (Fig. 10H – J). Finally, in all three reaches the majority of reach-wide biomass came from run and pool mesohabitat types, with riffle communities contributing relatively less overall. Although patterns of areal biomass were less clear in CR than in SMR, CR areal biomass contributions were more unique across reaches than within reaches for all three meso-habitat types.

IV. DISCUSSION

Relative influence of distance from springhead and mesohabitat conditions on benthic community composition

In this thesis, I examined the primary question of whether variation in benthic macroinvertebrate community composition in the upper physicochemically stable segment (~5 km) of two spring-fed rivers was more a function of distance from spring origin (upstream to downstream continuum) or the local mesohabitat conditions (discrete habitat associations). Contrary to my prediction that local mesohabitat conditions would outweigh downstream distance from springhead on benthic community structure, I found that distance from spring outflow was of greater or equivalent importance than local habitat characteristics in the SMR and the CR, respectively. In the SMR, distance from springhead had a primary role in shaping benthic composition. In the CR, the upstream - downstream gradient in community composition was less marked, but the difference in the relative importance of longitudinal distance from springhead between the two river systems may be due to differences in the physical configuration of the two spring-fed river systems. In the SM, springs emerge into a human-made lake (Spring Lake) and all the study reaches were located downstream from the headwater lake. However, the uppermost study reach in the CR (R1) was located upstream from a small human-made lake (Landa Lake) and the remaining two reaches were located downstream from this lake. The influence of downstream distance on community composition in the CR may be at least partially driven by the presence of the lake and dam. The presence of dams can disrupt lotic longitudinal gradients, leading to alteration of benthic community composition and lower diversity below dams (Ellis & Jones, 2013). Indeed, the two

reaches below Landa Lake in the CR (R2 and R3) were similar in composition and did not show the same longitudinal changes observed in the SMR.

Larger scale riverine studies have examined longitudinal changes in community structure, shaping the way ecologists study lotic ecosystems. (e.g., Vannote 1980). The RCC proposes that there are large-scale differences in community composition related to changing geomorphological setting and the relative importance of allochthonous and autochthonous resources; however, the RCC also posits that localized conditions can cause shifts in community composition along the continuum, but these local mechanisms are of less importance than processes occurring at the basin scale. Due to the scale at which these conceptual models such as the RCC were first created, they should not be applied to smaller stream reaches (a few km, like in the current study). Thus, despite the presence of longitudinal shifts in community structure in the present study, the proposed mechanisms driving these changes by continuum-based models likely have little applicability to relatively short (<10 km) segments found in spring-dominated lotic systems.

Previous research examining similar spatial scale patterns in spring systems suggest that spring influenced lower order streams are more akin to higher order streams in that their groundwater inputs , and there should exist environmental and community composition gradients as distance from groundwater source increases (Resh, 1983). Changes in benthic community composition with increasing distance from spring sources is well documented and can occur even within the first few meters from the groundwater discharge location (Barquin & Death 2011; Koperski, 2011; Smith, 2003; Von Fumetti, 2007). In the current study, I found longitudinal change in community composition at a

scale of 100s of meters. However, environmental conditions which drive benthic community compositional changes are highly variable and can vary greatly among lotic systems. For example, physicochemically stable springs and spring brooks contain distinct community structures and abundances, especially at sites closest to the source (Barquin & Death, 2011; Koperski, 2011). In general, as downstream distance from springhead increases, there is increasing variability in temperature, which drives community compositional turnover (Barquin and Death 2004; Barquin & Death 2011). In addition, discharge consistency and magnitude, variation in organic matter quality, and changes in water chemistry downstream from springheads can also act as driving factors in spatial changes in taxonomic composition (Glazier & Gooch, 1987; Smith, 2003; Koperski, 2011).

In the present study, there were relatively small changes in physicochemical conditions with increasing distance downstream (a $\sim 2^{\circ}\text{C}$ decrease in temperature, 5 – 20 mS/cm change in conductivity from R1 to R3), indicating that the downstream reaches of both study rivers were subjected to greater influence from the surrounding environment. However, it is unlikely that such small changes in physicochemical conditions influenced the observed changes in community composition in both rivers. Recent work has found that even small changes in temperatures can have sizeable effects on the survival and fitness of spring-associated invertebrates (e.g., Nair et al., in revision). However, most of the longitudinal community composition changes in the present study were not associated with loss of spring-associated fauna and replacement by more cosmopolitan taxa (as I initially predicted). Rather, turnover and changes in community composition was largely among more widespread (e.g., *H. azteca*) and non-native taxa

(e.g., *T. granifera*), indicating that there is more research needed to determine the mechanisms behind the observed compositional changes in the first several km of the SMR and CR.

Although longitudinal patterns in benthic macroinvertebrate communities were an unexpectedly important predictor of macroinvertebrate community structure, the present study also found that variation in community structure was also related to local mesohabitat conditions. Indeed, the only significant difference in taxonomic richness in both river systems occurred at the mesohabitat level in both rivers, with pools having distinctly lower richness than runs or riffles. Differences in benthic community composition and diversity across local habitat types is not novel and is well established in the literature (Poff and Ward 1990). Abiotic conditions at the scale of local mesohabitat units, such as flow velocity and physical habitat structure can shape benthic community composition (Brown & Brussock, 1991; Buffagni, 2021; Doisy, 2001). However, spring-fed systems may exhibit different responses to the influence of discrete habitat units and continuous longitudinal distances than surface water streams. For example, Bonada (2006) found that macroinvertebrate community composition was more influenced by hydrogeomorphic habitat type (pools versus riffles) than by reach-level characteristics in a groundwater-based stream. It has been hypothesized that local habitat variables are more important in shaping benthic community structure when flow conditions are stable (Karaouzas, 2019). In the present study, I found that downstream distance from springhead was equally or more influential on community composition than mesohabitat conditions, even under the stable flow conditions at two high-magnitude spring discharge rivers.

Spatial patterns of richness and β -diversity

Consistent with my predictions, I found that patterns in β -diversity within mesohabitat types among the study reaches was mostly driven by replacement of taxa for most of the mesohabitat types in both rivers. Taxa replacement occurs when taxa are simultaneously lost and gained between locations and contrasts with richness difference, which occurs when a site has more or fewer taxa than another (Legendre, 2014). In the present study, there was a loss of the amphipod *H. azteca* and *E. comalensis* at more downstream reaches and an increase in more widespread surface taxa (elmids and caddisflies) and non-native species (*T. granifera*). Other studies regarding taxa or species turnover in freshwater benthic macroinvertebrate communities have focused on gradients of anthropogenic stress (Gutierrez-C, 2013; Krynak 2019), altitude (Castro et. al, 2019) and latitude (Murria, 2017) and have often examined this process at regional or floodplain levels. Spring- specific studies examining β -diversity have often examined stygobiont and crenic species within and around spring openings and have been performed at the regional and metacommunity level (Hutchins et. al 2021; Cibik, 2022). Thus, there is clearly a need to examine patterns of β -diversity with increasing distances downstream from springheads in larger discharge spring systems to determine if patterns observed in the present study are widespread, and what possible mechanisms are driving these patterns.

Patterns in community composition observed in this study have several possible underlying mechanisms rooted in metacommunity ecology, including dispersal and species sorting (Leibold, 2014; Heino 2015). Dispersal is the movement of taxa between “patches” of a larger metacommunity, is observed across multiple scales, and is often

most important when dispersal abilities of taxa are high with a high degree of habitat fragmentation (Heino, 2015; Datry 2016; Crabot, 2020). Conversely, species sorting occurs when environmental conditions are the primary mechanism driving community composition, which often takes place in relatively more stable, well-connected environments (Leibold, 2014; Heino 2015). For freshwater benthic macroinvertebrates in particular, drying events and intermittent flow along with varying dispersal abilities of specific taxa (i.e., taxa with winged life stages or resistance to desiccation) can lead to dispersal rather than “environmental filtering” driving richness difference among sites both spatially and temporally (Datry, 2016; Crabot 2020). Indeed, a study performed partly within my own study range reported that stable, strongly spring influenced river reaches contained taxa like *H. azteca* with low dispersal abilities and lack of resistance to desiccation, which led to increased richness close to spring sources (Zawalski, 2019). The current study found similar patterns in communities containing low resistance and low resilience taxa in SM R1, including *H. azteca* and *E. comalensis* and in CR R1, with *H. azteca* and *Microcylloepus*. However, we also found poor dispersing, low resistant taxa at the furthest downstream reaches; the snail *E. comalensis* was found in the reaches furthest from springs source in both SMR and CR. This suggests that dispersal may not be the primary driving community composition within the first 4 km of these two large discharge spring systems. Rather, there was substantial covariation between reach identity and local mesohabitat characteristics (as observed in the global RDAs for both rivers), suggesting that local habitat conditions were changing among reaches and that communities were responding to these local and reach-scaled gradients. Thus, it may be more likely that species sorting is a more influential mechanism determining species

composition and turnover within the upper stable and physicochemically consistent portions of spring-fed rivers.

Spatial patterns in non-native invertebrates in spring systems

I predicted that because of the physicochemical consistency of the two high discharge spring systems examined by this study, that non-native benthic taxa would have a more continuous distribution throughout the study reaches. I found that the non-native snail *T. granifera* was found in both the CR and the SMR, but contradictory to my predictions *T. granifera* was found at its highest densities in the lower study reaches. Indeed, *T. granifera* contributed greatly to community wide biomass and had very high densities in the lower reaches of both rivers and was associated largely with the replacement of *H. azteca* in both rivers and *E. comalensis* in the SMR. Spring-influenced ecosystems have been postulated to be among the most vulnerable to invasion by non-native species due to high levels of environmental consistency and lack of disturbance (Moyle and Light 1996). *T. granifera* has successfully invaded many subtropical freshwater waterways in Central America, North America, South Africa, and the West Indies (Karayatev, 2009; Makherani, 2022; Moslemi, 2012; Weyl, 2020). It has been known to displace native snails, potentially through chemical cues (Appleton, 2009; Raw, 2013), and can reach incredible densities in invaded waterways (>10,000 individuals/m²) (Abbott, 1952; Appleton, 2009). The present study found that *T. granifera* densities could be as high as 2,950/m², with some per-site estimates reaching > 7,000/m² (sites in the Comal River, reach 2). In addition, *T. granifera* dominated reach-wide density and biomass in four out of six study reaches in both rivers, mostly occurring in pool and run sites. Given the spatial distribution patterns of both *T. granifera* and *E. comalensis*, it is

possible that these species may be spatially segregating habitats across flow regime, but this intriguing possibility requires greater exploration.

Spatial distribution of invertebrate biomass in spring-fed rivers

The findings of this study show that, while usually positively correlated, macroinvertebrate densities are not always reflective of the biomass composition of communities. Indeed, in the present study several taxa which were not top contributors to reach-wide density estimates contributed largely to reach biomass, and vice versa. For example, across R1 run habitats, leeches (Hirudinea) were among the top five biomass contributors in the SMR, but were not in the top group of reach-wide density. In contrast, the mayfly nymph *Leptohyphidae thraulodes* was one of the top contributors to reach-wide community density but was not in the top in terms of its biomass contribution. Ortiz (2007) similarly found a higher “whole community” density to biomass ratio in a sample reach downstream of a point source pollution site. This sample reach had density values far larger than biomass values, indicating an abundance of small-bodied individuals. Although most studies of macroinvertebrate community structure report densities, the use of per capita, population, and community-wide biomass is critical when considering multiple aspects of stream ecosystem functioning. Benthic macroinvertebrates can function as important components of in-stream nutrient cycling, acting directly by consuming food resources containing nutrients, sequestering nutrients into biomass, and excreting dissolved nutrients into the environment (Atkinson et al., 2017; Vanni et al., 2002). Metabolic ecology and the ecological stoichiometric method are widespread approaches to examining the effects of consumers on ecosystem nutrient dynamics and rely on the use of estimates of individual per-capita biomass of species (i.e., body size)

and community-wide estimates of biomass (Brown et al., 2004; Sterner & Elser, 2008; Vanni et al., 2002). In the context of the upper San Marcos and Comal rivers, the benthic macroinvertebrate biomass estimates presented here provide a foundation for future nutrient cycling and other ecosystem impact studies in these ecosystems.

Conclusions

Overall, this study found that within the upper few kilometers of two groundwater-based river systems, upstream to downstream gradients and distance from springhead explained an equal or greater amount of variation in benthic macroinvertebrate community composition than localized abiotic conditions found at the scale of discrete mesohabitat units. This result indicates that there can be substantial longitudinal changes in benthic community composition and turnover in spring-influenced rivers, even when physicochemical conditions remain relatively consistent. In addition, this study revealed that the upper reaches of both study systems had lower densities of a non-native snail, indicating that the presence of native species may provide resistance to further spread of non-native taxa (e.g., Howeth et al. 2017). The findings of this study have substantial implications for the conservation and protection of spring-fed rivers and their invertebrate fauna.

San Marcos River Reach Locations

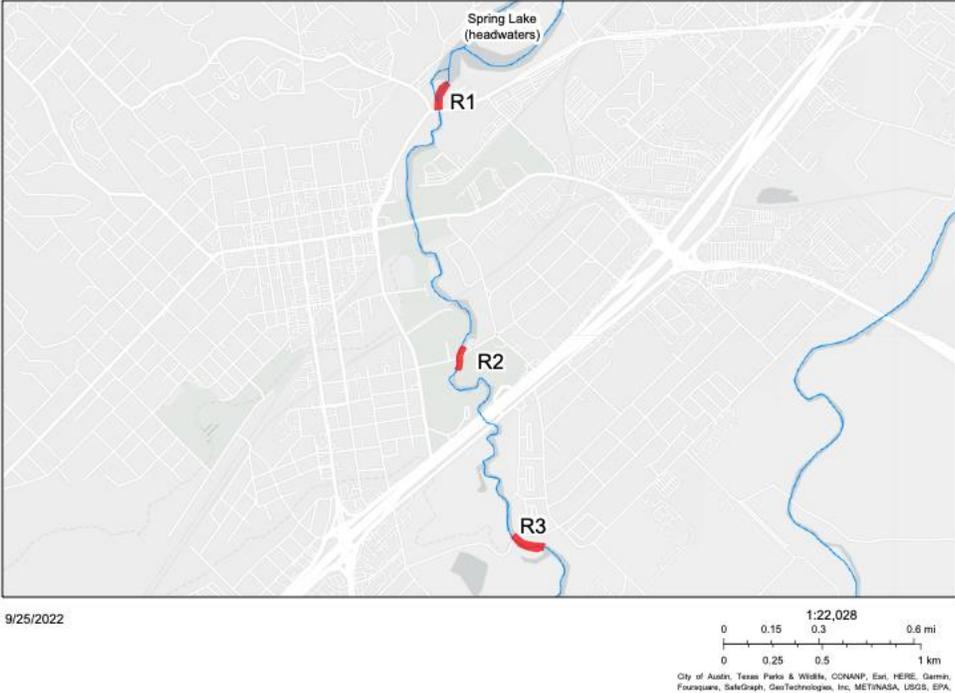


Figure 1. Map of the upper San Marcos River with the three study reaches (R1, R2, and R3) indicated.

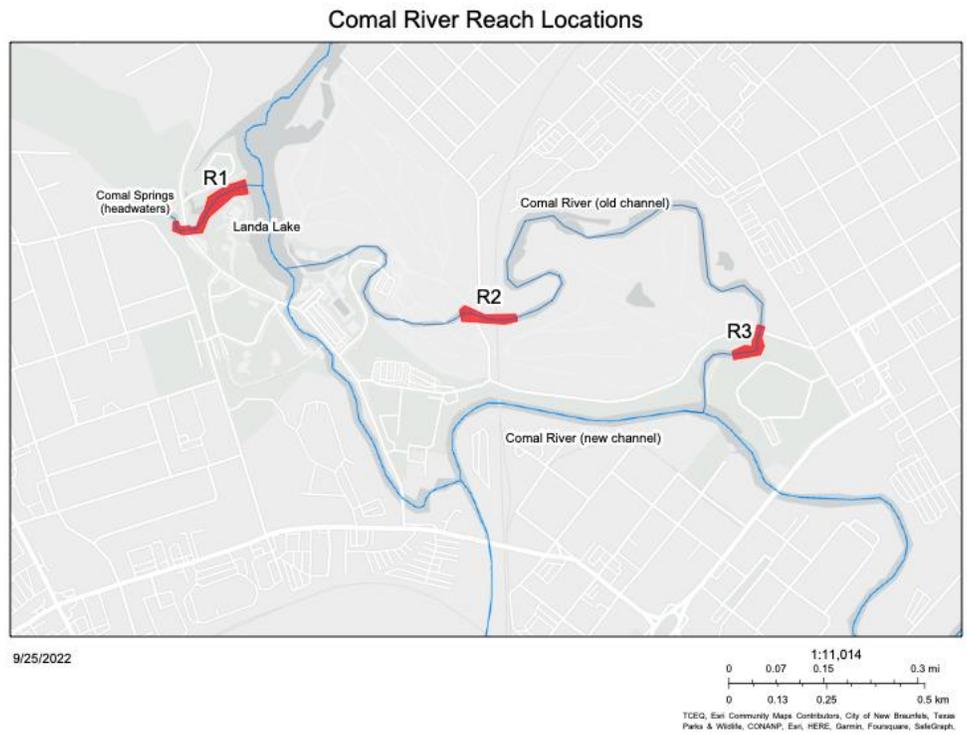


Figure 2. *Map of the upper Comal River with the three study reaches (R1, R2, R3) indicated.*

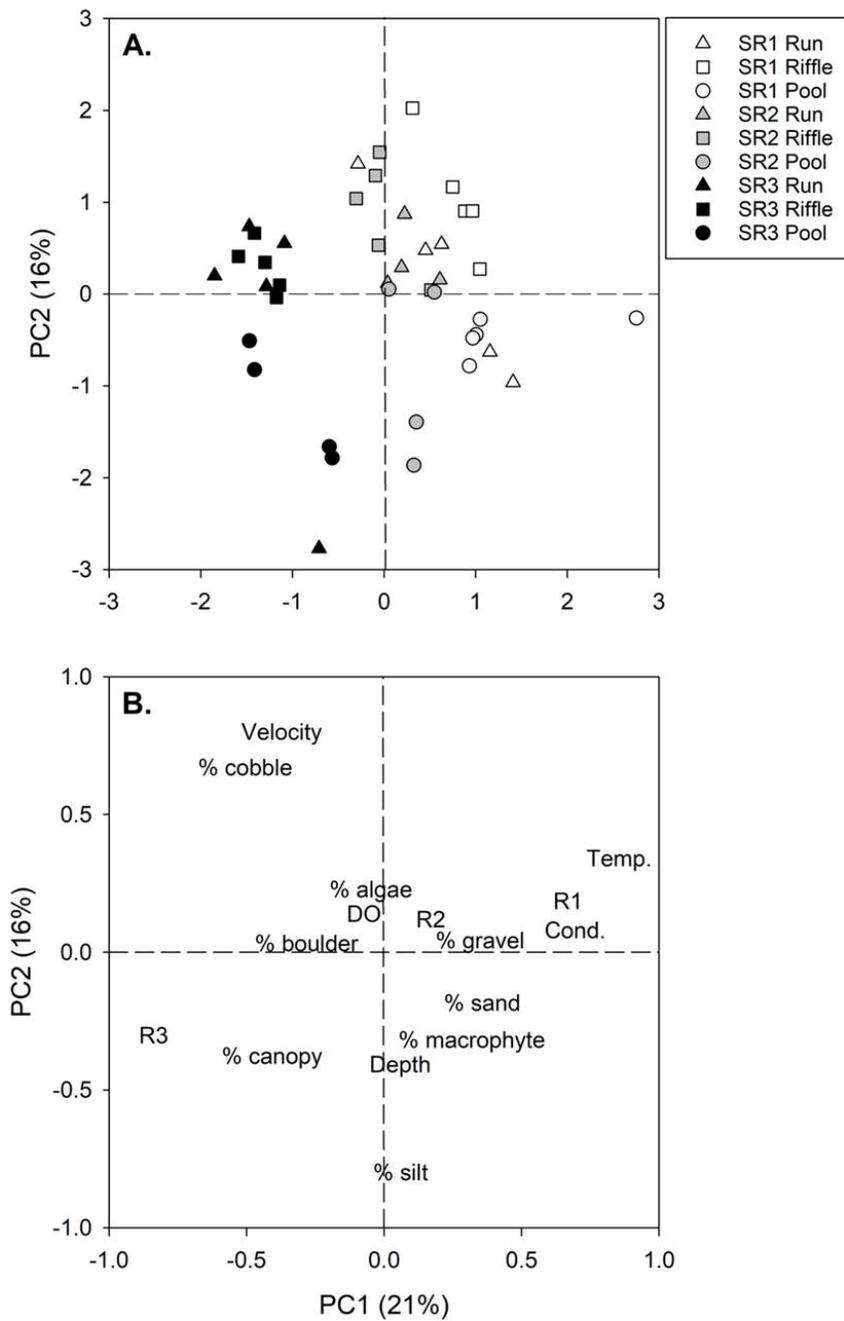


Figure 3. *Principal component analysis of mesohabitat and reach-level conditions in the upper San Marcos River. The site scores with the associated mesohabitat types (A.) and the environmental loading plots (B.) are presented.*

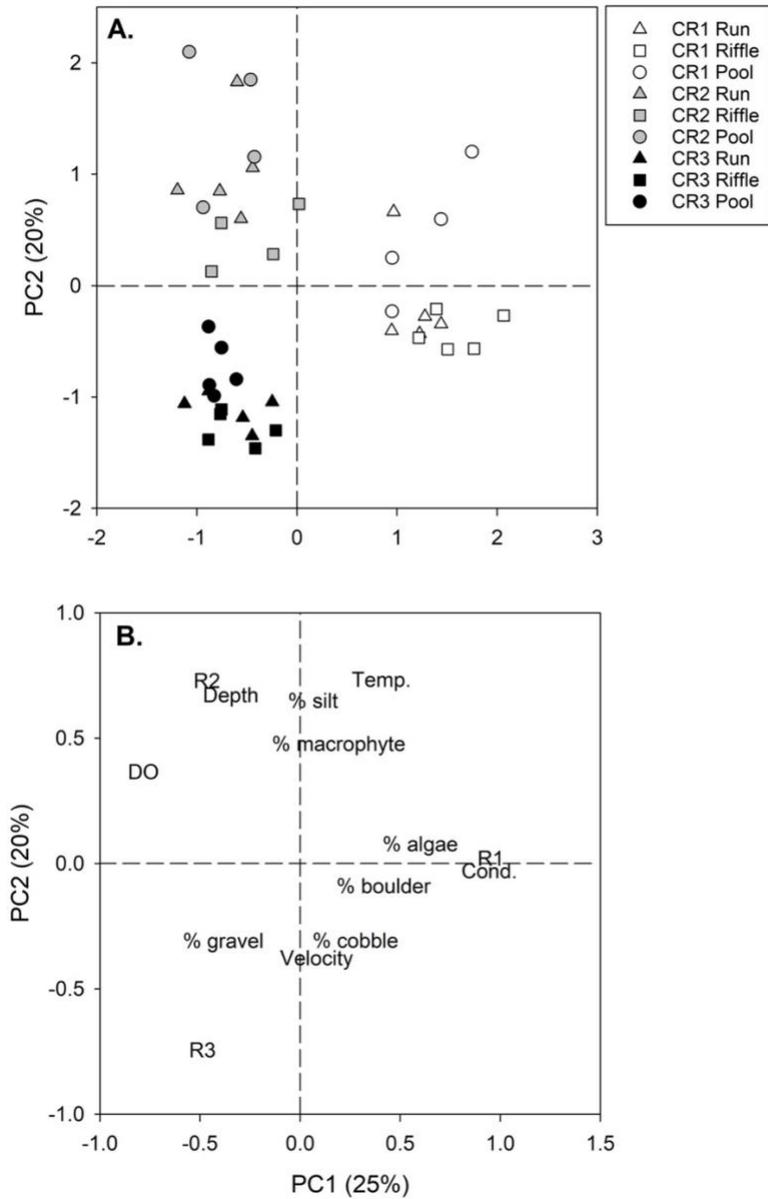


Figure 4. *Principal component analysis of mesohabitat and reach-level conditions in the upper Comal River. The site scores with the associated mesohabitat types (A.) and the environmental loading plots (B.) are presented.*

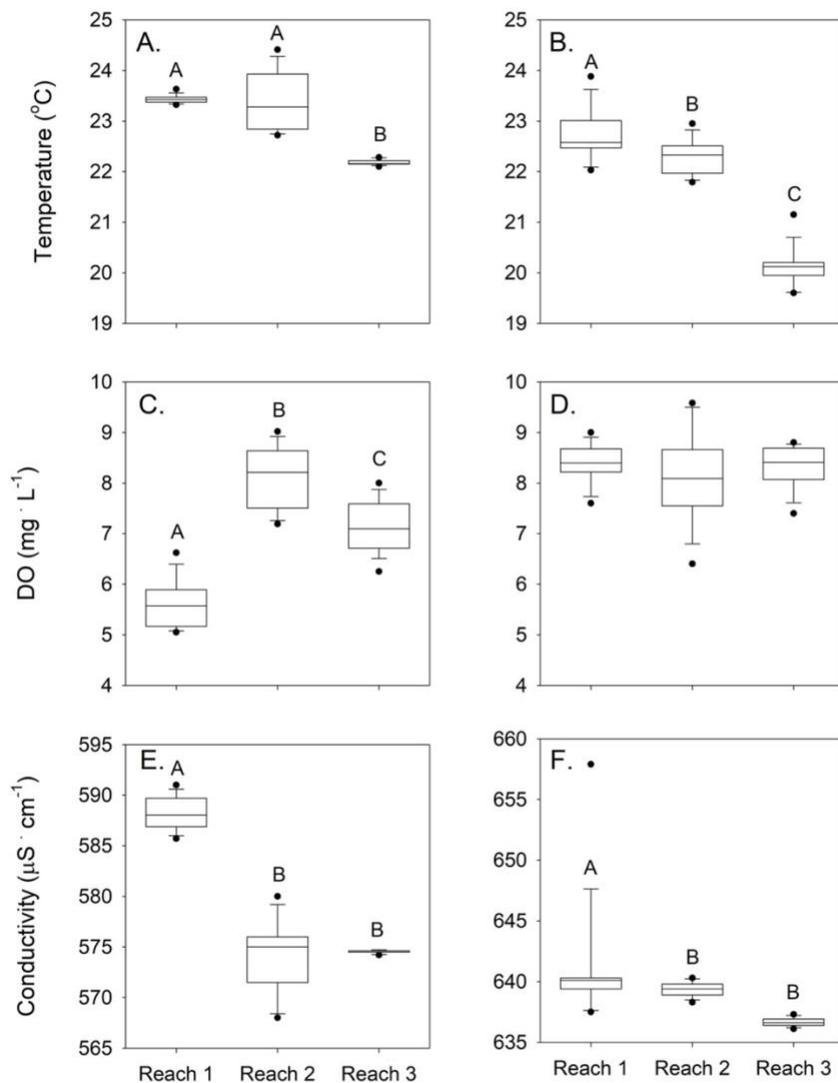


Figure 5. *Box and whisker plots of water physicochemistry*. Box and whisker plots of water temperatures (panels A and B), conductivity (panels C and D), and dissolved oxygen concentration (panels E and F) for the San Marcos River (left side) and the Comal River (right side). Homogeneous subsets determined from Tukey's HSD tests are indicated by letters above the plots.

San Marcos Redundancy Analyses

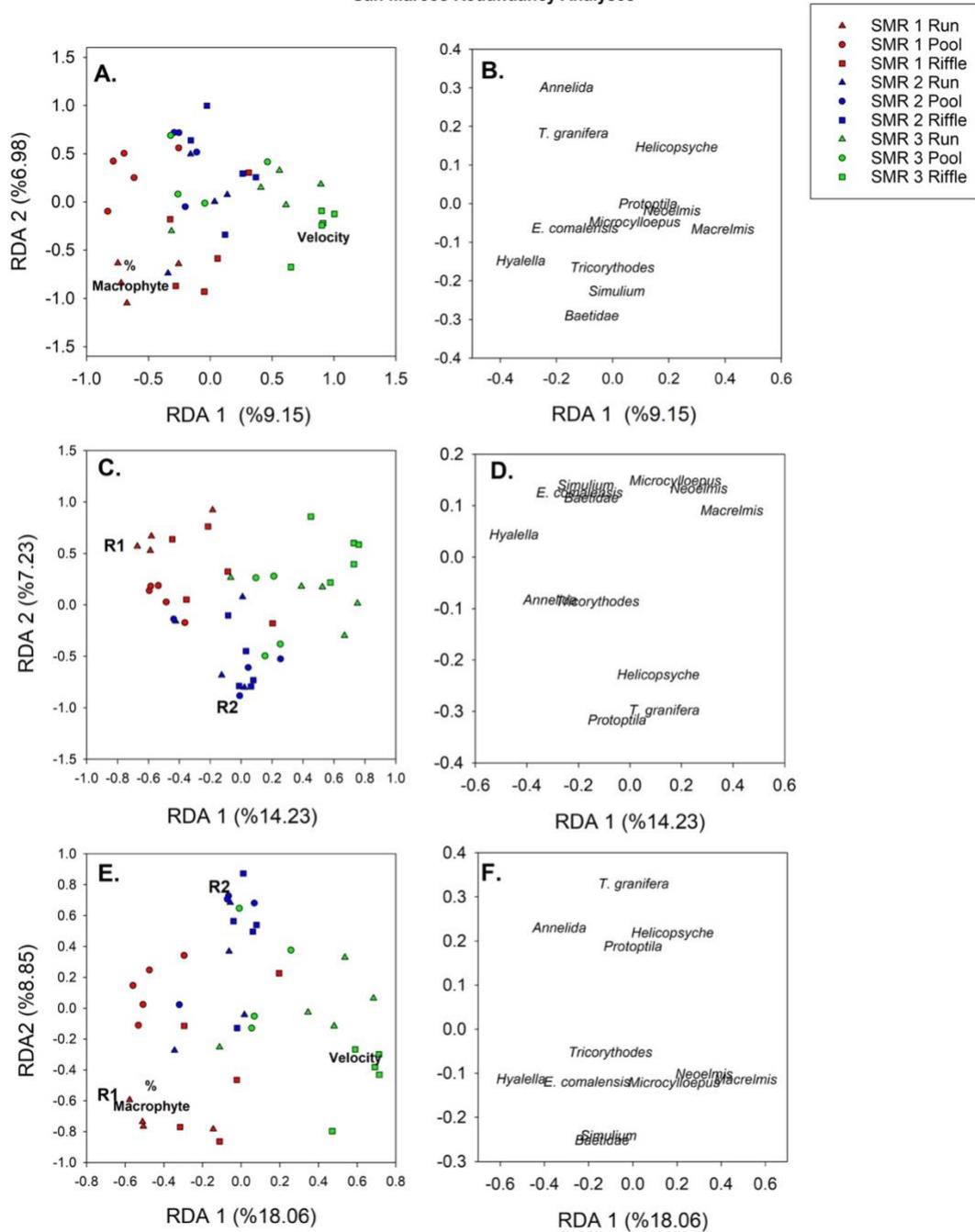


Figure 6. *Partial and global redundancy analysis (RDA) plots of community composition for the upper San Marcos River. The partial RDA for reach identity (A and B), local mesohabitat conditions (B and C) and the global analysis (D and E) are presented.*

Comal River Redundancy Analyses

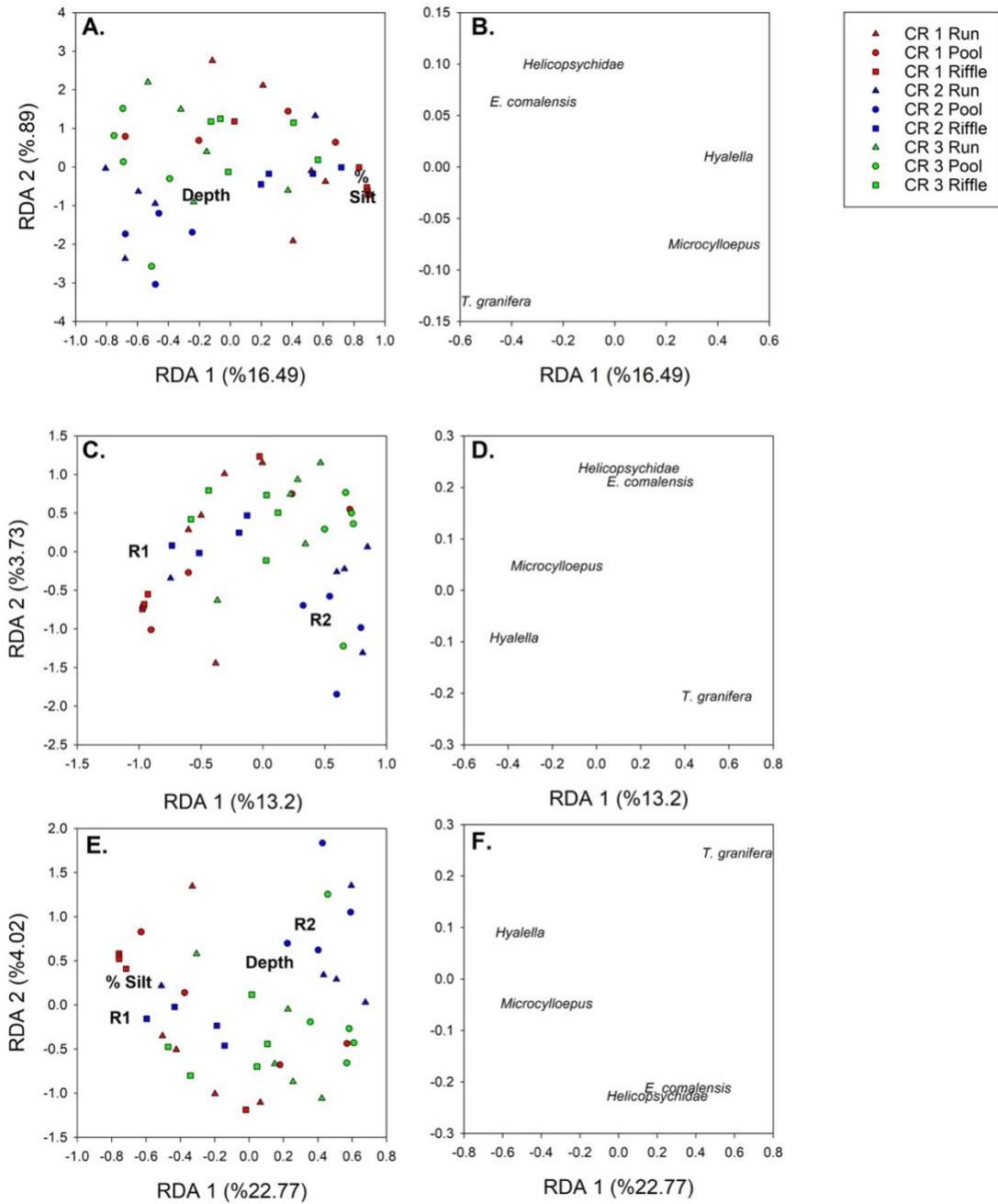


Figure 7. *Partial and global redundancy analyses (RDA) plots of community composition for the upper Comal River. The partial RDA for reach identity (A and B), local mesohabitat conditions (B and C) and the global analysis (D and E) are presented.*

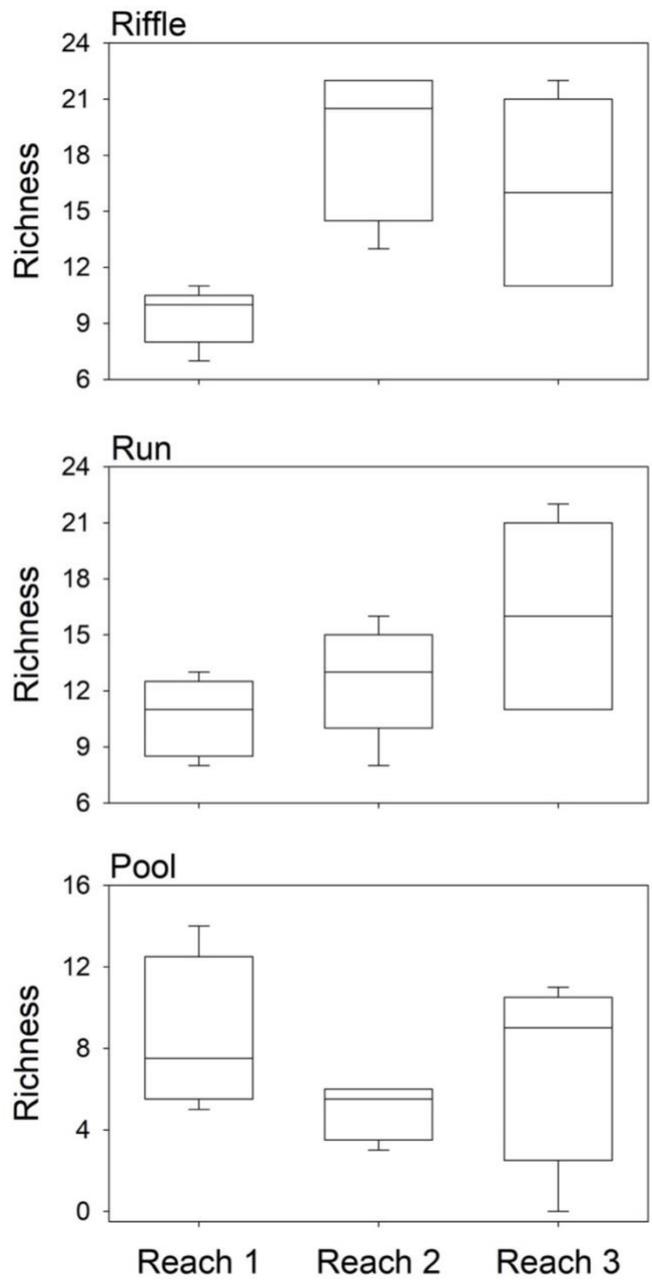


Figure 8. Box and whisker plots of taxonomic diversity for each mesohabitat type for the San Marcos River. Taxonomic diversity for riffles, pools, and runs for Reach 1 (A), Reach 2 (B) and Reach 3 (C).

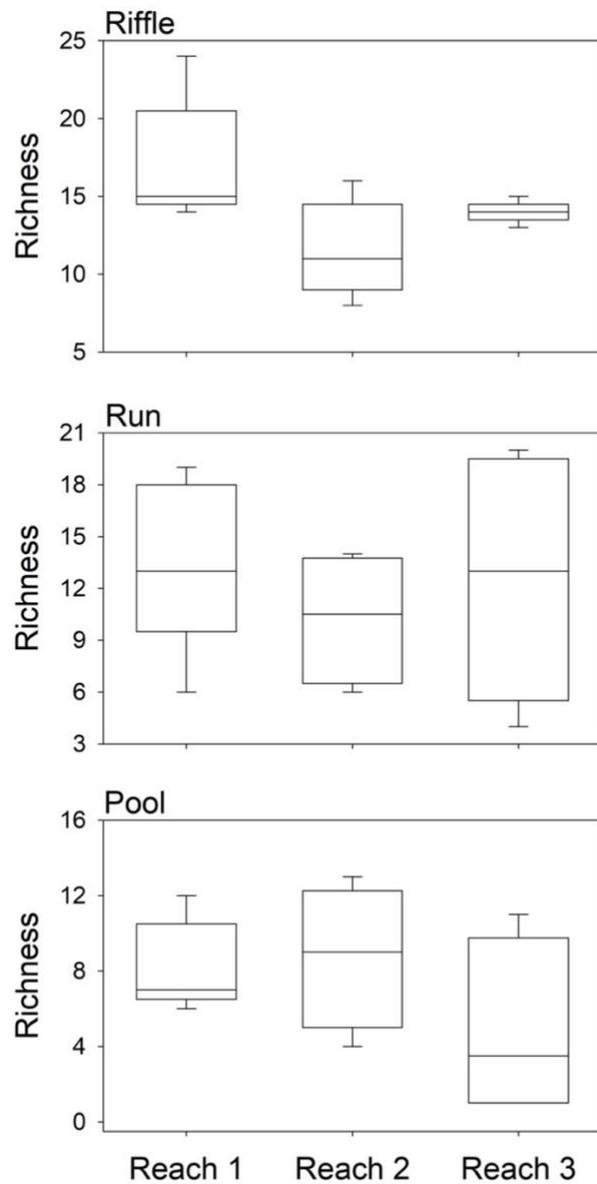


Figure 9. Box and whisker plots of taxonomic diversity for each mesohabitat type for the Comal River. Taxonomic diversity from riffles, pools, and runs for Reach 1 (A), Reach 2 (B) and Reach 3 (C).

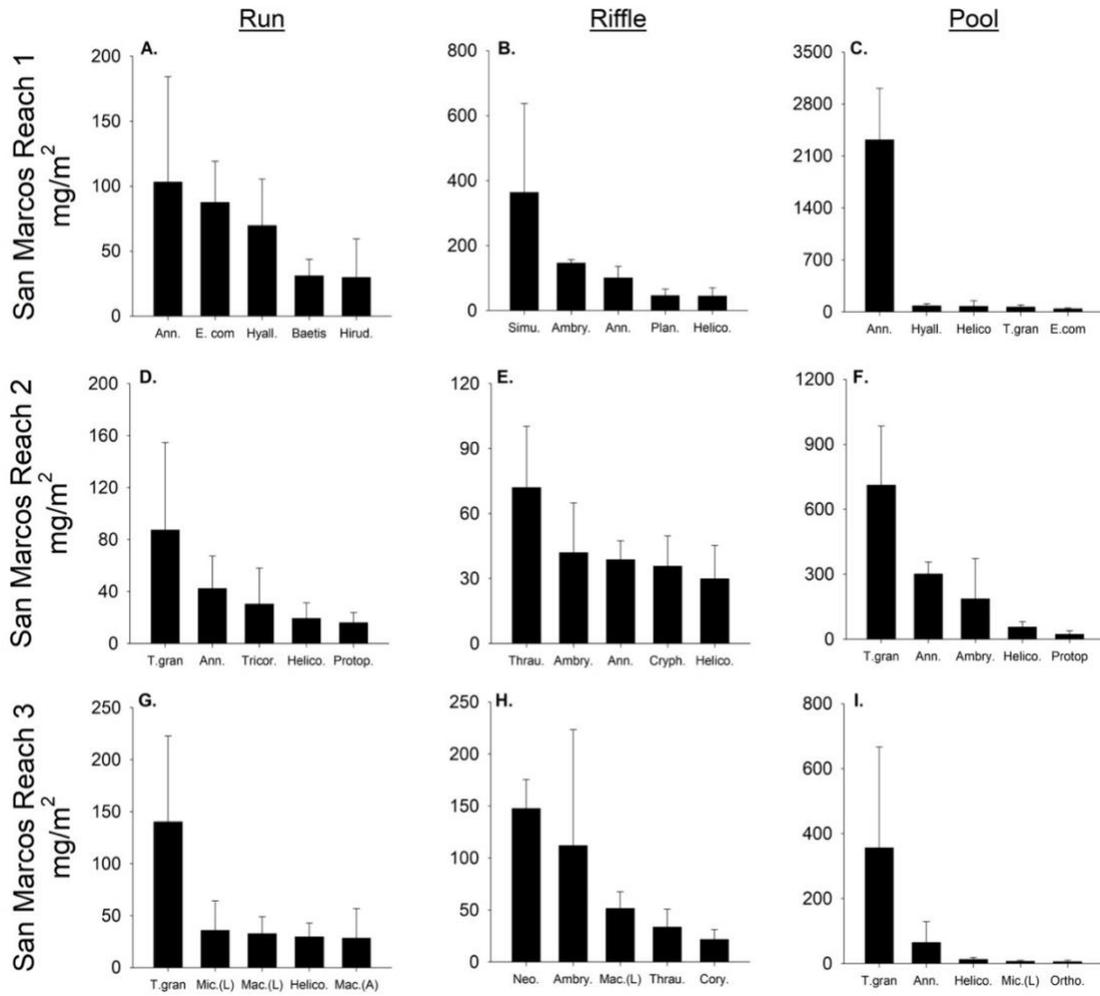


Figure 10. Areal biomass estimates for the top five contributing taxa in the San Marcos River for mesohabitat types across reaches. Areal biomass estimates from riffles, runs, and pools for Reach 1 (A – C), Reach 2 (D – F) and Reach 3 (H – J).

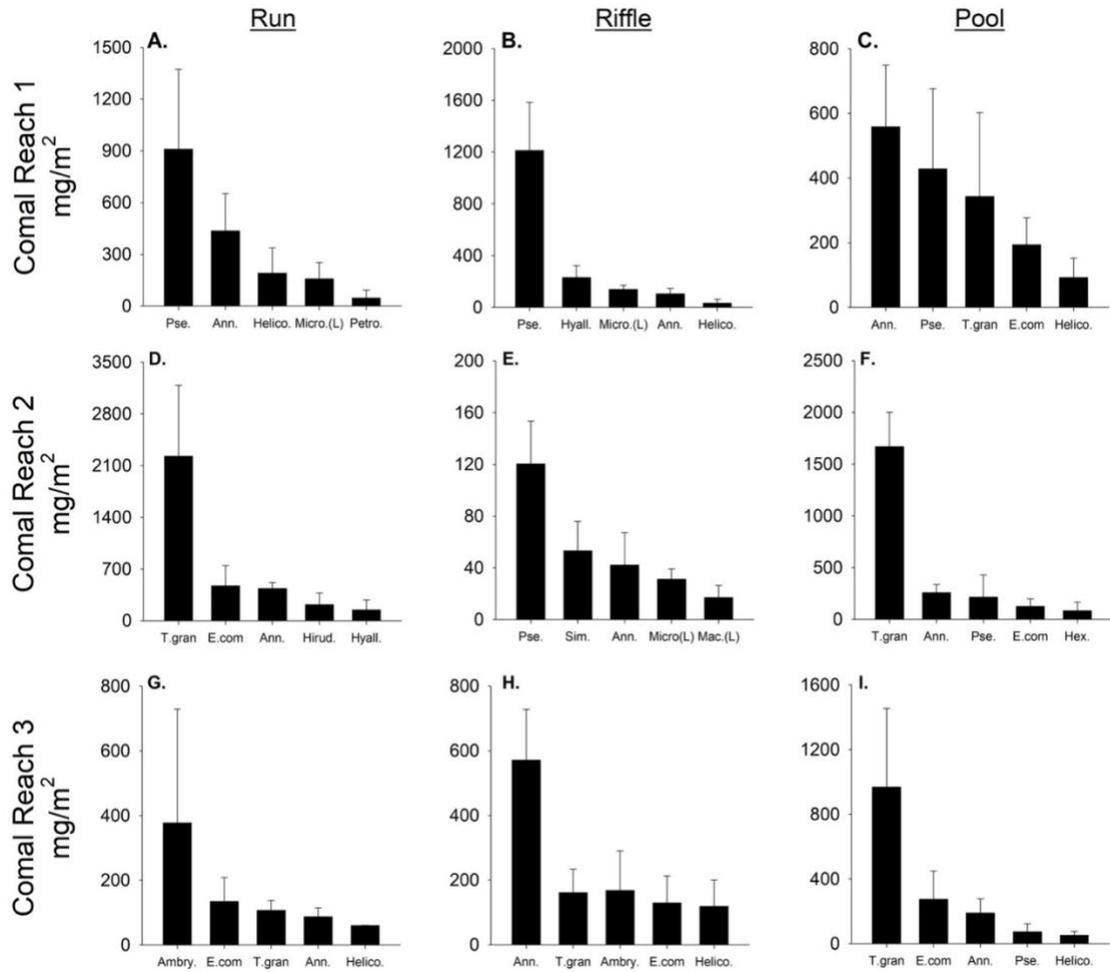


Figure 11. Areal biomass estimates for the top five contributing taxa in the Comal River for mesohabitat types across reaches. Areal biomass estimates from riffles, runs, and pools for Reach 1 (A – C), Reach 2 (D – F) and Reach 3 (H – J).

Table 1. Proportion of variation explained by reach and local predictor variable sets for benthic macroinvertebrate community composition for the upper San Marcos (SMR) and Comal (CR) rivers. The shared and residual variation are also presented.

River	Proportion of Variation Explained			
	Reach	Local	Shared	Residual
SMR	14.30%	8.70%	3.12%	73.87%
CR	7%	7%	6%	80%

Table 2. Total β -diversity and the percent contribution of taxon replacement and richness difference to β -diversity in each mesohabitat across the three upstream-to-downstream study reaches in the San Marcos and Comal rivers.

River	Mesohabitat Type	Total β-Diversity	% Replacement	% Richness Difference
San Marcos	Run	0.275	58.27%	41.73%
	Pool	0.344	87.36%	12.64%
	Riffle	0.26	61.52%	38.48%
Comal	Run	0.319	73.24%	26.75%
	Pool	0.325	44.61%	55.38%
	Riffle	0.308	55.24%	44.76%

Table 3. The top three contributor taxa to species contributions to b-diversity (SCBD) for the San Marcos and Comal Rivers. SCBD values are expressed as the proportional contribution of each taxon to overall b-diversity.

River	Taxa	TCBD
San Marcos	<i>H. azteca</i>	0.108
	<i>T. granifera</i>	0.165
	<i>Helicopsyche</i>	0.095
	<i>E. comalensis</i>	0.09
Comal	<i>T. granifera</i>	0.091
	<i>Helicopsyche</i>	0.089
	<i>H. azteca</i>	0.069
	<i>Macrelmis</i>	0.061

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