

Intercontinental analysis of temperate steppe stream food webs reveals consistent autochthonous support of fishes

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Abstract

Quantifying the trophic basis of production for freshwater metazoa at broad spatial scales is key to understanding ecosystem function and has been a research priority for decades. However, previous lotic food web studies have been limited by geographic coverage or methodological constraints. We used compound-specific stable carbon isotope analysis of amino acids (AAs) to estimate basal resource contributions to fish consumers in streams spanning grassland, montane and semi-arid ecoregions of the temperate steppe biome on two continents. Across a range of stream sizes and light regimes, we found consistent trophic importance of aquatic resources. Essential AAs of heterotrophic microbial origin generally provided secondary support for fishes, while terrestrial carbon did not seem to provide significant, direct support. These findings provide strong evidence for the dominant contribution of carbon to higher-order consumers by aquatic autochthonous resources (primarily) and heterotrophic microbial communities (secondarily) in temperate steppe streams.

KEYWORDS

amino acid, carbon, compound-specific stable isotope analysis, food web, steppe

INTRODUCTION

Characterising the trophic basis of production supporting metazoan consumers in lotic food webs is central to describing ecosystem dynamics. In particular, quantifying the relative proportions of aquatic, autochthonous resources (produced within the system) versus terrestrial, allochthonous resources (produced outside of the system) supporting consumer production benefits our conceptual understanding of stream ecosystems and therefore, our ability to protect their critical functions. The river continuum concept (RCC) (Vannote et al., 1980) provided a theoretical explanation for carbon processing in rivers occurring along a continuum from headwaters to mouths. According to the RCC, trophic bases of production in forested headwater streams should be dominated by terrestrial carbon, based on simultaneous light limitation on primary production and high abundance of terrestrial organic litter fall from the riparian canopy. Aquatic-derived resources were thought to be the most important energy sources in mid-order streams lacking this extensive canopy shading.

A number of studies have been conducted to quantify resource contributions to consumers in streams and rivers, but support for various food web frameworks has often reflected the types of systems studied. Some field studies have provided support for the reliance of stream consumers on terrestrial resources in forested watersheds (Culp & Davies, 1982; Curtis et al., 2018; Grubaugh et al., 1996; Hawkins & Sedell, 1981; Rosi-Marshall & Wallace, 2002). However, fewer studies of consumer resource use have occurred in streams draining watersheds of climatically different ecoregions. In tropical forested streams, some studies have found support for the general framework of the RCC (Greathouse & Pringle, 2006; Tomanova et al., 2007), but Neres-Lima et al. (2016) clearly demonstrated the importance of autochthonous resources even in shaded reaches. Because few streams located outside north temperate forested watersheds have received sufficient research attention despite their widespread global occurrence (González-Bergonzoni et al., 2018), broad patterns of resource use occurring in streams located outside of temperate forested regions are generally not well understood. While the authors of the RCC noted that autochthonous resources may be more important than allochthonous resources in non-forested headwater streams with open canopies (Minshall et al., 1983, 1985; Vannote et al., 1980), this idea was proposed as a contextual qualification of a concept developed for forested systems. The stream biome gradient concept (SBGC), proposed by Dodds et al. (2015), integrated concepts from terrestrial and lotic ecosystems to predict differences in stream ecosystem function across biomes, positing that autochthonous resources would be most important in streams of drier desert and grassland ecoregions, respectively, even in headwaters. This concept could provide an important foundation

for understanding the trophic bases of production in streams of diverse climatic regimes.

Independent of study area, conclusions drawn about basal resource use reflect the abilities and assumptions of the analytical technique used. For example, much previous support for allochthonous basal resource use in headwater streams was established based on defined invertebrate functional feeding group designations (e.g. Jiang et al., 2011) or the enumeration of diet items in the alimentary tract of invertebrates (e.g. Rosi-Marshall et al., 2016), neither of which provide direct integrated information about carbon assimilated into consumer tissues. Alternatively, bulk-tissue stable isotope analyses enable researchers to quantify the resources that have been integrated into tissues (Guo et al., 2016). Still, problems arising from: (a) overlapping $\delta^{13}\text{C}$ values among materials of terrestrial and algal origin (Phillips et al., 2014); (b) widespread changes in algal $\delta^{13}\text{C}$ values over even small spatiotemporal scales (Finlay, 2001; Finlay et al., 1999); (c) constraints on collecting sufficient quantities of labile food sources present at low standing stocks; and (d) difficulties matching bulk-tissue metazoan isotope values to reflect those of their consumed food resources (Dodds et al., 2014) all raise obstacles to interpretation. The recent application of compound-specific stable isotope analysis of amino acids (CSIA-AA) has the potential to clarify complex resource dynamics occurring in streams because carbon isotope values of essential amino acids ($\delta^{13}\text{C}_{\text{EAA}}$ values), which are diagnostic of broad resource groups (e.g. algae, plants, fungi and bacteria, among others; Besser et al., 2022), are highly conserved across time and space (e.g. Elliott Smith et al., 2022a; Larsen et al., 2012, 2013, 2015; Liew et al. 2019a). This means that resource samples do not necessarily need to be collected in situ. Additionally, $\delta^{13}\text{C}_{\text{EAA}}$ values undergo minimal modification when moving between resources and their consumers (Whiteman et al. 2019). Therefore, CSIA-AA allows for partitioning of specific energetic pathways (i.e. autochthonous aquatic carbon, allochthonous terrestrial carbon and associated heterotrophic microbial carbon) that are usually entangled in freshwaters (Demars et al., 2021; Harada et al., 2022).

We quantified the relative importance of aquatic, bacterial, fungal and terrestrial energetic pathways in composing the trophic basis of production for 31 fish taxa from three feeding groups in a set of 61 streams representing grassland, montane and semi-arid ecoregions of the temperate steppe biome on two continents. We then tested the foundational predictions of the RCC as modified by the SBGC by asking how the relative importance of basal resources for stream fishes varied across ecoregions and with site habitat. Specifically, we predicted that essential amino acids (EAAs) from terrestrial detritus (i.e. allochthonous resources) and associated heterotrophic microbes would be most important for fish food webs in low-order, narrow streams with higher coverage of riparian vegetation, while EAAs of aquatic origin

(i.e. autochthonous resources) would be more important to consumers collected in wider, mid-order grassland and desert streams with open canopies. We used CSIA-AA to quantify the trophic basis of production in low- to mid-order streams of each ecoregion by quantifying basal resource assimilation for the major fish species at each site. Our study, which contributes the largest CSIA-AA dataset of freshwater consumers to date, presents new insights about broad spatial patterns of basal resources for higher-order consumers in lotic systems.

METHODS

Study sites

We sampled a total of 61 stream sites (2016–2019) distributed across nine drainages, representing three climatic regions (grassland, montane and semi-arid terminal basin) located within two globally significant stretches of the temperate steppe biome (the Great Plains of the United States and the Eurasian Steppes of Mongolia; Olson et al., 2001; Figure 1; Table S1). Grassland rivers sampled included the following: (a) the Kherlen River of Mongolia (a tributary of the Amur River), which is mostly precipitation-fed and therefore controlled by flashy hydrological regimes with alternate periods of flooding

and drying; and (b) the Niobrara River (Nebraska, USA, a tributary of the Missouri and Mississippi Rivers), which is fed by a combination of precipitation and groundwater inputs from the Ogallala Aquifer. Montane sites in the United States (located within tributaries of the Yellowstone River, Wyoming, which is ultimately a tributary of the Mississippi River) and Mongolia (on the Delgermörön River, a tributary of the Selenge River) were represented by high-elevation headwaters that ranged from steep-gradient forested sites to meandering streams in mountain meadows, both fed primarily by snowmelt. Finally, semi-arid terminal basin sites, represented by the Bear, Carson and Humboldt Rivers (flowing from the mountains surrounding the United States Great Basin) and the Khovd and Zavkhan Rivers of northwestern Mongolia (flowing from the Altai and Khangai Mountains), are endorheic systems that are sourced from melting, high-elevation snowpack and glaciers, and then ultimately flow to terminal lakes or dry basins, rather than into the ocean.

Stream habitat assessments

Stream habitat characteristics (wetted width, mid-stream canopy cover and canopy cover at stream bank) were surveyed at each site using standard methods (Lazorchak et al., 1998). Average wetted width was estimated at each site as the mean of 10 measurements taken along equidistant transects spanning the sample site. Canopy cover was measured at mid-stream and stream banks using a convex spherical densiometer at a distance of ~0.3 m above the stream surface and averaged at the site level. Strahler stream order was also remotely designated for each site in ArcGIS as established by Maasri et al. (2021). Briefly, digital elevation models were used to inform flow direction and accumulation defined at the 10 km² scale, and stream orders were classified using the Strahler method (Strahler, 1957).

Fish collections

Fishes were collected in the summer season (July–September) during 2016, 2017, 2018, or 2019, depending on sample site (Ball State University IACUC permit #126193). We collected representative fish community samples using single-pass backpack electrofishing (Model ABP-2, ETS), supplemented by seining, gill netting and angling, following guidelines of the American Fisheries Society standard methods (Bonar et al., 2009). Fishes were collected from reach lengths measuring a minimum of 20× the average wetted stream width. All fishes were weighed to the nearest 0.1 g, and their standard lengths were measured to the nearest 1 mm.

We collected fish tissue samples from three similarly sized individuals of the most dominant species

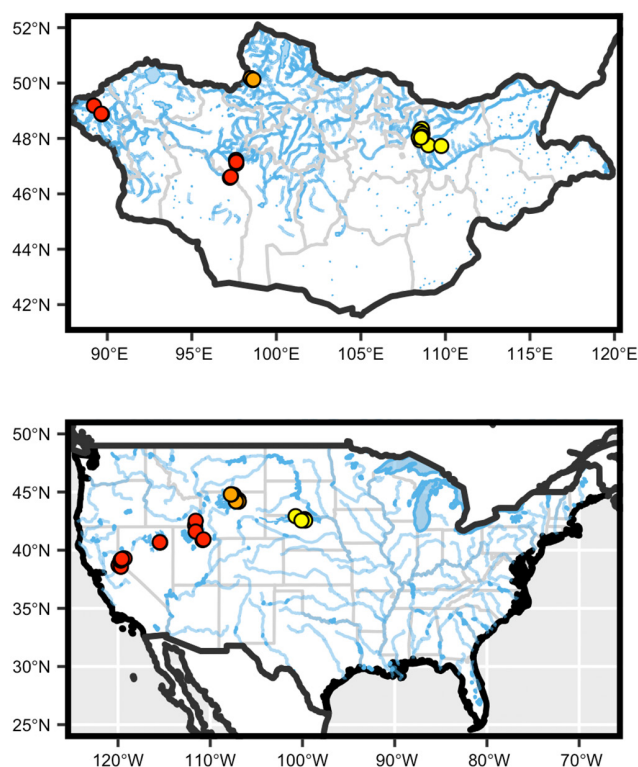


FIGURE 1 Map of study sites ($n = 61$) in Mongolia ($n = 22$, left) and the United States ($n = 39$, right). Point colour corresponds to ecoregion type within the temperate steppe biome, with grassland sites in yellow, montane sites in orange and semi-arid terminal basin sites in red. Note the difference in scale between panels.

representing at least two unique feeding groups (when possible) to characterise fish communities comparably across geographically dispersed sites (Table S2). In instances when three species were equally dominant within a reach, three replicates of an additional species were also analysed. Fishes were identified in the field and later assigned functional feeding groups based on species and size according to FishBase (Froese & Pauly, 2000) or Mendsaikhon et al. (2017). We extracted muscle samples using a clean scalpel and stored them in 70% ethanol until analysis. Based on previous demonstrations, preservation in ethanol is unlikely to affect interpretations of $\delta^{13}\text{C}_{\text{AA}}$ values (Chua et al., 2020).

Basal resource collections

In addition to fish tissues, we analysed representative primary producers, including terrestrial C_3 plants (*Salix* spp. and *Pinus* sp.; $n = 21$), terrestrial C_4 plants (*Zea* sp. and *Panicum* sp.; $n = 6$), microalgae (*Chlorella* sp., and a diatom-dominated biofilm; $n = 6$), filamentous algae (*Spirogyra* sp.; $n = 3$), cyanobacteria (*Spirulina* sp.; $n = 3$) and an aquatic macrophyte (*Hydrilla* sp.; $n = 3$). Primary producers were either collected in situ or purchased from commercial suppliers (Table S3). Diatom-dominated biofilms and *Spirogyra* sp. were harvested in situ from the Zavkhan River, Mongolia, with care taken to collect samples highly dominated by specific taxa. Sample purity was then confirmed by separating and sorting samples in stream water under a microscope to remove invertebrates, coarse organic material and other taxa. Diatoms were filtered to further improve sample purity, and filamentous algae samples were agitated and rinsed with stream water to wash out epiphytes and associated algae of other species. Although we did not observe large amounts of fungal material in these samples, we note that we cannot completely rule out bacterial contamination. All other field-collected samples, including *Hydrilla* sp., *Salix* spp., *Pinus* sp., *Zea* sp. and *Panicum* sp. were rinsed and examined for purity in the laboratory after collection.

Amino acid carbon isotope analysis

We dried muscle tissues and resource samples at 60°C for 48 h and then homogenised them into fine powders using a Wig-L-Bug mixer/amalgamator (Rinn/Crescent Dental Mfg.). Homogenised samples were weighed into quantities of 2 mg (fish tissues) or 20–30 mg (resources) and placed into glass vials that had been pre-combusted at 400°C for 8 h. Processed samples were sent to the Louisiana State University Stable Isotope Ecology Laboratory for carbon CSIA-AA.

Samples were acid hydrolyzed at 110°C for 20 h to isolate constituent amino acids (AAs). For samples such as

plant material and algae, AA hydrolysates were cleaned and columned using a Dowex 50WX8-400 cation exchange resin. Then, AAs were derivatised with methyl chloroformate following methods described by Walsh et al. (2014) and injected in duplicate as methoxycarbonyl (MOC) AA esters onto a constant flow column on a Trace 1310 gas chromatograph (GC; Thermo Fisher Scientific) for AA separation. Values for $\delta^{13}\text{C}_{\text{AA}}$ were obtained using a Thermo Scientific Delta V Advantage isotope ratio mass spectrometer (IRMS; Thermo Fisher Scientific) coupled to the GC with a GC IsoLink II (Thermo Fisher Scientific).

Following Walsh et al. (2014) we used triplicate injections of an L-AA mix standard that was co-measured with samples to correct for the non-analyte carbon incorporated into AAs through derivatisation and kinetic isotope effects. The L-AA mix standard was comprised of the individual AAs ($\geq 99.5\%$; Sigma-Aldrich) alanine (Ala), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), proline (Pro), threonine (Thr) and valine (Val). We corrected for non-analyte carbon effects using the equation of Docherty et al. (2001): $n_{\text{cd}} \delta^{13}\text{C}_{\text{cd}} = n_{\text{c}} \delta^{13}\text{C}_{\text{c}} + n_{\text{d}} \delta^{13}\text{C}_{\text{dcorr}}$, where n is the number of moles of carbon, C_{c} is the compound of interest (AA), C_{cd} is the derivatised compound (MOC AA ester), and C_{dcorr} is the empirically determined correction factor. We determined correction factors by comparing the $\delta^{13}\text{C}$ values of each underivatized individual L-AA obtained with Elemental Analyser IRMS (EA-IRMS) and the measured $\delta^{13}\text{C}$ values of their derivatised forms.

L-norleucine ($\geq 99.5\%$; Sigma-Aldrich) was used as an internal reference material. In addition, triplicate injections of a D-AA mix standard and a well-described red drum (*Sciaenops ocellatus*) fish muscle laboratory reference material were used to assess instrument precision and assure the quality of sample runs. The D-AA mix standard was comprised of the same individual AAs ($\geq 99.0\%$; Alfa Aesar) in the L-AA mix standard and the $\delta^{13}\text{C}$ value of the underivatized L-norleucine reference material, and each underivatized D-AA was obtained using EA-IRMS. Isotope values are expressed in delta (δ) per mil (‰) notation according to the following formula, as calculated for each AA in the sample tissue: $\delta^{13}\text{C} (\text{‰}) = \left(\left(\frac{^{13}\text{C}/^{12}\text{C}}{^{13}\text{C}/^{12}\text{C}} \right)_{\text{sample}} / \left(\frac{^{13}\text{C}/^{12}\text{C}}{^{13}\text{C}/^{12}\text{C}} \right)_{\text{standard}} - 1 \right) \times 1000$. The 10 AAs listed above were recovered for all samples. Mean analytical error (SD) was 0.27‰ for all AAs in samples and 0.24‰ for EAAs in samples. Mean analytical error (SD) for reference materials and mix standards was 0.45‰ for all AAs and 0.39‰ for EAAs.

Statistical analysis

All statistical analyses were conducted in R version 4.2.0 (R Core Team, 2022). Following the isotopic ‘fingerprinting’ approach (sensu Larsen et al., 2009), we

focused our analyses on the $\delta^{13}\text{C}$ values for AAs that are essential for fishes: Ile, Leu, Phe, Thr and Val (Table S4). Because our objective was to identify broad energetic pathways contributing to consumer basal diets, we supplemented our small sampled resource dataset with previously published resource datasets containing $\delta^{13}\text{C}_{\text{EAA}}$ values of pure aquatic (i.e. algae, cyanobacteria and aquatic vegetation), bacterial, fungal or terrestrial (i.e. C_3 and C_4 plants) materials from work by Larsen et al. (2013), Paolini et al. (2015), Jarman et al. (2017), Thorp and Bowes (2017), Liew et al. (2019b), Moyo et al. (2020), Pollierer et al. (2020), Besser (2022) and Elliott Smith et al. (2022b). Samples of leaf litter detritus or other non-pure samples ($n = 39$) were excluded from the aggregated dataset because we wanted to trace specific energetic pathways. We also excluded resource data from taxonomic groups that could not be found in a freshwater environment (e.g. saltmarsh grasses, red algae and kelp). In addition, 21 samples were eliminated from subsequent analyses because they contained missing values for at least one of the five EAAs. Therefore, the final aggregated resource dataset ($n = 371$) used for analyses included 350 total samples.

We normalised resource $\delta^{13}\text{C}_{\text{EAA}}$ values by subtracting the mean $\delta^{13}\text{C}$ of all EAAs ($n = 5$) for a particular sample from each $\delta^{13}\text{C}_{\text{EAA}}$ value in that sample. This method of normalisation allows for the comparison and aggregation of source data by maximising the biomolecular differences among AAs and simultaneously reducing the noise introduced by environmental variability (Larsen et al., 2020). Normalised resource and consumer data were examined for normality using Q-Q plots and Shapiro–Wilk tests. We ran a principal component analysis (PCA) on normalised $\delta^{13}\text{C}_{\text{EAA}}$ values using the ‘prcomp’ function in the *stats* package in R (R Core Team, 2022). Then, using the *MASS* package in R (Venables & Ripley, 2002), we ran a linear discriminant analysis (LDA) to classify samples into food source categories that were pre-determined based on PCA visualisation (Larsen et al., 2013). We used a leave-one-out cross validation approach to ensure that resource groups were statistically distinguishable from one another, as indicated by a high classification accuracy (>80%; Fox et al., 2019).

To infer contributions of basal resources to consumers, we first ran a PCA and plotted normalised consumer $\delta^{13}\text{C}_{\text{EAA}}$ profiles along with normalised resource $\delta^{13}\text{C}_{\text{EAA}}$ profiles in principal component space with 95% confidence ellipses as a preliminary visualisation. We then used two models to determine the origin of EAAs in consumer tissues. First, we ran a second LDA using resource $\delta^{13}\text{C}_{\text{EAA}}$ values as a training set and fish $\delta^{13}\text{C}_{\text{EAA}}$ values as a test set to determine the probability of classification of each fish into each resource category. Next, we ran a Bayesian isotope mixing model using the *MixSIAR* package in R (Stock et al., 2018) to estimate proportional

resource use by consumers, using the same inputs as we did for the LDA. Bayesian mixing models can estimate proportional dietary contributions even in cases of complex, multichannel feeding (Manlick & Newsome, 2022). The *MixSIAR* model, which explicitly included site and species as nested factors, was set to run for 300,000 iterations (burn-in = 200,000) on three parallel Monte Carlo Markov chains with a thinning interval of 100 using non-informative (generalist) priors (Equ. S1). We used the multiplicative error method (i.e. both process and residual error) as recommended by Stock and Semmens (2016) for food web studies in natural ecosystems. To account for any minimal trophic discrimination between diet and consumer $\delta^{13}\text{C}_{\text{EAA}}$ values, we used small, non-zero values for trophic discrimination (mean $\pm$ SD; $0.1 \pm 0.1\text{‰}$; McMahon et al., 2010). The model was assessed using Geweke and Gelman–Rubin convergence statistics, MCMC standard error and visualisation of trace and density plots (Table S5).

Probabilities of consumer classification into each resource group (LDA) and proportional resource contributions of each resource category to consumers (*MixSIAR*) were summarised (mean $\pm$ SD) at the levels of site, ecoregion and country. Linear mixed-effects models were used to identify influences of local habitat (i.e. canopy coverage, wetted width and stream order) on proportional basal resource assimilation, with country, ecoregion and site, and fish taxonomic family and species, included as nested random effects. We fit linear mixed-effects models using the *lme4* package in R (Bates et al., 2015). Model diagnostics were evaluated using the *lmerTest* R package (Kuznetsova et al., 2017). Significance of model terms were assessed with Satterthwaite's method using the ‘summary’ function in the *performance* R package (Lüdtke et al., 2021), with p -values <0.05 considered statistically significant.

RESULTS

Site habitat characteristics

More across-site variability in canopy coverage was observed at stream banks than at mid-stream (Figure 2). Most sites were characterised by low (<25%) canopy coverage at mid-stream, with just a few United States sites having mid-stream canopy coverage above 48% (USSA1, USMT1, USMT2 and USMT3). Mongolia stream sites had lower mean canopy coverage at both mid-stream (2.1%) and stream bank (6.9%) than US sites (14.6% at mid-stream and 39.1% at stream bank, respectively). These differences in canopy coverage were related to country-level differences in Strahler stream order and average wetted width of sampled streams, with the US having smaller and narrower sites overall (with a mean Strahler stream order of 2.3 and a mean site wetted width of 9.4 m) and Mongolia having larger and wider sites

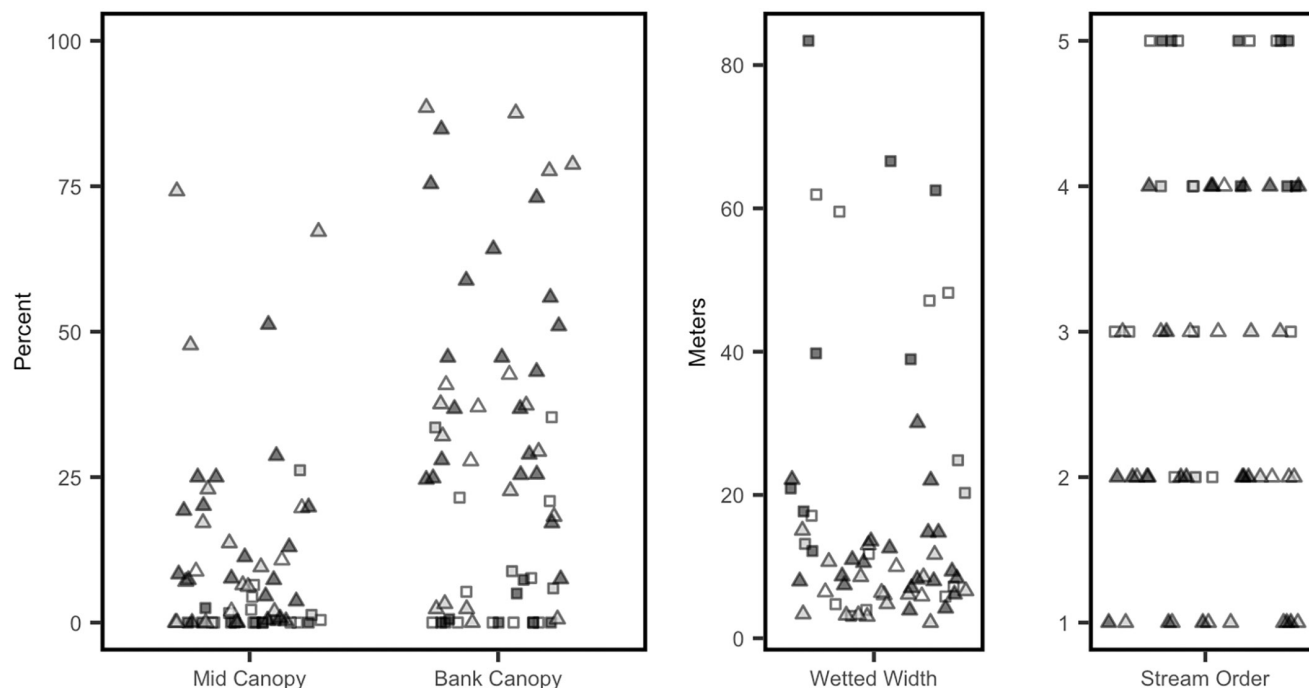


FIGURE 2 Habitat characteristics for 61 stream sites in grassland (white), montane (grey) and semi-arid terminal basin (black) ecoregions in Mongolia (squares) and the United States (triangles). Characteristics include mean percent canopy coverage at mid-stream (mid canopy), mean percent canopy coverage at stream bank (Bank canopy), mean stream wetted width in meters (wetted width) and Strahler stream order.

(with a mean Strahler stream order of 4.0 and a mean site wetted width of 30.5 m; Table S1).

Fish communities

We analysed a total of 356 individual fishes representing three ecoregional types in two countries: Mongolia grassland ($n = 83$), Mongolia montane ($n = 24$), Mongolia semi-arid ($n = 35$), US grassland ($n = 29$), US montane ($n = 73$) and US semi-arid ($n = 112$; Table S3; Table S4). Of the 31 unique fish taxa (29 distinct species) collected, three feeding guilds were represented: piscivores ($n = 59$), invertivores ($n = 222$) and omnivores ($n = 75$). US fish assemblages included 11 native species, 7 species that were nonnative within at least one portion of their collected range and one hybrid (*Salmo trutta* x *Salvelinus fontinalis*). One species (*Oncorhynchus clarki*) was represented by two subspecies (*Oncorhynchus clarki bouvieri*, which was collected in the US montane region, and *Oncorhynchus clarki utah*, which was collected in the US semi-arid region). Mongolia was represented by 11 species, which were all native. One fish species, *Esox lucius*, was shared between Mongolia and US sites.

Resource categorisation

The first two principal components accounted for the majority of the variation within a large, aggregated food source dataset, with PC1 explaining 56.3% and PC2

explaining an additional 20.0% of variation (Figure S2). Separation along PC1 in principal component space confirmed four putative basal resource categories for use in subsequent analyses: aquatic resources ($n = 117$), bacterial resources ($n = 12$), fungal resources ($n = 37$) and terrestrial resources ($n = 184$). Normality tests indicated some deviation from normality, but these instances seemed to be heavily influenced by sample size with a relatively small number of samples driving minor skews in the distribution of the data, so we moved forward with LDA. High overlaps among resource groups in principal component space (based on 95% confidence ellipses) were resolved in linear discriminant space, where LD1 explained 69.8% of the proportion of trace among groups, and LD2 explained an additional 27.4%; Figure S3. Of the five EAAs used as $\delta^{13}\text{C}_{\text{EAA}}$ biotracers in this study, Leu had the linear discrimination coefficient with the highest absolute value (-0.41), followed by Phe (0.26), Val (-0.25), Ile (0.09) and Thr (0.07). LDA with leave-one-out cross validation classified source samples into their respective groups with 87.1% accuracy overall. The bacterial resource category had the best reclassification rate (100%), followed by terrestrial (92%), while aquatic and fungal resources had relatively lower reclassification accuracy (79% and 76%, respectively).

Basal resource estimation

When resource $\delta^{13}\text{C}_{\text{EAA}}$ values were plotted alongside fish $\delta^{13}\text{C}_{\text{EAA}}$ values in principal component space, fishes

tended to cluster within 95% confidence intervals of both aquatic and fungal resource spaces, with PC1 explaining 55.9–58.3% of variation and PC2 explaining an additional 20.0–21.3% (Figure S4).

LDA posterior classifications based on LD1 and LD2 indicated that all fish individuals classified within the aquatic resource category (Figure 3). LD3 was not considered in our models because it explained only 2.8% of the variability among source groups. On average, the mean $\pm$ SD probabilities that fishes classified with aquatic, bacterial, fungal or terrestrial resources were as follows: $92.3 \pm 6.0\%$, $0.15 \pm 0.3\%$, $3.9 \pm 4.1\%$ and $3.6 \pm 4.5\%$, respectively, based on LDA (Figure 4).

Results for proportional resource assimilation from Bayesian mixing models were consistent with LDA

results overall, in that aquatic sources were most important for all fishes at all sites on average ($59.9 \pm 21.2\%$), followed by heterotrophic resources belonging to fungal ($24.4 \pm 16.0\%$) and bacterial ($11.3 \pm 8.7\%$) groups, and then terrestrial ($4.4 \pm 5.2\%$) sources. For all ecoregions, *MixSIAR* models suggested that fishes obtained most of their EAAs from aquatic sources, but the predicted importance of aquatic resources differed. Fishes collected in Mongolia grassland ($75.6 \pm 17\%$) and United States montane ($73.7 \pm 13.9\%$) streams showed the highest aquatic resource assimilation estimates overall, while semi-arid streams of Mongolia and the United States were characterised by relatively lower estimates ($48.1 \pm 11.8\%$ and $45.9 \pm 19.6\%$, respectively; Figure 5; Table S6).

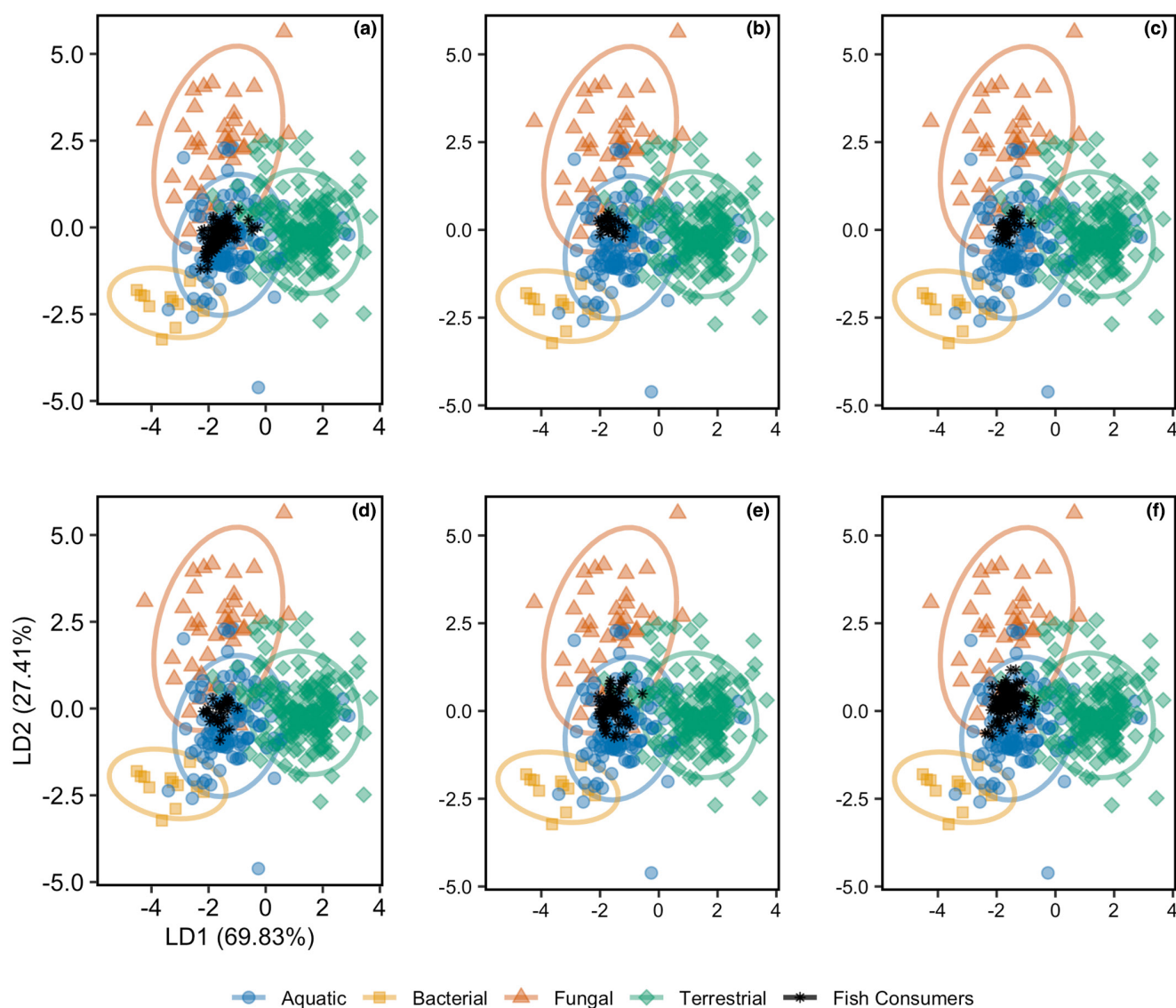


FIGURE 3 Normalised $\delta^{13}\text{C}_{\text{EAA}}$ values for putative basal resources and fish consumers plotted in linear discriminant space, separately for six ecoregional types in two countries: Mongolia grassland (a), Mongolia montane (b), Mongolia semi-arid terminal basin (c), US grassland (d), US montane (e) and US semi-arid terminal basin (f). Points in colour represent basal resources (aquatic, bacterial, fungal or terrestrial; $n = 350$), and fishes collected in each ecoregion are represented by small black asterisks ($n = 356$). Ellipses represent 95% confidence intervals for group membership. All figures share the same linear discriminant axes given in plot d.

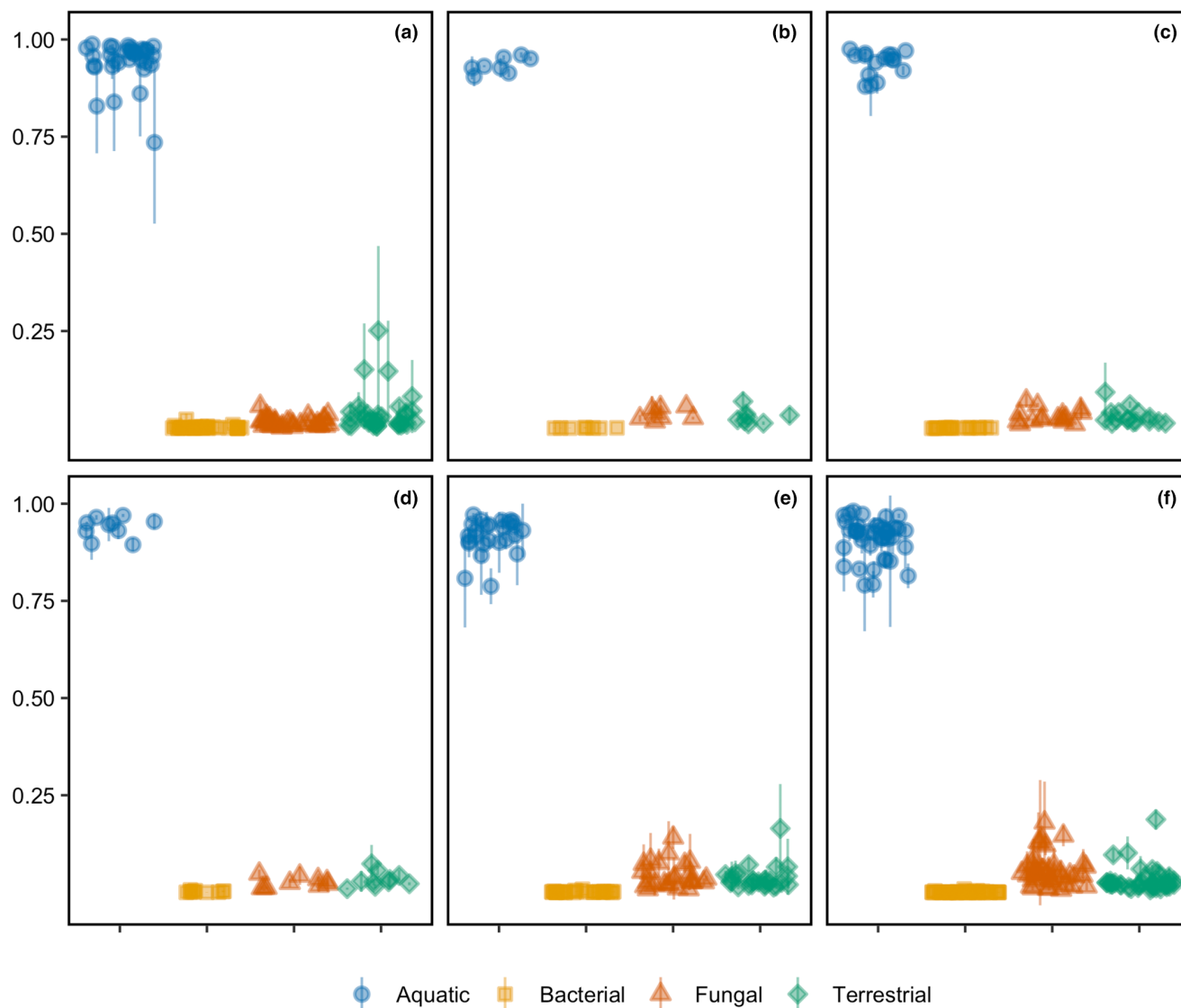


FIGURE 4 Linear discriminant model posteriors (mean $\pm$ SD) indicating the probability of classification into aquatic, bacterial, fungal, or terrestrial basal resource categories for fishes collected in six ecoregions: Mongolia grassland (a), Mongolia montane (b), Mongolia semi-arid terminal basin (c), US grassland (d), US montane (e) and US semi-arid terminal basin (f; $n = 356$).

According to linear mixed-effects models, neither canopy coverage nor wetted width had a meaningful influence on proportional resource assimilation by fish consumers ($p > 0.05$ for all four resource categories). Stream order had a significant positive effect on bacterial resource assimilation only ($p = 0.029$). A square root transformation was applied to the response variable to correct for heteroskedasticity in the terrestrial model.

DISCUSSION

Through our analysis of the largest amino acid carbon isotope dataset for freshwater fishes to date, we tested the foundational predictions of the RCC (Vannote et al., 1980) with modified hypotheses in the SBGC (Dodds et al., 2015) by directly quantifying the contributions of

terrestrial (allochthonous), aquatic (autochthonous) and heterotrophic microbial energetic pathways to stream consumers in first to fifth order streams of grassland, montane and semi-arid ecoregions of the understudied temperate steppe biome. We found that resources of aquatic origin provided the dominant basal support for fish food webs in all comparable ecoregions on two continents. This pattern was consistent across a gradient of stream sizes and riparian canopy cover regimes, in contrast with our hypothesis that allochthonous resources would be increasingly important in low order, narrow streams with higher riparian canopy coverage. In fact, while terrestrial resources may have been important for a few individual fishes, we found no patterns of significant, direct assimilation of terrestrially derived carbon at the site level, even at four United States sites with the highest instances of canopy shading. Heterotrophic microbial

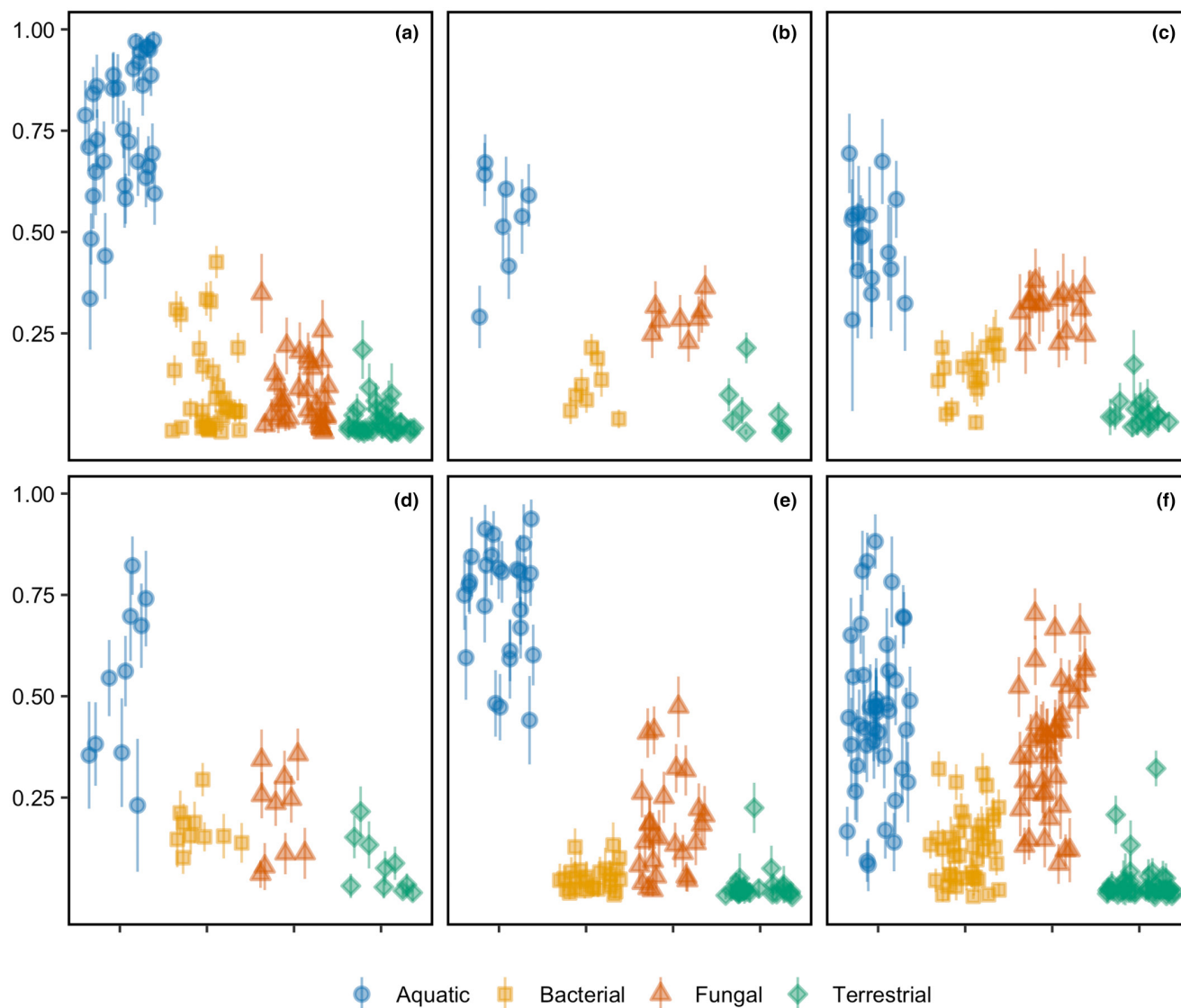


FIGURE 5 Bayesian stable isotope mixing model (*MixSIAR*) posteriors (mean $\pm$ SD) indicating the relative proportional contribution of each basal resource category (aquatic, bacterial, fungal and terrestrial) assimilated by fishes collected in six ecoregions: Mongolia grassland (a), Mongolia montane (b), Mongolia semi-arid terminal basin (c), US grassland (d), US montane (e) and US semi-arid terminal basin (f; $n = 356$).

resources provided substantial secondary support for fish consumers as inferred by EAA origin.

We found that carbon from aquatic resources consistently provided most EAAs to fishes overall, in both Mongolia and the United States. Further, this general trend was consistent across results generated by two models despite their different underlying algorithms (LDA and *MixSIAR*). Given the high assimilation efficiency and nutrition of aquatic (especially algal) resources (Brett et al., 2017; Guo et al., 2018; Marcarelli et al., 2011), their importance as basal resources for higher-order fish consumers is not surprising from a physiological standpoint. Many producers of aquatic origin are characterised by low C:N ratios and contain high amounts of essential dietary components for metazoans including AAs (Ruess & Müller-Navarra, 2019) and long-chain polyunsaturated fats (Ebm et al., 2020; Labed-Veydert et al., 2021).

Our results confirm the importance of autochthonous resources in streams with relatively sparse canopy cover, even in headwater reaches. Therefore, SBGC modifications of the trophic predictions of the RCC could expand stream ecology frameworks predicting large-scale patterns of ecosystem function in streams across climatic regions.

Our results for temperate steppe streams, together with studies from temperate forested watersheds (Hayden et al., 2016; Mayer & Likens, 1987; McNeely et al., 2007), streams in subarctic and alpine regions (Ebm et al., 2020; Fujibayashi et al., 2019), tropical stream ecosystems (Hayden et al., 2021; Neres-Lima et al., 2016), large rivers (Bowes et al., 2020; Thorp & Bowes, 2017), desert floodplains (Bunn et al., 2003) and a cave river ecosystem (Liew et al. 2019b) provide united evidence that while some consumers may assimilate allochthonous

resources (e.g. omnivorous minnows of Mongolia grassland streams in the present study), many are nutritionally dependent on resources of aquatic origin. We note that this does not suggest that fishes are consuming these aquatic resources directly. Instead, it confirms that while fish possess diverse feeding strategies in terms of what they ingest, the selective retention of autochthonous carbon and transfer through trophic levels is likely to occur regardless of this functional diversity, due to its higher assimilation efficiency into metazoan tissues (Brett et al., 2017; Guo et al., 2016; Kühmayer et al., 2020). This mechanism explains the widespread assimilation of autochthonous resources into stream food webs across this range of climatic regimes and habitat characteristics.

Terrestrial plant matter did not provide significant direct energetic support for fishes in temperate steppe streams, even in the montane and semi-arid regions of the United States where stream canopy cover was the highest. This highlights the discrepancy that can occur between ingested and assimilated resources in that only the most nutritious components of the diet are likely to be integrated into consumer tissues (Dodds et al., 2014). Fishes that did assimilate EAAs of terrestrial origin were mostly minnows or large salmonids, which may have been most likely to consume terrestrial detritus or terrestrial insects, respectively. However, terrestrial invertebrates known to provide important resource subsidies to stream consumers (Nakano & Murakami, 2001) were not observed in significant amounts in fish stomachs at the time of sampling (Minder et al., 2020, 2021). Because we did not analyse the $\delta^{13}\text{C}_{\text{EAA}}$ profiles of invertebrates, we cannot be certain about the value of direct terrestrial assimilation for organisms that feed directly on decomposing terrestrial organic matter (e.g. Taipale et al., 2016). Finally, while the strength of the conclusions that we can draw about the influence of canopy shading on the trophic basis of production are limited by the range of canopy coverage that we observed at our sites, aquatic resources contributed the majority of EAAs to fish consumers at US montane sites with 49–74% mid-stream canopy coverage (USMT1, USMT2 and USMT3). However, we also note that canopies at the most heavily forested sites included coniferous trees, which are less palatable than some deciduous species (Webster & Benfield, 1986).

Heterotrophic microbes (fungi, primarily, and secondarily, bacteria) were consistently important as secondary basal resources for all ecoregions, and they were the primary basal resource category at seven of 61 total sites. It is unknown whether the heterotrophic microbial energy pathway to higher-order consumers is sustained by autochthonous or allochthonous carbon (or what proportion of AAs are routed through gastrointestinal tract microbes; Kelly & Martínez del Río, 2010; Newsome et al., 2011). However, our observation of a distinct heterotrophic signal in consumer tissues (as opposed to a $\delta^{13}\text{C}_{\text{EAA}}$ profile characteristic of terrestrial carbon)

suggests that any contribution of allochthonous carbon is likely reworked and facilitated into metazoan food webs primarily via the microbial loop (Arsenault et al., 2022; France, 2011). In fact, the $\delta^{13}\text{C}_{\text{EAA}}$ profiles of terrestrial resources did not overlap with those of fish communities, or with any other resource category, despite being the most thoroughly characterised resource group. This lends support to the paradigm that fungi and other microbes provide the labile ‘peanut butter’ on a recalcitrant detrital ‘cracker’ (Cummins, 1974; Graça, 2001), as evidenced by the assimilation of microbial carbon by shredding invertebrates (e.g. Chung & Suberkropp, 2009; Findlay et al., 1986). We found evidence of a positive influence of stream order on assimilation of bacterial carbon, which could be driven in part by feeding activity on large ungulate excrement in mid-order streams of the Mongolia grassland ecoregion. Further characterisation of bacterial (and fungal) $\delta^{13}\text{C}_{\text{EAA}}$ profiles is needed to fully reconcile the interactions among these energy pathways.

We conclude that aquatic, autochthonous resources and heterotrophic microbes are responsible for producing the EAAs that support higher-order fish consumers in temperate steppe streams. Our results, obtained using new tools, are consistent with the biome-gradient view of the SBGC and other previous modifications to the RCC (e.g. Minshall et al., 1985), where rivers and streams in drier habitats with lower canopy cover rely more heavily on autochthonous sources. These findings further underscore the importance of protecting freshwater ecosystems within the context of their relatively undisturbed historic character given that higher trophic levels of lotic food webs rely on in-stream basal resources.

AUTHOR CONTRIBUTIONS

ERA and JHT designed the study with input from co-authors. ERA wrote the original draft. ERA conducted data analyses with input from MJP. MJP and HB conducted analytical work and provided methodological expertise. JHT, MJP, MM, WKD, FT, AM and MP provided major input to the writing of the manuscript. ERA, MM, MP, BM, AO, SA, RS and CA collected samples. MP, BM, AO, SA, MM, RS, CA and SC contributed expert knowledge of fish natural history and taxonomy. All authors approved the final version.

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Data and code are available on Dryad (<https://doi.org/10.5061/dryad.dncjsxm21>).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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