General comments.

Nice job on this revision, the ms is really coming together nicely.

1. It helps to use consistent terminology. We use “δ15N values” 27 times and “δ15N signatures” 11 times in the ms. If these are synonymous, let’s stick with a single term. I prefer “values” but can go either way. If these terms are not synonymous, then we need to define how they differ.

2. Look at a recent article in Hydrobiologia to see how citations are handled. For citations in parentheses, a comma is used after the author’s names, and a semicolon is used to separate different references):

“Reducing light levels could reduce the effectiveness of aquatic predator vision (Helfman, 1981; Verweij et al., 2006).”

3. For citations within the text, no comma is used after the author’s names:

“A similar result was documented by Nakamura et al. (1997) who studied the fish of Lake Kasumigaura, Japan.”

4. Be sure references are in correct format. Hydrobiologia uses a common not a period after the last author’s name. Also, they use “&” instead of “and” for the last author in a series:

Eighani, M., S. Y. Paighambari, M. Taquet & J.-C. Gaertner, 2019. Introducing nearshore fish aggregation devices (FAD) to artisanal Persian Gulf fisheries: a preliminary study. Fisheries Research 212: 35–39.

5. Hydrobiologia does not capitalize the common names of fishes.

6. Hydrobiologia uses “Fig.” not “Figure” when referring to figures in the text.

Simuliidae is misspelled in Fig 2, 3 and 5.

Based on Fig.6, omnivores remove the environmental gradient effect for 5 of 6 fish species (which is as good as Simuliidae). Why don’t we consider them as suitable baselines? What are their pros and cons?

Journal: *Hydrobiologia*

Type: Article

**Assessing a standardized method to identify optimal baselines for trophic position estimation in stable isotope studies of stream ecosystems**

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**Statement of authorship**: Conceptualization: NTB, BMM. Developing methods: NTB, BMM. Data analysis: NTB, BMM. Preparation of figures and tables: NTB. Conducting the research, data interpretation, writing: NTB, BMM, FJR. All authors contributed critically to the drafts and gave final approval for publication.

**Data accessibility statement**: The data and code supporting the results will be archived on GitHub and in the Zenodo public repository.

**Conflict of Interests**: None to declare.

**Abstract (200 max for Hydrobiologia)**

Nitrogen stable isotope ratios (δ15N) are widely used to quantify trophic position in aquatic ecosystems. Comparing trophic position across space requires identifying baselines to account for large variation in δ15N signatures of basal resources, but few standardized methods exist for identifying suitable baselines. We evaluated a standardized method for identifying optimal isotopic baselines in streams spanning the Rocky Mountains–Great Plains ecotone. We assessed candidate taxonomic groups and functional feeding groups following four criteria: 1) organisms should be easy to collect and widely distributed, 2) within-site δ15N variation should be low, 3) δ15Nvalues should be correlated with geographic variability in δ15N values, and 4) trophic position of consumers calculated from the baseline should be independent of system-specific δ15N variability when there is no change in diet. Simuliidae (obligate, sestonic filter feeders) met all four criteria. Trophic position estimates using Simuliidae baselines for five fish species were consistent with dietary changes across space. The four-criteria screening method is suitable for temperate streams in North America, and support the recommendation to use Simuliidae or filter feeders as baseline organisms for stable isotope studies quantifying fish trophic position.

**Keywords**: stable isotope analysis, trophic level, isotopic baselines, food chain, food web

**Introduction**

One major goal in ecology is to understand how trophic relationships vary over space and time, which is often done by quantifying an organism’s trophic position on a continuous scale. Stable isotope analysis is widely used to assess trophic position (Post 2002, Martínez del Rio et al. 2009). In particular, the ratio of stable isotopes of nitrogen (15N:14N, δ15N) is used to estimate trophic position (TP) of consumer organisms because of changes in the proportion of light to heavy nitrogen isotopes during trophic transfers from one trophic level to the next (DeNiro and Epstein 1981, Minagawa and Wada 1984). This difference in isotope transfer results in consumers having tissues enriched with the heavy isotope relative to their food resources. Nitrogen isotope analysis can be used to quantify shifts in trophic structure from species invasions (Vander Zanden and Rasmussen 1999, Hickerson et al. 2019, Kirk et al. 2022) or to understand contaminant exposure or biomagnification (McHuron et al. 2018, Lepak et al. 2019). However, in aquatic ecosystems, large spatial variability in nitrogen isotope ratios at the base of local food webs can make comparisons of TP among sites or along environmental gradients problematic (Anderson and Cabana 2005, Barnes et al. 2008).

Variability in δ15N values arises from natural (e.g., in situ denitrification and nitrogen fixation) and anthropogenic (i.e., land use) processes (Chappuis et al. 2017). Agricultural runoff, sewage effluent, or livestock manure increase denitrified components of nitrogen in the system through bacterial decomposition. During decomposition, bacteria preferentially assimilate 14N over 15N which results in elevated δ15N values that enter the system and propagate up the food web (Di Lascio et al. 2013). In the Saint Lawrence lowlands in Quebec, Canada, δ15N values of primary consumers increased up to 15 ‰ with regional nitrogen loading from synthetic fertilizers and manure from pig, chicken and cattle operations (Anderson and Cabana 2005). In contrast, watersheds in the northeastern United States dominated by natural forest cover exhibit variability in δ15N of primary consumers on the order of 2 ‰ (Mayer et al. 2002). In streams in northeastern Spain, δ15N values of basal resources are highest in human-impacted mainstem locations (Pastor et al. 2013), and anthropogenic land-use practices have been shown to increase δ15N values in lacustrine systems in Rhode Island (Lake et al. 2001) and boreal streams in south-central Sweden (Bergfur et al. 2009). Accordingly, comparing TP estimates without correcting for these baseline differences in δ15N values among locations can lead to erroneous inferences of food web structure and trophic relationships.

To account for the effects of geographic variation in isotope levels, TP is typically estimated relative to an isotope “baseline” (i.e., material representing geographic variation) using simple formula transformations or statistical analysis (Kjeldgaard et al. 2021). These approaches generally use a proxy organism as a baseline to correct for background δ15N variation across space (Cabana and Rasmussen 1996, Post 2002, Jardine et al. 2014, Kristensen et al. 2016). While this proxy organism approach has been applied in food web studies, it requires careful consideration before a proxy baseline is selected. Ideally, researchers should collect all potential basal primary resources at a site (i.e., suspended and benthic organic matter, biofilms, filamentous algae, macrophytes, and riparian vegetation in aquatic ecosystems) multiple times during a given time interval (e.g., three times over the growing season) and use the average δ15N values of the primary producers as a baseline (Vinagre et al. 2008, Govender et al. 2011). However, collecting primary producers in streams is arduous and expensive because it requires many samples over many dates to adequately characterize baseline variation. Therefore, long-lived primary consumers such as bivalves, with tissue turnover rates closer to higher level consumers are more appropriate than primary producers (Vander Zanden and Rasmussen 2001, Jardine et al. 2014). Bivalves also exhibit a uniform, specialized filter feeding strategy that can reduce additional variation from omnivorous feeding habits. However, bivalves and similar long-lived primary consumers can be sparsely distributed in temperate stream ecosystems, rendering them impractical as baseline proxies.

Only two studies evaluated standardized approaches for selecting primary consumer taxonomic groups as baselines in temperate streams where long-lived bivalves are not widely distributed (Anderson & Cabana 2007, Kristensen et al. 2016). Both standardized approaches have limitations, and the selected baselines have not been compared to find consensus between regions. Anderson & Cabana, (2007) recommended that baselines be broadly distributed and use the same basal resources across sites (i.e., low omnivory). Kristensen et al. (2016) included two additional criteria: the baseline’s δ15N values must track δ15N variation, and the baseline should remove the influence of δ15N variation on TP estimates for common higher order consumers (i.e., fish). One limitation was that Anderson & Cabanana (2007) used low mean δ15N values as the metric for low omnivory (i.e., using the same basal resource across locations). But there is a large range of δ15N values within basal resource compartments. Taxonomic groups that feed on a single resource with high δ15N values would meet the low omnivory criteria but might not be considered as baseline proxies because they have high δ15N signatures relative to omnivorous taxonomic groups that feed on a variety of resources with low δ15N values. Alternatively, Kristensen et al. (2016) used the mean coefficient of variation (CV) of taxonomic groups, which likely better represents omnivory. However, when determining if baselines track systematic δ15N variation in a system, Kristensen et al. (2016) correlated δ15N only to land-use (i.e., a gradient form natural land use to human land uses) which may ignore other sources of geographic variation in δ15N values. Additionally, when checking if baselines accounted for δ15N variation on TP estimates of common higher order consumers, Kristensen et al. (2016) assumed that TP should not change along the environmental gradient, which may not be valid for consumers that exhibit diet shifts along geographic gradients. Updated criteria and regional comparisons are needed to develop a consensus for determining optimal baseline proxies in food web studies.

Here, we build on the standardized method proposed by Kristensen et al. (2016), select a suitable baseline in streams that span the Rocky Mountain—Great Plains ecotone in western North America, and compare our selected baselines to those found in other regions. To test if candidate baseline proxies tracked δ15N spatial variation, we used a synthetic variable that includes several potential sources of δ15N variation including land-use changes along an ecotone. We also performed a separate stomach content analysis to inform our expectation of how TP of common higher order consumers might change if these consumers exhibit systematic changes in diet composition. We also compare our baselines to those in Danish Lowland streams (Kristensen et al. 2016) to look for consensus between regions. Improving standardized methods for finding baselines that are applicable across regions should help future studies better identify and understand drivers of trophic structure across space and time.

**Methods**

*Study system and site selection*

We sampled sixteen 2nd-5th order stream sites distributed along the Rocky Mountain—Great Plains ecotone. Sites spanned the longitudinal (i.e., upstream-downstream) gradient of the three primary tributaries to the North Platte River in Wyoming, USA (Figure 1). The Sweetwater, Medicine Bow, and Laramie rivers are snowmelt dominated with peak flows occurring in the spring and base flows occurring by mid-summer. Along these tributaries, sites were selected to represent an environmental gradient based on elevation and land-use that resulted in a wide variation of δ15N values at the base of local food webs. Sites located at high elevation were dominated by natural forested land at high elevations, and sites at low elevation were dominated by agricultural with some urbanization. These streams vary in land-use characteristics moving from upstream headwaters to downstream reaches, making them an opportune system to identify a widely usable and reliable baseline indicator.

*Longitudinal gradient and land use index variable*

To account for all sources of background variability in δ15N values, we used a synthetic variable for a site’s location along the longitudinal stream gradient (Maitland & Rahel, 2023). Previous studies used percent natural land cover alone to compare δ15N values among potential baselines. Changes in natural land cover can track elevated δ15N values caused by agriculture and urbanization (Kristensen et al. 2016) but these are not the only sources of background variation in δ15N values. Geographic context also drives background variability in δ15N values. Our synthetic variable combined seven metrics of environmental conditions at a study site into an index of longitudinal stream position using the Axis 1 site score from a principal component analysis (PCA). Input variables to the PCA were elevation (m a.s.l.), stream slope (km/km), distance to North Platte River (km), Strahler stream order, mean August water temperature (℃), upstream drainage area (km2), and stream width (m). We recorded hourly water temperature at each reach from June to September each year with loggers (Onset Computer Corporation, Bourne, Massachusetts) to quantify mean August water temperature (August, ℃). Stream slope (km/km), upstream drainage area (km2), and downstream distance to the North Platte River (km) were estimated using ArcGIS. Channel width (m) was measured at 10 equally spaced transects at each reach in 2016The longitudinal gradient index (PC1) explained 78% of site-level variation among the 16 study sites and described changes in environmental conditions from upstream, high-elevation, cold-water streams to downstream, low-elevation, warm-water streams. Upstream sites were more likely to be surrounded by natural land cover (e.g. forest, grassland, shrub land, and wetland land types (Maitland & Rahel, 2023). In a previous study, PC1 was positively correlated with δ15N values of basal resources and primary consumers (r = -0.32; Maitland & Rahel, 2023), consistent with previous research (Cabana and Rasmussen 1996, Lake et al. 2001, Kristensen et al. 2016). We used PC1 score as a measure of local variation in δ15N values for all future analyses.

*Sample collection and preparation*

We sampled basal resources, macroinvertebrates, and fishes during the summer of 2016. Basal resources and macroinvertebrates were sampled once in June, July, and August to account for fine-scale temporal variation in δ15N (Jardine et al. 2014), but fishes were only sampled in the last sampling event in August. Seston was collected by filtering three replicates of up to 10 L of water onto pre-combusted (550 °C, 4 hours) filters (Whatman GF/F, 47 mm O) in the field using a modified portable drill pump (Kelso and Baker 2016). Biofilm was collected by scraping five cobble-sized rocks within the reach. Nine replicates of fine benthic organic matter (FBOM) were collected using a food baster by sucking material off the stream bed from randomly selected pools at each site. Filamentous algae was collected by hand. To collect a representative sample of the macroinvertebrate assemblage, we used a D-framed kick net in a variety of habitats (i.e., riffle, pools, macrophytes, and river margins). Macroinvertebrates were kept in filtered stream water for 24 hours to allow for gut clearance, then frozen. We collected fish using a backpack electrofishing unit (Smith-Root, Vancouver, WA) to obtain muscle tissue samples for stable isotope analysis. To minimize the number of fish euthanized, a muscle plug using a 5-mm biopsy punch was taken from fish greater than 300 mm in length. The stomach contents of fish greater than 300 mm were collected using pulsed gastric irrigation (Light et al. 1983), preserved in individual containers with 95% ethanol, and transported to the laboratory for identification. The remaining fish used for stable isotope analysis or stomach content analysis were euthanized with a lethal dose of MS-222, placed on ice and then frozen upon returning to the lab. Fish larger than 300 mm in length were returned to the stream. Macroinvertebrate and fish samples were then processed in the lab.

We characterized macroinvertebrates into feeding groups for statistical analyses, but processed macroinvertebrates for stable isotope analysis at the taxonomic level (here after referred to as ‘taxonomic group’), The taxonomic groups were identified to family level using an invertebrate guide (i.e., taxonomic groups, Merritt et al. 2008), and we characterized each taxonomic group into feeding groups using a trait-based method. Because larvae and adults have different feeding behaviors, Elmidae were separated by life stage during identification. We obtained invertebrate trophic trait data from the USA Freshwater Biological Traits Database (Vieira et al. 2006), and then assigned trait affinity scores from 0 (absent) to 3 (strong affinity) using “fuzzy-coding” to each feeding group (Chevene et al. 1994, Maitland 2020). For stable isotope analysis, we used individuals from each taxonomic group whenever possible but for small taxa we pooled individuals to ensure enough material for stable isotope analysis.

In addition to the muscle plugs from the field, we obtain muscle fillets from the euthanized fish in the lab, but we only used tissue samples from the widely distributed fish species for statistical analysis. We removed muscle filets and the stomachs from each fish. Muscle filets were removed from the anterior dorsal portion of each fish prior to stable isotope analysis. Brown Trout *Salmo trutta* (Linnaeus, 1758), Creek Chub *Semotilus atromaculatus* (Mitchill, 1818), Longnose Dace *Rhinichthys cataractae* (Valenciennes, 1842), Longnose Sucker *Catostomus catostomus* (Forster, 1773) and White Sucker *Catostomus commersonii* (Lacépède, 1803) were used because these species were found at greater than 50% of the sites.

For stable isotope analysis, samples were oven dried (60 °C, 48 hours), ground into a homogenous powder, weighed to the nearest 0.001 mg, then sent off to obtain isotopic values. Animal samples (~1.0 mg) and basal resources (~2.0 mg) were weighed into 8 x 5-mm tin capsules and analyzed for δ15N, δ13C, %C, %N and C:N ratio at the University of Wyoming Stable Isotope Facility using a Delta Plus XP Continuous Flow Stable Isotope Ratio Mass Spectrometer (Thermo Finnigan, Bremen, Germany) coupled to a Costech Analytical 4010 elemental analyzer. Nitrogen (15N:14N) isotope ratios (R) were estimated relative to atmospheric nitrogen. Working internal standards were run as controls throughout the analyses to ensure accurate measurements. Isotopic values are reported in per mill delta notation (Eq. 1):

where δ1 is 15N, and R is the ratio of the heavy to lighter isotope. Analytical error (i.e., 1 SD of lab standard) of sample runs was estimated at 0.18‰ (δ15N) for the invertebrates, and 0.1‰ (δ15N) for fish.

*Data analysis*

Suitability of various taxonomic groups and feeding groups as baselines were based on the four updated criteria: 1) organisms should be easy to collect and widely distributed, 2) within-site δ15N variation should be low, 3) δ15Nvalues should be correlated with geographic variability in δ15N values, and 4) trophic position of consumers calculated from the baseline should be independent of system-specific δ15N variability when there is no change in food consumption. Although all analyses were performed for all taxonomic and feeding groups, selection occurred progressively from each criterion. For example, if a taxonomic group met the first criterion, it would stay in the list of potential baselines and be evaluated for the second criterion, but if it did not meet the first criterion then it would be removed from the list. A common method for baselines corrections is to use an average of all basal resources, so we also evaluated if using basal resources would meet these criteria.

The geographic distribution was expressed as the percentage of the 16 sites that contained a given taxonomic or feeding group. We used a cut off value of 75% of sites in the selection process as a reasonable value for identifying a taxonomic or feeding group with a wide distribution. Although 75% was chosen arbitrarily, Kristensen et al. (2016) included two taxa that would fall below this mark, and the optimal baseline identified by Kristensen et al. (2016) was found at 78.9% of sites. This makes our cutoff a more conservative measure of distribution while retaining the distribution levels of the best baselines in other studies. Taxonomic or feeding groups present at greater than 75% of sites were considered widely distributed.

We calculated the mean coefficient of variation (CV) of δ15N values for each taxonomic and feeding group across sites. We calculated CV at the site level by dividing the standard deviation of δ15N signatures by the mean δ15N signature of each taxonomic or feeding group at each site. We then found the mean and 95% confidence intervals for each taxonomic and feeding groups across sites. A low coefficient of variation for δ15N indicates that a baseline candidate has a non-omnivorous diet.

We used one-way analysis of variance (ANOVA) to test for differences in CV values among taxonomic or feeding groups. Separate analyses were done on taxonomic and feeding groups. The mean CV values were square root or log transformed to ensure normality. We used the Tukey HSD post hoc tests for pairwise-comparison using an alpha of 0.05. The taxonomic or feeding groups with the lowest mean CV relative to the other groups were considered suitable baselines.

For taxonomic and feeding groups, we used least squares linear regression to assess relationships between each taxonomic or feeding group’s δ15N position at sites along the longitudinal stream gradient (PC1 score). We wished to compare each group’s relationships to the longitudinal gradient with those of the basal resources and fishes. Therefore, we did separate least squares linear regressions for four of the basal resources at our sites and the five widely distributed fish species. Taxonomic and feeding groups with significant (*P* ≤ 0.05) relationships with the longitudinal gradient were considered to sufficiently track background δ15N variation.

We compared uncorrected TP of the five fish species to the TP estimates of the fish species after correction by various taxonomic and feeding groups. We calculated trophic positions for Brown Trout, Creek Chub, Longnose Dace, Longnose Sucker, and White Sucker using each taxonomic or feeing group as baselines using the following equation (Post, 2002):

where TPcorrected is the corrected trophic position of each individual fish at a site, δ15Nconsumer is the δ15N signature of each individual fish, δ15Nbaseline is the mean δ15N signature of the taxonomic or feeding group at each site, Δ15N is the discrimination factor for each trophic transfer, and TLbaseline is the expected trophic level of the baseline. To assess the difference between corrected TP estimates and uncorrected TP estimates of Brown Trout, Creek Chub, Longnose Dace, Longnose Sucker and White Sucker, we modified the above equation by removing the baseline correction. The resulting formula was:

where TPuncorrected is the uncorrected of each individual fish at a site, δ15Nconsumer is the δ15N signature of each individual fish, and Δ15N is the discrimination factor for each trophic transfer. Discrimination factors were estimated using the methods of Caut et al. (2009).

To inform our expectations of how TP might change across the environmental gradient, we looked for changes in diet composition for Brown Trout, Creek Chub, Longnose Dace, Longnose Sucker, and White Sucker by using stomach content analysis. The diet items from each stomach sample were split into seven groups: algae, amphibians, aquatic invertebrates, terrestrial invertebrates, fish, crayfish, and debris. The stomach content groups were counted and weighed. To assess the relative importance of the diet items in the stomachs, we used an index of relative importance (%IRI). The index of relative importance considers the proportional number of diet items, the proportional weight of the items, and the frequency of occurrence of the items in diets (Cortés 1997). We then used analysis of covariance (ANCOVA), to identify if fish changed their diet composition along the environmental gradient.

We compared the TP estimates of the fish species corrected by each taxonomic and feeding group to the uncorrected TP estimates to assess if each taxonomic or feeding group removed the influence of background variability. We regressed the uncorrected TP estimates and the TP of each fish species corrected by each taxonomic/feeding group across the longitudinal gradient to obtain the effect of the longitudinal gradient on TP estimates (slope or β1). If the slopes of the corrected TP estimates were lower than the slopes of the uncorrected TP estimates, then we inferred that environmental variability was removed. We then used our stomach content data to infer if fish diet composition changed along the gradient. If fish consumed more fish and crayfish along the environmental gradient, then we expected that TP should increase along the gradient even after baseline correction (i.e., β1 > 1). If there were no changes in consumption of the different diet items, then we expected TP to not change along the environmental gradient (i.e., β1 = 0). Taxonomic or feeding groups that removed background variability and gave TP conditions consistent with stomach content changes were considered suitable baselines.

**Results**

*Criteria 1: Geographic Distribution*

Of the 21 taxonomic groups and 6 feeding groups, 8 taxonomic groups and 5 feeding groups were found at greater than 75% of sites (Criterion 1; Figure 2). Chironomidae, Elmidae-larvae, Elmidae-adult, Heptaganeidae, and Hydropyschidae were the most common taxa and were found at all sites (Figure 2). Perlidae was the least common taxon and was found at six sites. Collectors, Grazers, Filterers, Omnivores and Predators were the most common feeding groups and were found at all sites. Shredders were only found at 63% of sites. Thirteen baselines (8 taxonomic groups, 5 feeding groups) were considered sufficiently distributed and used in further analysis.

*Criteria 2: Low Mean Coefficient of Variation*

Mean CV varied by taxonomic group (ANOVA; F7,103 = 6.748, *P* < 0.001; Figure 2). Simuliidae had the smallest CV across sites and Elmidae-adult had the largest (Figure 2). Simuliidae were 1.57 CV units lower than Elmidae-adult, 1.59 CV units lower than Elmidae-larvae, 1.47 CV units lower than Chironimidae, 1.27 CV units lower than Dytiscidae, 1.20 CV units lower than Baetidae, and, although marginally significant, 1.01 CV units lower than Hydropyschidae (Tukey HSD test compared to Simuliidae CV; *P* < 0.001 for Elmidae-adult; *P* < 0.001 for Elmidae-larvae, *P* = 0.001 for Chironomidae; *P* = 0.007 for Dytiscidae, *P* = 0.013 for Baetidae, *P* = 0.070 for Hydropyschidae; Figure 2). The CV for Simuliidae was not different from Heptaganeidae (Tukey HSD test, *P* = 0.999). Overall, Simuliidae and Heptaganeidae had the lowest mean CV values relative to the other taxonomic groups.

Mean coefficient of variation also varied by feeding group, (ANOVA; F4,71 = 21.52, *P* < 0.001; Figure 2). Filterers had the smallest CV across sites and Collectors had the largest. The CV for Filterers was lower than all other feeding groups (ΔCV units ≥ 1.16; Tukey HSD tests; P < 0.001; Figure 2). Overall, filterers had the lowest mean CV relative to the other feeding groups.

*Criteria 3: Correlation with Environmental δ15N*

We evaluated how δ15N signatures for the candidate baseline groups and fishes changed along the environmental gradient. All basal resources (i.e., biofilm, seston, filamentous algae, and FBOM) had δ15N signatures that increased with the longitudinal stream gradient (PC1), though the increase was only marginally significant for filamentous algae (R2 ≥ 0.105, *P* ≤ 0.068; Figure 3). All eight of the well distributed taxonomic groups and all five feeding groups had δ15N signatures that increased with PC1 (R2 ≥ 0.476; *P* < 0.001 Figure 3). δ15N signatures increased with PC1 for all fish species (OLS regression, R2 ≥ 0.092; *P* < 0.001 Figure 3).

*Criteria 4: Trophic Position Estimates Independent of Environmental δ15N*

Of the five widely distributed fishes, only Brown Trout showed a statistically significant diet shift along the environmental gradient, decreasing their consumption of benthic invertebrates and increasing their consumption of crayfish downstream (Figure 4). Creek chub appeared to shift in diet along the gradient but the change was insignificant (P > 0.05). Longnose Dace relied on benthic invertebrates and rarely consumed other food groups (Figure 4). White Sucker and Longnose Sucker mostly relied on benthic invertebrates, algae, and debris across the gradient (Figure 4). Based on these results, after correction of TP by each taxonomic group, brown trout should still have an increasing slope, while the other species should have exhibit no change.

Uncorrected TP estimates for all five fish species were positively correlated with the longitudinal gradient (Figure 5). These positive correlations emphasize the need for baseline correction.

For Brown Trout, the effect of the longitudinal gradient on TP was generally reduced (i.e., decreased slope) when baseline corrections were done using either taxonomic groups (Figure 5A,B) or functional feeding groups (Figure 6 A,B). However, the relationship of TP and PC1 became statistically insignificant or was even reversed when correcting by the taxonomic groups Heptaganeidae and Chironomidae, respectively (Figure 5A,B).

For Creek Chub, the effect of the longitudinal gradient on TP was generally reduced (i.e., decreased slope) when baseline corrections were done using either taxonomic groups (Figure 5 C,D) or functional feeding groups (Figure 6 C,D). However, the positive correlation between TP and PC1 remained when correcting by the taxonomic groups Baetidae, Heptaganeidae and Chironomidae, but was reversed when correcting by the taxonomic group Dytiscidae (Figure 5C,D). Also, the positive correlation between TP and PC1 remained when correcting by the feeding group Grazers, but was reversed when correcting by the feeding group Predators (Figure 6 C,D). When correcting by basal resources, positive correlations between TP and PC1 remained when correcting by the basal resource compartments biofilm, filamentous, FBOM and bulk basal resources (Figure 5C,D; Figure 6 C,D).

For Longnose Dace, the correlation between TP and the longitudinal gradient was removed after correction for most of the taxonomic groups and functional feeding groups (Figure 5 E,F, Figure 6 E,F). The correlation between TP and PC1 was reversed when correcting by the taxonomic Chironomidae. When correcting by basal resources, the correlation between TP and PC1 remained after correcting by the basal resource compartments filamentous and FBOM (Figure 5 E,F, Figure 6 E,F).

For Longnose Sucker, the correlation between TP and the longitudinal gradient was reduced or became insignificant for most taxonomic groups and functional feeding groups (Figure 5 G,H, Figure 6 G,H). However, the positive correlation between TP and PC1 remained when correcting by the taxonomic group Dytiscidae (Figure 5G,H). When correcting by basal resources, the correlation between TP and PC1 remained for all basal resource compartments and for bulk basal resources (Figure 5 G,H, Figure 6 G,H).

For White Sucker, the correlation between TP and the longitudinal gradient was reduced or became insignificant after correction by taxonomic groups and functional feeding groups (Figure 5 I,J; Figure 6 I,J). However, the positive correlation between TP and PC1 was reversed when correcting by the taxonomic groups Elmidae-larvae and Dytiscidae (Figure 5 I,J). Also, the positive correlation between TP and PC1 was reversed when correcting by the feeding groups Omnivores, Collectors and Predators (Figure 6 I,J). When correcting by basal resources, the correlations between TP and PC1 remained for the basal resource compartments filamentous, FBOM, and bulk basal resources (Figure 5 I,J; Figure 6 I,J).

**Discussion**

Obtaining consensus about suitable baseline indicators that account for geographic variation in δ15N values is a key step toward improving the use of stable isotope in aquatic ecosystems. We evaluated the effectiveness of a standardized method for identifying optimal isotopic baselines previously developed for lowland temperate streams (Kristensen et al. 2016) by applying it to stream systems spanning the Rocky Mountains–Great Plains ecotone. The four screening criteria appear useful for selecting a suitable baseline for estimating trophic positions of consumer organisms. Simuliidae emerged as the taxonomic group that met all four of the criteria in our system and in Danish lowland streams (Kristensen et al., 2016).

Contrary to many studies that use aquatic herbivores as baselines (Kjeldgaard et al., 2021), the standardized method we evaluated indicated that some filterers such as Simuliidae, may also serve as a reliable baseline when sufficiently distributed in stream ecosystems.

*Assessment of Criteria*

The distribution of aquatic macroinvertebrates poses a limitation when selecting suitable baselines. Widely distributed baselines are preferred to ensure that baselines cover the geographic extent of the study. But sixty-two percent of the taxonomic groups found within our region were not present at 75% of our sites. Similarly, 87% of taxonomic groups in Danish Lowland streams were not sufficiently distributed to be useful for baseline corrections (Kristensen et al. 2016). Although frustrating for selecting baselines, typical metacommunities consist of many locally distributed taxonomic groups and few widely distributed taxonomic groups (REF).

Combining taxonomic groups into feeding groups can increase the spatial coverage of a suitable baseline, but consideration is needed when choosing how aggregate. Often, studies have used bulk primary consumers or bulk basal resources as baselines to increase spatial coverage, but our synthesis indicates that bulked groups do not reliably remove background variability of δ15N on TP estimates (Table 1). This may be because taxonomic groups may include species with different feeding ecologies that change in abundance along environmental gradients. We found that basal resources responded differently to the geographic variations, which also may explain why bulk basal resources are unreliable as well (Figure 3).

TOPIC SENTENCE – larger continuum To increase spatial coverage, feeding groups present a promising alternative to taxonomic groups (Table 1). Increases in spatial coverage will likely decrease with even larger study regions because the classic River Continuum Concept (RCC) indicates that feeding ecologies change predictably along the longitudinal gradient based upon the relative amount and type of autochthonous or allochthonous resources (Vannote et al. 1980). Indeed, at our regional scale, we found that Shredders were insufficiently distributed because they were primarily located in the upper reaches of our region where riparian vegetation cover was highest. In the St. Lawrence watershed, the feeding groups Filterers and Shredders were also removed from consideration as baselines for TP estimation because of low spatial distribution (Anderson and Cabana 2007). Without grouping methods that do not affect TP estimation, distribution will remain a major limitation when selecting suitable baselines.

Also bivalves bad distribution

For suitable baselines to track background variation in δ15N, taxonomic groups should exhibit low omnivory which can be estimated by measuring mean within site variation in δ15N. In our study, Simuliidae had the lowest mean within site variability in δ15N relative to other taxonomic groups indicating it had the lowest degree of omnivory. Exploring low CV relative to other taxonomic or feeding groups appears to be an effective criterion when identifying suitable baselines of background variation in δ15N signatures.

The δ15N signatures for all macroinvertebrate groups were all correlated with the environmental gradient which is consistent with results from Danish Lowland streams (Kristensen et al. 2016). This criterion was not used when selecting suitable baselines of background variation of δ15N in the St. Lawrence watershed (Anderson and Cabana 2007). Correlations of δ15N signatures with the environmental gradient affirm that the taxonomic or feeding groups are tracking background variation in δ15N. Tracking appears to be relatively common even among basal resource compartments and fishes (Table 1, Figure 3). Such tracking of environmental variation in δ15N values confirms the need for baseline corrections when assessing trophic positioning in aquatic food web studies.

Finally, assessing if baselines give TP estimates independent of environmental influence for higher order consumers (i.e., fishes) serves as a crucial check that the baseline is working as intended. But interpretation of “independence of the environmental gradient” is subjective to how TP is (or is not) expected to change with the environmental gradient. We echo the call by (Kjeldgaard et al. 2021) for the need of independent information regarding dietary composition to inform these expectations. Previous methods for selecting suitable baselines have either not used this criterion (Anderson and Cabana 2007) or have used mixing models to assess if diets changed along the environmental gradient which are still dependent on stable isotopes (Kristensen et al. 2016). Based on stable isotope data, brown trout exhibited substantial shifts in resource use along the environmental gradient even after baseline corrections using most of the taxonomic groups and functional feeding groups. Stomach content analysis, which is independent of stable isotope information, corroborated this shift in resource use, indicating the shift was not an artifact of environmental variation in δ15N along the longitudinal stream gradient.

*Optimal Baselines*

A recent review found that most studies in aquatic systems that calculate TP using stable isotopes use herbivores (e.g., Grazers) as baselines (Kjeldgaard et al. 2021). In our study, the grazer feeding group would be considered a suitable baseline for four of the five fish species (the exception being creek chub, Fig. 6B). But the grazer feeding group was not as effective a baseline as Simuliidae in our system and in Danish Lowland streams (Kristensen et al. 2016). Simuliidae (detritivores/filterers) fit all four criteria for optimal baselines (Table 1) and in both stream systems removed or greatly reduced the effect of environmental variation in δ15N on TP estimates of fishes. The prevalence of using herbivores in the literature may be a result of previous work that selected Phesonid grazing snails as optimal because they exhibited the lowest within site mean δ15N as an indicator of low omnivory (Anderson and Cabana 2007). But grazing snails were not well distributed in our study system, occurring at only 5 of 16 study sites. Our results coupled with those in Danish Lowland streams indicate that taxonomic groups that exhibit filter feeding, such as Simuliidae, may be particularly good baseline indicators (Kristensen et al. 2016). Another filter feeding group sometimes considered an optimal baseline are long-lived bivalves with longer tissue turnover rates (Jardine et al. 2014). However, as with snails, bivalves did not meet the criteria for wide distribution as they occurred in only x of our 16 sites. Filterers may serve as good baselines because their main food source, seston, was the best basal resource compartment for removing the influence of the environmental gradient on TP of fishes (Table 1).

Although Simuliidae fit all four criteria, Hydropyschidae, the third taxonomic group with the lowest CV, effectively removed the influence of the longitudinal gradient on the TP of the five fishes in our study (Table 1). Successful removal of the influence of the longitudinal gradient on TP suggests that Hydropsychidae could be an effective baseline indicator even though it had higher within site CV values than Simuliidae. Hydropsychidae were categorized as omnivores in our study, but some species exhibit filter feeding strategies (Ficsor & Csabai, 2021). Even though Hydropyschidae appears to effectively remove the influence of the longitudinal gradient on TP in our system, we advise caution when using Hydropyschidae in other systems.

*Conclusions*

Studies calculating TP using stable isotopes have used 10 major groups of baselines and 8 different methods (Kjeldgaard et al. 2021)*.* More work on standardizing procedures will reduce variation in choices of baselines and methodology that complicates across-system syntheses of TP. Kjeldgaard et al. (2021) recommended that the first decision when calculating TP should be the selection of an appropriate baseline. The four criteria proposed by (Kristensen et al. 2016) combined with our adjustments (i.e., assessments along geographic not just land-use gradients, and evaluation of fish diet composition using stomach contents) present a promising standardized approach for selecting a suitable baseline. Using this approach, we found evidence that the taxonomic group Simuliidae (Filterers) may be an adequate baseline for temperate streams where other macroinvertebrate baseline candidates are not widely distributed. Additional studies in other regions are needed to further validate the use of this approach.

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**References**

Anderson, C., and G. Cabana. 2005. N in riverine food webs: effects of N inputs from agricultural watersheds. Canadian Journal of Fisheries and Aquatic Sciences 62:333–340.

Anderson, C., and G. Cabana. 2007. Estimating the trophic position of aquatic consumers in river food webs using stable nitrogen isotopes. Journal of the North American Benthological Society 26:273–285.

Barnes, C., S. Jennings, N. V. Polunin, and J. E. Lancaster. 2008. The importance of quantifying inherent variability when interpreting stable isotope field data. Oecologia 155:227–235.

Bergfur, J., R. K. Johnson, L. Sandin, and W. Goedkoop. 2009. Effects of nutrient enrichment on C and N stable isotope ratios of invertebrates, fish and their food resources in boreal streams. Hydrobiologia 628:67–79.

Cabana, G., and J. B. Rasmussen. 1996. Comparison of aquatic food chains using nitrogen isotopes. Proceedings of the National Academy of Sciences of the United States of America 93:10844–10847.

Chappuis, E., V. Seriñá, E. Martí, E. Ballesteros, and E. Gacia. 2017. Decrypting stable-isotope (δ13C and δ15N) variability in aquatic plants. Freshwater Biology:1–12.

Chevene, F., S. Dolédec, and D. Chessel. 1994. A fuzzy coding approach for the analysis of long‐term ecological data. Freshwater Biology 31:295–309.

Cortés, E. 1997. A critical review of methods of studying fish feeding based on analysis of stomach contents: application to elasmobranch fishes. Canadian Journal of Fisheries and Aquatic Sciences 54:726–738.

DeNiro, M. J., and S. Epstein. 1981. Influence of diet on the distribution of nitrogen isotopes in animals. Geochimica et cosmochimica acta 45:341–351.

Di Lascio, A., L. Rossi, P. Carlino, E. Calizza, D. Rossi, and M. L. Costantini. 2013. Stable isotope variation in macroinvertebrates indicates anthropogenic disturbance along an urban stretch of the river Tiber (Rome, Italy). Ecological Indicators 28:107–114.

Govender, N., A. J. Smit, and R. Perissinotto. 2011. Trophic functioning of the St. Lucia estuarine lake during a drought phase assessed using stable isotopes. Estuarine, Coastal and Shelf Science 93:87–97.

Hickerson, B. T., B. M. Maitland, and A. W. Walters. 2019. Effects of multiple nonnative fish on an imperiled cyprinid, Hornyhead Chub *Nocomis biguttatus*. Transactions of the American Fisheries Society 148:1132–1145.

Jardine, T. D., W. L. Hadwen, S. K. Hamilton, S. Hladyz, S. M. Mitrovic, K. A. Kidd, W. Y. Tsoi, M. Spears, D. P. Westhorpe, and V. M. Fry. 2014. Understanding and overcoming baseline isotopic variability in running waters. River Research and Applications 30:155–165.

Kelso, J. E., and M. A. Baker. 2016. Filtering with a drill pump: An efficient method to collect suspended sediment. Journal of the American Water Resources Association 52:262–268.

Kirk, M. A., B. M. Maitland, B. T. Hickerson, A. W. Walters, and F. J. Rahel. 2022. Climatic drivers and ecological impacts of a rapid range expansion by non-native smallmouth bass. Biological Invasions 24:1311–1326.

Kjeldgaard, M. K., J. A. Hewlett, and M. D. Eubanks. 2021. Widespread variation in stable isotope trophic position estimates: patterns, causes, and potential consequences. Ecological Monographs 91.

Kristensen, P. B., T. Riis, H. E. Dylmer, E. A. Kristensen, and M. Meerhoff. 2016. Baseline identification in stable-isotope studies of temperate lotic systems and implications for calculated trophic positions. Freshwater Science 35:909–921.

Lake, J. L., R. A. McKinney, F. A. Osterman, R. J. Pruell, J. Kiddon, S. A. Ryba, and A. D. Libby. 2001. Stable nitrogen isotopes as indicators of anthropogenic activities in small freshwater systems. Canadian Journal of Fisheries and Aquatic Sciences 58:870–878.

Lepak, R. F., J. C. Hoffman, S. E. Janssen, D. P. Krabbenhoft, J. M. Ogorek, J. F. DeWild, M. T. Tate, C. L. Babiarz, R. Yin, E. W. Murphy, D. R. Engstrom, and J. P. Hurley. 2019. Mercury source changes and food web shifts alter contamination signatures of predatory fish from Lake Michigan. Proceedings of the National Academy of Sciences 116:23600–23608.

Maitland, B. M. 2020. Isotopic Ecology of Aquatic Communities Along the Rocky Mountains— Great Plains Ecotone. University of Wyoming.

Martínez del Rio, C., N. Wolf, S. A. Carleton, and L. Z. Gannes. 2009. Isotopic ecology ten years after a call for more laboratory experiments. Biological Reviews 84:91–111.

Mayer, B., E. W. Boyer, C. Goodale, N. A. Jaworski, N. Van Breemen, R. W. Howarth, S. Seitzinger, G. Billen, K. Lajtha, K. Nadelhoffer, and others. 2002. Sources of nitrate in rivers draining sixteen watersheds in the northeastern US: Isotopic constraints. Biogeochemistry 57:171–197.

McHuron, E. A., S. H. Peterson, and T. M. O’Hara. 2018. Chapter 2 - Feeding Ecology Tools to Assess Contaminant Exposure in Coastal Mammals. Pages 39–74 *in* M. C. Fossi and C. Panti, editors. Marine Mammal Ecotoxicology. Academic Press.

Merritt, R. W., K. W. Cummins, and M. B. Berg. 2008. An introduction to the aquatic insects of North America. Kendall/Hunt Publishing Company, Dubuque, Iowa.

Minagawa, M., and E. Wada. 1984. Stepwise enrichment of 15N along food chains: Further evidence and the relation between δ15N and animal age. Geochimica et Cosmochimica Acta 48:1135–1140.

Paine, R. T. 1980. Food Webs: Linkage, Interaction Strength and Community Infrastructure. Journal of Animal Ecology 49:666–685.

Pastor, A., M. Peipoch, L. Cañas, E. Chappuis, M. Ribot, E. Gacia, J. L. Riera, E. Martí, and F. Sabater. 2013. Nitrogen stable isotopes in primary uptake compartments across streams differing in nutrient availability. Environmental science & technology 47:10155–10162.

Post, D. M. 2002. The long and short of food-chain length. Trends in Ecology and Evolution 17:269–277.

Thompson, R. M., U. Brose, J. A. Dunne, R. O. Hall, S. Hladyz, R. L. Kitching, N. D. Martinez, H. Rantala, T. N. Romanuk, D. B. Stouffer, and J. M. Tylianakis. 2012. Food webs: Reconciling the structure and function of biodiversity. Trends in Ecology and Evolution 27:689–697.

Vander Zanden, M. J., and J. B. Rasmussen. 1999. Primary consumer δ13C and δ15N and the trophic position of aquatic consumers. Ecology 80:1395–1404.

Vander Zanden, M. J., and J. B. Rasmussen. 2001. Variation in δ15N and δ13C trophic fractionation: Implications for aquatic food web studies. Limnology and Oceanography 46:2061–2066.

Vannote, R. L., G. W. Minshall, K. W. Cummins, J. R. Sedell, and C. E. Cushing. 1980. The river continuum concept. Canadian Journal of Fisheries and Aquatic Sciences 37:130–137.

Vieira, N. K. M., N. L. Poff, D. M. Carlisle, I. Moulton, Stephen R., M. L. Koski, and B. C. Kondratieff. 2006. A database of lotic invertebrate traits for North America. U.S. Geological Survey Data Series 187.

Vinagre, C., J. Salgado, M. Costa, and H. Cabral. 2008. Nursery fidelity, food web interactions and primary sources of nutrition of the juveniles of Solea solea and S. senegalensis in the Tagus estuary (Portugal): a stable isotope approach. Estuarine, Coastal and Shelf Science 76:255–264.