General comments.

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

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.

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

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**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 values 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 values 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 surveyed 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 (Fig. 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.

*Environmental gradient*

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 2016. The 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 & 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 minimize the number of fish euthanized for stable isotope analysis (Maitland and Rahel 2021), 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.

We identified macroinvertebrates in the lab to the 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 sufficient sample sizes for stable isotope 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. We focus on 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) because these species are widely distributed in the region (Maitland and Rahel 2023).

In total we analyzed 1,391 invertebrate, fish, and basal resource samples which were oven dried (60 °C, 48 hours), and ground into a homogenous powder. Animal samples (~1.0 mg) and basal resources (~2.0 mg) were weighed into 8 x 5-mm tin capsules and analyzed for δ15N 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 heavier 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 dietary change. 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. Common methods for baselines corrections in the literature are to either use indivdual basal resource compartments, the average of all primary consumers, or the averages of all basal resources. We also evaluated if using composite averages of primary consumers or 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 compared differences in within-site variation (coefficient of variation = CV) of the mean δ15N values of single taxa and feeding groups with 1-way analysis of variance (ANOVA). We calculated CV as SD/mean. The mean CV values were log transformed to ensure normality. We used the Tukey HSD post hoc tests for pairwise-comparison using an alpha of 0.05.

We used least squares linear regression to assess relationships between stream compartment δ15N and trophic position values (see below) versus the longitudinal stream gradient (PC1 score). 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 dietary changes 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 values consistent with stomach content changes were considered suitable baselines.

**Results**

*Criteria 1: Geographic Distribution*

Of the 62 taxonomic groups and 6 feeding groups, 12 taxonomic groups and 5 feeding groups were found at greater than 75% of sites (Criterion 1; Fig. 2). Elmidae-larvae, Elmidae-adult, Heptaganeidae, and Hydropyschidae were the most common taxa and were found at all sites (Fig. 2). Veliidae, Seiomyzidae, Potamanthidae, Philopatamidae, Philopotamidae, Nemouriidae, Lymnaeidae, Limnephilidae, Libellilidae, Lepidostomatidae, Empididae, Corbiculidae, Capnidae, Belostomatidae, and Baetiscidae were the least common taxon and were found at one site. Collectors, Grazers, 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 (12 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; F11,152 = 7.2052, *P* < 0.001; Fig. 3). Simuliidae had the smallest CV across sites and Elmidae-adult had the largest (Fig. 3). Simuliidae had statistically lower CVs from Elmidae-adult, Elmidae-larvae, Chironomidae, Dytiscidae, Leptohyphidae, Gomphidae, Hydropyschidae, Perlidae, and Ephemeridae (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* < 0.001 for Dytiscidae, *P* < 0.001 for Leptohyphidae, *P* < 0.001 for Gomphidae, *P* < 0.001 for Perlidae, *P* = 0.001 for Ephemeridae, and *P* = 0.005 for Hydropyschidae; Fig. 3). The CV for Simuliidae was not different from Baetidae nor Heptaganeidae (Tukey HSD test, *P* = 0.114; P = 0.932, respectively). Overall, Simuliidae, Baetidae, and Heptaganeidae had the lowest mean CV values relative to the other taxonomic groups.

Mean coefficient of variation also varied by feeding group, (ANOVA; F4,72 = 24.886, *P* < 0.001; Fig. 3). Filterers had the smallest CV across sites and Collectors had the largest. The CV for Filterers was lower than all other feeding groups (Tukey HSD tests; P < 0.001; Fig. 3). Overall, filterers had the lowest mean CV relative to the other feeding groups.

*Criteria 3: Correlation with Environmental δ15N*

We evaluated how δ15N values 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 values that increased with the longitudinal stream gradient (PC1), though the increase was only marginally significant for filamentous algae (R2 ≥ 0.105, *P* ≤ 0.068; Fig. 4). All eight of the well distributed taxonomic groups and all five feeding groups had δ15N values that increased with PC1 (R2 ≥ 0.252; *P* < 0.001 Fig. 4). δ15N values increased with PC1 for all fish species (R2 ≥ 0.092; *P* < 0.001 Fig. 4).

*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, marginally decreasing their diet of benthic and terrestrial invertebrates (benthic invertebrate: R2 = 0.205, *P* = 0.059; terrestrial invertebrate: R2 = 0.197, *P* = 0.063; Fig. 5). Brown trout increased their diet of crayfish downstream (R2 = 0.362, *P* = 0.134; Fig. 5). Creek chub appeared to follow a similar pattern to brown trout but was not statistically significant (P ≥ 0.244). Longnose dace, longnose sucker, and white sucker did not change their diets along the longitudinal gradient (R2 ≤ 0.139, P ≥ 0.233). 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 exhibit no change.

Uncorrected TP estimates for all five fish species were positively correlated with the longitudinal gradient (Fig. 6). 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 (Fig. 6 A,B) or functional feeding groups (Fig, 7 A,B). However, the relationship of TP and PC1 was statistically insignificant when corrected by the taxonomic groups Heptaganeidae and Chironomidae (Fig. 6A,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 (Fig. 6 C,D) or functional feeding groups (Fig. 7 C,D). However, the positive correlation between TP and PC1 remained when correcting by the taxonomic groups Baetidae, Hydropyschidae, and Chironomidae. The relationship was reversed when correcting by the taxonomic group Dytiscidae, and Gomphidae (Fig. 6 C,D). Also, the positive correlation between TP and PC1 remained when correcting by the feeding group Grazers, Omnivores, and Averages of Basal Resources. The relationship was reversed when correcting by the feeding group Predators (Fig. 7 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 average of all basal resources (Fig. 6 C,D; Fig. 7 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 (Fig. 6 E,F, Fig. 7 E,F). The correlation between TP and PC1 was reversed when correcting by the taxonomic Chironomidae and Leptohyphidae. When correcting by basal resources, the correlation between TP and PC1 remained after correcting by the basal resource compartments filamentous, FBOM, and average of all basal resources (Fig. 6 E,F, Fig. 7 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 (Fig. 6 G,H, Fig 7 G,H). However, the positive correlation between TP and PC1 remained when correcting by the taxonomic group Dytiscidae and Gomphidae (Fig. 6G,H). When correcting by basal resources, the correlation between TP and PC1 remained for all basal resource compartments and when correcting using the average of all basal resources (Fig. 6 G,H, Fig. 7 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 (Fig. 6 I,J; Fig. 7 I,J). However, the positive correlation between TP and PC1 was reversed when correcting by the taxonomic groups Elmidae-larvae, Heptaganeidae, Gomphidae, Perlidae, Leptohyphidae, Ephemeridae, and Dytiscidae (Fig. 6 I,J). Also, the positive correlation between TP and PC1 was reversed when correcting by the feeding groups Collectors and Predators (Fig. 7 I,J). The average of all aquatic invertebrates reversed the correlation between TP and PC1 for white sucker (Fig 7). When correcting by basal resources, the correlations between TP and PC1 remained for the basal resource compartments filamentous, FBOM, and average of all basal resources (Fig. 6 I,J; Fig. 7 I,J).

*Summary*

We started with 62 candidate taxonomic groups, but only twelve taxonomic groups were present at ≥ 75% of sites (Fig. 2). Simuliidae, Baetidae, and Heptaganeidae had the lowest CVs relative to the other 9 widely distributed taxonomic groups (Fig. 3; Table 1). The δ15N values of Simulliidae, Baetidae, and Heptaganeidae were correlated with the environmental gradient (Fig. 4; Table 1). When estimating TP for the five fish species, Simuliidae was the only taxonomic group that removed or reduced the background variation associated longitudinal gradient *and* retained slopes consistent with changes in diet (Fig. 6; Table 1). Baetidae removed or reduced the background variation associated with the longitudinal gradient but did not completely remove the background variation for creek chub which did not exhibit changes in diet along the gradient (Fig.5, Fig. 6, Table 1). Heptaganeidae removed or reduced the background variation associated with the longitudinal gradient but completely removed the effect of the longitudinal gradient on brown trout which exhibited substantial changes in diet along the gradient (Fig. 5, Fig. 6, Table 1). Also, Heptaganeidae overcorrected TP estimates for white sucker resulting in a negative slope along the longitudinal gradient, but white sucker did not show any changes in diet along the longitudinal gradient (Fig. 5, Fig. 6, Table 1).

We started with 6 candidate feeding groups, and five were present at ≥ 75% of sites (Fig. 2). Filterers had the lowest CV relative to the other 4 feeding groups and had δ15N values that were correlated with the longitudinal gradient (Fig. 3; Fig. 4; Table 1). When estimating TP of the 5 fish species, Filterers also removed or reduced the background variation associated longitudinal gradient *and* retained slopes consistent with changes in diet.

To relate our data to commonly used baselines in the literature, we assessed the basal resource compartments individually, the average of the basal resource compartments, and the average of all primary consumers (Fig. 4, Fig 6, Fig. 7, Table 1). Except for filamentous algae, the δ15N values of each basal resources compartment was correlated with the longitudinal gradient (Fig. 4; Table 1). Generally, the individual basal resource compartments removed or reduced the background variation associated with the longitudinal gradient, but rarely removed the gradient to the same magnitude of the taxonomic groups (Fig. 6; Table 1). The weaker reduction capacity resulted in many TP estimates with positive slopes along the longitudinal gradient for the fish species that exhibited no change (Fig. 6; Table 1). This pattern was also seen when using the average of all the basal resources (Fig 7; Table 1). However, the TP estimates for 4 out of the 5 fish species (all but longnose dace) that were corrected by seston had slopes along the longitudinal gradient consistent with the dietary changes (Fig. 6, Table 1). Using the average of all primary consumers to correct TP estimates of fish species was generally better at removing the longitudinal gradient and was better at retaining slopes consistent with changes in diet than the average of all basal resources (Fig. 7; Table 1). However, using the average of all primary consumers to estimate TP overcorrected and produced a negative slope with the longitudinal gradient for white sucker which exhibited no change diet (Fig. 7).

**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 nineteen 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 (Rosi-Marshall et al., 2002).

Combining taxonomic groups into feeding groups can increase the spatial coverage of a suitable baseline, but consideration is needed when choosing how to aggregate. Often, studies have used averages of all primary consumers or averages of all basal resources as baselines to increase spatial coverage, but our synthesis indicates that averages of all primary consumers or basal resources 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 averages of all basal resources are unreliable as well (Fig. 4). Generally, averaging all the primary consumers did a better job at correctly reducing or removing the background variation than the average of all the basal resources (Fig 7, Table 1). But averaging all the primary consumers resulted in overcorrected TP estimates and a negative slope along the longitudinal gradient for white sucker (Fig. 7). Additionally, averaging all the primary consumers did not successfully remove the background variation on TP estimate of the two fish species analyzed in Danish Lowland Streams (Table 1; Kristensen et al., 2016). Feeding groups (particularly Filterers) present a promising alternative to averaging all the basal resources or primary consumers (Table 1). Filterers correctly reduced or removed the background variation associated with the longitudinal gradient in our study and was the only feeding group to remove the background variation for one of the fish in Danish Lowland Streams (Table 1, Kristensen et al., 2016).

Our study region was primary located in the Rocky Mountain and upper regions of the Great Plains, so in the middle to lower reaches of the Great Plains it is likely that optimal baseline will change as dominant taxonomic and feeding groups change along the larger longitudinal gradient. For example, the relative dominance of certain taxonomic groups nested within feeding groups may change. The optimal baseline in our study region, Simuliidae, is also the dominant filterer. Other filterers like Bivalves are uncommon and rare in our region (see, Corbiculidae, Fig. 2). Because of temperature and habitat preference, Simuliidae become less common and abundant at lower reaches and bivalves typically become more common and abundant. Similar things occur with feeding groups. 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 lower elevation St. Lawrence watershed, the feeding groups Filterers and Shredders were insufficiently distributed, so Grazers became important baseline (Anderson and Cabana 2007). Because optimal baselines may change further down the longitudinal gradient, further studies should identify suitable baselines for these regions.

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, Baetidae, and Heptaganeidae 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 values.

The δ15N values 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 values with the environmental gradient affirm that the taxonomic or feeding groups are tracking background variation in δ15N. Consistent with a large body of literature (Anderson & Cabana 2005, Mayer et al., 2002, Pastor et al., 2013, Lake et al., 2001, Bergfur et al., 2009), tracking background variation is common and confirms the need for baseline corrections when estimating TP.

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 Phesonidae 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 1 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).

*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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