# You are not always what we think you eat: selective assimilation across multiple whole-stream isotopic tracer studies

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*Abstract.* Analyses of 21 $^{15}$N stable isotope tracer experiments, designed to examine food web dynamics in streams around the world, indicated that the isotopic composition of food resources assimilated by primary consumers (mostly invertebrates) poorly reflected the presumed food sources. Modeling indicated that consumers assimilated only 33–50% of the N available in sampled food sources such as decomposing leaves, epilithon, and fine particulate detritus over feeding periods of weeks or more. Thus, common methods of sampling food sources consumed by animals in streams do not sufficiently reflect the pool of N they assimilate. Isotope tracer studies, combined with modeling and food separation techniques, can improve estimation of N pools in food sources that are assimilated by consumers. Food web studies that use putative food samples composed of actively cycling (more readily assimilable) and refractory (less assimilable) N fractions may draw erroneous conclusions about diets, N turnover, and trophic linkages of consumers. By extension, food web studies using stoichiometric or natural abundance approaches that rely on an accurate description of food-source composition could result in errors when an actively cycling pool that is only a fraction of the N pool in sampled food resources is not accounted for.

*Key words:* $^{15}$N; consumer; food resources; food web; label mismatch; nitrogen cycling; stable isotope tracer addition.

## Introduction

Trophic relationships and food web structure remain a central focus of ecological research. Early food webs were constructed using simple trophic links (e.g., Elton 1927), but functional representations of food webs are more often based on quantitative flows of energy and carbon (e.g., Lindeman 1942, Paine 1966, Polis and Hurd 1995, Hall et al. 2000, Cross et al. 2013) and/or biologically active elements such as nitrogen (N) and phosphorus (P; e.g., Fry 1991, Cabana and Rasmussen 1996, Mulholland et al. 2000). Variation in the natural abundance of stable isotopes and the stoichiometry of an organism's elemental composition have been widely used to examine the flow of elements in food webs and how the composition of resources and diets of consumers constrain productivity and a variety of ecological processes (e.g., Elser et al. 2000). An accurate under-

standing of what food resources are ingested and assimilated by consumers is central to quantitative food web studies. Identifying specific foods used by consumers is critical for quantifying the sources and fluxes of energy and nutrients across trophic levels, determining consumer growth efficiencies, and characterizing potential elemental imbalances between consumers and their food (Frost et al. 2005, 2006).

Gut-content analyses have long been used to quantify consumer diets, but the method has shortcomings because the temporal and spatial variability in food resources consumed requires frequent sampling (Rosi-Marshall and Wallace 2002, Wellard Kelly et al. 2013); quantification is time-consuming, especially for very small animals; detrital particles in diets are difficult to identify microscopically (e.g., amorphous detritus could be derived from leaves or algae); and assimilation efficiency varies among foods and diet proportions. Thus, estimates of assimilation based on gut-content data need to be adjusted to allow quantitative estimates of energy or material flow (Benke and Wallace 1980). While gut-content analyses can reveal functional roles of consumers associated with foraging and ingestion, additional information is required to understand which food resources are actually assimilated (e.g., Altig et al. 2007).

Variation in the natural abundances of stable isotopes (e.g., $^{13}C$, $^{15}N$, $^{34}S$, and $^2H$) has been increasingly used to characterize trophic relationships and provides an alternative (or complement) to gut-content analysis. This method relies on accurate isotopic characterization of consumers and their food resources and knowledge of trophic enrichment of the consumer relative to its diet (Layman et al. 2012). The $^{15}N$ enrichment at each trophic level generally averages 3–4‰ although it can be less (Minagawa and Wada 1984, Cabana and Rasmussen 1996, Vander Zanden and Rasmussen 1999). Therefore, N isotope ratios in consumers can indicate trophic level, an approach that offers advantages over gut-content analyses because it is logistically simpler, integrates diets over space and time, and directly reflects food that is assimilated, not simply ingested. Similarly, other isotopes have been used as food web tracers and these isotopes are often used simultaneously as natural labels to trace food web sources and material flow pathways (Peterson et al. 1985). However, natural abundance isotope approaches also have shortcomings, including uncertainties in estimating the isotopic composition of basal food web compartments that often have rapid turnover and high spatiotemporal variability; high spatial and temporal variability in isotopic content of some consumers and/or their prey; and uncertainty in the trophic enrichment of $^{15}N$ (and other isotopes) associated with specific trophic steps (e.g., Cabana and Rasmussen 1996, Evans-White et al. 2001, Martínez del Río and Anderson-Sprecher 2008, Jardine et al. 2014).

Experimental tracer additions of stable isotopes can also be used to understand trophic relationships within food webs and to estimate elemental fluxes through food web compartments (e.g., Mulholland et al. 2000, Peterson et al. 2001). During $^{15}N$ tracer additions of enriched ammonium or nitrate, stream biota, able to assimilate dissolved inorganic N directly from the water column (i.e., algae, bacteria, and fungi in biofilms), become enriched in $^{15}N$, and over time that enrichment is transferred up the food web as these primary uptake compartments are consumed. The temporal pattern of isotopic enrichment in a consumer depends on the isotopic signature of its food, its assimilation efficiency, and the N turnover time of its tissues. In the analysis of data from isotope tracer addition experiments, diet and consumer isotope ratios are normally corrected for background isotope ratios (sampled at an upstream reference site), thereby factoring out the uncertain effect of trophic enrichment on diet–consumer comparisons. Furthermore, trophic fractionation of isotopes is generally small compared to the isotope signatures created by the tracer additions.

Numerous $^{15}N$-isotope addition experiments have been conducted to examine food webs in terrestrial (e.g., Nadelhoffer et al. 1999), marine (e.g., Veuger et al. 2007), and freshwater (e.g., Mulholland et al. 2000) environments. Such studies hold promise for clarifying trophic relationships by experimentally tracking elemental fluxes through food webs. However, few isotope tracer studies have addressed trophic dynamics of food webs in detail, in part, due to difficulties in interpreting the pattern of isotopic labeling between basal food resources and consumers. This study focuses on $^{15}N$, but our conclusions can be extended to other isotopic tracers that might be employed in food web studies.

Results from the lotic intersite nitrogen experiment (LINX) and subsequent experiments based on similar $^{15}N$ tracer addition approaches, have revealed an apparent and consistent discrepancy between the tracer $^{15}N$ enrichment of basal food resources and consumers of those resources, in that consumers often become more enriched with the tracer isotope than sampled food resources (e.g., Tank et al. 2000, Hamilton et al. 2004). This "label mismatch" may result from inaccurate sampling of food resources and/or differential ingestion or assimilation of materials within the food samples. For example, epilithon (biofilm growing on rocks) is commonly assumed to be a food source for grazers in streams and consists of a complex mixture of algae, heterotrophic microbes, and non-living (detrital) organic matter. Epilithon is often sampled by scraping bulk material off rock surfaces, but grazing invertebrates may selectively ingest and/or assimilate the more actively cycling components of the epilithon, which can result in consumer isotope labeling that exceeds that observed in the bulk food resource.

Collective analyses of data from the LINX studies and a growing number of similar experiments indicate numerous cases of label mismatch in which the maximum tracer $^{15}N$ enrichment of animals (particu-

TABLE 1.   Site location and discharge of the study sites during the tracer releases.

| Acronym | Stream name | Latitude (°) | Longitude (°) | Discharge (L/s) | Citation |
|---------|-------------|--------------|---------------|-----------------|----------|
| BBNY | Blues Brook, USA | 43.67 | −74.94 | 5.0 | |
| BBNH | Bear Brook, USA | 43.93 | −71.75 | 9.1 | |
| BCNC | Upper Ball Creek, USA | 35.05 | −83.43 | 129.6 | Tank et al. (2000) |
| CBNY | Combs Brook, USA | 43.67 | −74.94 | 5.0 | |
| E1AK | E1 Alaska, USA | 68.63 | −149.63 | 134.0 | |
| ECMI | Eagle Creek, USA | 42.33 | −85.33 | 202.0 | Hamilton et al. (2001) |
| EVNT | Rio Maria, Panama | 8.64 | −80.04 | 22.9 | Whiles et al. (2013) |
| EVWT | Rio Maria, Panama | 8.64 | −80.04 | 22.4 | Whiles et al. (2013) |
| GCNM | Gallina Creek, USA | 36.58 | −105.58 | 4.0 | |
| KCKS | S. Kings Creek, USA | 39.08 | −96.58 | 15.8 | Dodds et al. (2000) |
| KGNZ | Kye Burn, New Zealand | −44.95 | 170.35 | 22.3 | Simon et al. (2004) |
| KTNZ | Kye Burn, New Zealand | −44.95 | 170.35 | 34.6 | Simon et al. (2004) |
| LIDK | Lilleaa, Denmark | 56.25 | 10.05 | 63.2 | Riis et al. (2012) |
| MCOR | Mack Creek, USA | 44.20 | −122.15 | 56.6 | Ashkenas et al. (2004) |
| QBPR | Quebrada Bisley, USA | 18.32 | −65.75 | 20.2 | Merriam et al. (2002) |
| SBIC | Steinbogalaekur, Iceland | 65.53 | −17.02 | 156.4 | |
| SCAZ | Sycamore Creek, USA | 33.75 | −111.5 | 43.0 | |
| ULTD | Upper La Laja, Trinidad and Tobago | 10.49 | −61.30 | 14.0 | |
| WBTN | Walker Branch, USA | 35.97 | −84.28 | 17.5 | Mulholland et al. (2000) |

*Notes:* The last two letters of the acronym are abbreviations for the state or country of the site. Citations of original studies are indicated. For latitude, positive values are north and negative values are south. For longitude, positive values are east and negative values are west.

larly primary consumers) ranged from 10‰ to over 200‰ (delta [$\delta$] units or per mil [Fry 2006]) above their presumed food source. The logical explanation for the label mismatch is that field sampling of food resources in these studies did not accurately represent the N isotopic composition of the food assimilated by the consumer. The few studies that have applied techniques to separate algae from detrital organic matter using density separation by centrifugation in colloidal silica suggest that much of this discrepancy can be accounted for by the isotopic differences between highly active/labile portions of the food compared with more refractory material in the bulk resource (Hamilton et al. 2001, 2005).

Here, we explore the relationship between isotope tracer accumulation in stream consumers and their presumed food resources using data from 21 experimental $^{15}N$ tracer additions in streams ranging from tropical to arctic biomes. We address how well $^{15}N$ tracer measured in samples of putative food sources matches the $^{15}N$ tracer in consumers, and how we can correct for the contribution of less assimilable N that is included in samples of primary uptake compartments. In order to accurately describe the observed trends in the collective food web data, we used a modeling approach to fit observed data and draw conclusions about how well we sampled food resources for different consumer groups compared to what they ultimately assimilated. We then present recommendations for how future isotope enrichment experiments, natural abundance studies, and stoichiometric research on food webs can more accurately capture consumer–resource relationships.

## METHODS

Detailed methods and data from the majority of the 21 experiments included in this study have been

published previously, and methods from all experiments stem from a common set of protocols used in the original LINX study. Additional details can be found in the references in Table 1, particularly Mulholland et al. (2000), Tank et al. (2000), and Webster et al. (2003). Briefly, at each site $^{15}N$-ammonium ($^{15}N$-$NH_4$) was added to the stream for 5–42 days resulting in water column $\delta^{15}N$-$NH_4 > 100$‰ (most were >1000‰).

The study sites used in this analysis included streams from North America, the Caribbean, Central America, New Zealand, Denmark, and Iceland; sites ranged from tropical to arctic, but the majority were temperate. All $^{15}N$ tracer additions were conducted in relatively small streams (most discharges < 100 L/s; Table 1). Food resources and consumers (largely invertebrates) were sampled for tracer $^{15}N$ content as described in the papers referenced in Table 1, using techniques that are common in stream ecology research. Presumed food resources included biofilms scrubbed from rocks (epilithon for grazers), fine detritus suctioned from sediments or suspended in the water column (for collectors and filterers, respectively), pieces of detritus (leaves, wood for shredders), macrophytes, and macroinvertebrates for predators. Consumers were categorized based on functional feeding groups (e.g., collector-gatherers, filterers, grazers, shredders, and predators), and those that consumed more than one food source were classified as omnivores.

### Modeling

The model uses a linked dynamic compartment approach based on observed isotopic enrichment of food and consumers, hereafter referred to as food pool (FP) and consumer pool (CP). Note that we refer to ecosystem compartments as pools of N, not to be confused with pools and riffles found in streams. The

data were collected over time as the system was enriched with tracer $^{15}$N, and as $^{15}$N declined after the addition stopped (Wollheim et al. 1999, Dodds et al. 2000, Whiles et al. 2013). In this model, both the $^{15}$N and $^{14}$N mass in each pool (in units of mmol/m$^2$) were tracked over time, and gains and losses of isotopes were based on uptake ($U_{15N}$ and $U_{14N}$) and loss ($L_{15N}$ and $L_{14N}$) of both isotopes expressed as mass per unit area per unit time ($t$). These uptake and loss fluxes were calculated by tracking the $^{15}$N and $^{14}$N in the consumer pool (CP$_{15N}$ and CP$_{14N}$, respectively) as a function of the $\delta^{15}$N of up to three potential food pools labeled a, b, and c (FP$_{\delta15N,a}$; FP$_{\delta15N,b}$; FP$_{\delta15N,c}$).

The composite food pool $\delta^{15}$N (FP$_{\delta15N}$) was estimated by weighting the $\delta^{15}$N of individual diet sources (FP$_{\delta15N,a}$) by the proportion of that food source in the consumer diet ($P_a$; Eq. 1). The proportion of each food source was estimated using knowledge of consumer diets obtained through previous research examining gut contents. For the most part, these determinations were based on previously published research from each site (e.g., Evans-White et al. 2003, Frauendorf et al. 2013). For organisms that were presumed to eat fewer than three food sources, the number of sources was reduced accordingly

$$\text{FP}_{\delta15N} = \text{FP}_{\delta15N,a} \times P_a + \text{FP}_{\delta15N,b} \times P_b + \text{FP}_{\delta15N,c} \times P_c. \quad (1)$$

The $^{15}$N atomic ratios of the FP (ARFP) and CP (ARCP) were calculated from $\delta^{15}$N values to allow separate mass-balance tracking of $^{15}$N and $^{14}$N

$$\text{ARFP} = \frac{\text{FP}_{15N}}{\text{FP}_{14N} + \text{FP}_{15N}} = (\text{FP}_{\delta15N}/1000) \times 0.003663 + 0.003663 \quad (2)$$

where 0.003663 is the AR of the standard (atmospheric N$_2$).

At each sampling point, the $^{15}$N and $^{14}$N fluxes into the consumer pool were assumed to be proportional to the atomic ratio of the food pool(s), and N uptake and loss were the sum of the $^{15}$N and $^{14}$N uptake rates ($U_{15N}$ and $U_{14N}$, respectively)

$$U = U_{15N} + U_{14N}. \quad (3)$$

The change in the consumer pool of $^{15}$N, between time steps one and two, representing the time ($t$) between sampling, was used to calculate the new size of the consumer pool at time step two (CP$_{15N,t=2}$) from net uptake, which depends on ARFP and the uptake from the FP over time, as well as the loss from the CP ($L$), the atomic ratio of the consumer pool (ARCP), and time

$$\text{CP}_{15N,t=2} = \text{CP}_{15N,t=1} + (U \times \text{ARFP} \times t) - (L \times \text{ARCP} \times t). \quad (4)$$

Similarly, net uptake of $^{14}$N was calculated as

$$\text{CP}_{14N,t=2} = \text{CP}_{14N,t=1} + U \times [1 - \text{ARFP}] \times t) - (L \times [1 - \text{ARCP}] \times t). \quad (5)$$

The calculated ARCP can lead to model instability if the time steps are too large (i.e., many days between samples). Thus, ARCP was reset to the observed value at each sampled time step in the model. Equations of Laws (1984) were used to weight changes in ARCP and ARFP over time because straight means are not appropriate in this case.

The loss term ($L$) represents any loss from the consumer pool including excretion, drift, predation, and emergence. The ratio of the total uptake to total loss was used to determine if there was mass loss or gain. If the biomass of CP was constant over the time period, then $U = L$. In cases where there were changes in N mass over time, then the ratio of $U$ to $L$ required estimation.

We created a multiplier ($M$) to evaluate the degree to which we were not accounting for $\delta^{15}$N label mismatch in the food pool by adjusting the peak food source label to fit the observed peak animal isotopic signal. The bounds on the range of $M$ values were set such that the lowest possible value for $M$ adjusted the food source to match the $\delta^{15}$N of its consumer (CP$_{15N}$/FP$_{15N}$). In contrast, the highest $M$ value was dependent on the measured or calculated $\delta^{15}$N-NH$_4^+$ in the water column during the experiment (Water$_{15N}$/FP$_{15N}$).

The model was created in Microsoft Office Excel 2007 and the "Solver" function was used to fit observed to modeled values of $\delta^{15}$N by minimizing the sum of square of errors and changing $U$, $U/L$, and $M$. If there was no change in biomass over the experiment, then $U/L$ was set to one. If there was a change in biomass, a preliminary run was used to ensure that the total mass change was correct, and in this case $U/L$ and $U$ were changed to minimize the difference between observed and modeled final biomass based on the sum of squared errors and accumulation of $^{14}$N and $^{15}$N. However, the total mass change was very insensitive to $^{15}$N mass, as it was a small portion of the total N mass, even in highly labeled compartments. Once the $U/L$ was set, then the model was used to fine-tune the values of $U$ and $M$ to match observed and modeled $\delta^{15}$N.

In all cases, the model output was also observed graphically to assess the quality of fit. While we recognize this to be a somewhat subjective approach, mathematical search methods that are designed to minimize error can find locally stable solutions that do not match the observed data. In cases where this occurred, the initial estimates were adjusted manually to provide parameters that produced predicted values that more closely fit observed values and then the automated fitting procedure was reinitiated using the new seed values. We also recognize the criticisms associated with using the Solver function in Excel to fit functions (e.g., McCullough and Heiser 2008), and that solving for multiple parameters can lead to potential errors by changing the fit of one parameter to

compensate for another. Synthetic data sets were created with known multipliers (i.e., 1/[proportion of refractory N]) and a variety of animal turnover rates were used to test the model in an effort to address this concern. Using synthetic data sets with samples at approximately the same frequency maximum as sampled in most experiments (seven days), the model found the best fit value for $M$ and estimated parameters accurately (observed vs. expected fits of $M$ had a slope not significantly different from one and were highly significant, $P < 0.0001$, $r^2 \approx 1$) indicating the modeling approach we used was effective.

## RESULTS

The transfer of tracer $^{15}N$ from basal resources such as algae and bacteria into consumers could not always be accurately modeled without using a multiplier to correct for the mismatch between tracer $^{15}N$ observed in the presumed food sample and the consumer. Overall, across all our sites, 90 pairs of consumers and their food sources were modeled, and of those, 41 pairs (45%) had consumers whose $^{15}N$ enrichment exceeded that of their presumed food (Table 2), most by a considerable amount. Isotopic mismatches were observed at all but one site (Table 2) and included consumers eating all types of food resources. Overall, 58% of primary consumers became more labeled than their food sources. In contrast to the primary consumers, label mismatch in secondary consumers was much less common, with <5% of secondary consumers showing greater tracer $\delta^{15}N$ label than their presumed food sources (Table 2).

A representative plot of observed $\delta^{15}N$ in the grazing mayfly *Stenonema* spp. from Walker Branch, Tennessee, USA, (Fig. 1A) demonstrates how a consumer became more labeled than its putative food source after 35 days of $^{15}N$-$NH_4^+$ tracer addition. First, the measured $\delta^{15}N$ label in the epilithon indicated that the sampled food source had reached isotopic equilibrium in ~25 days, stabilizing at a value that was approximately one-sixth of the $\delta^{15}N$-$NH_4^+$ in the water column, and only decreasing after the tracer addition was terminated on day 42. Second, fitting a multiplier to the epilithon food source resulted in the modeled food source being more highly labeled than the consumer (Fig. 1B). The model yielded values of the $\delta^{15}N$ for *Stenonema* that matched the observed $\delta^{15}N$ after the multiplier ($M$) was included for the food source (Fig. 1C).

Overall, there was considerable variability in the degree of mismatch between consumers and their presumed food when consumers were examined across functional feeding groups, with higher multipliers in primary consumers (Fig. 2A) spanning groups that are likely feeding on diverse basal resources (e.g., epilithon, leaves, suspended particulate organic N, or fine benthic organic N). The median multiplier across primary consumers ranged from approximately two to three, suggesting that only about one-half to one-third of the N in food sampled (i.e., primary uptake compartments)

TABLE 2. Number of consumers modeled per site and number of cases where the tracer $^{15}N$ label in the focal animal exceeded that in its presumed food source.

| Stream abbreviation | Primary consumers | | Secondary consumers | |
|---|---|---|---|---|
| | Modeled | Exceeding food | Modeled | Exceeding food |
| BBNY | 2 | 1 | 1 | 0 |
| BCNC | 5 | 3 | 1 | 0 |
| CBNY | 2 | 0 | 1 | 0 |
| E1AK | 3 | 3 | | |
| ECMI | 4 | 4 | | |
| EVNT | 5 | 2 | 4 | 1 |
| EVWT | 5 | 1 | 4 | 0 |
| GCNM | 3 | 2 | | |
| KCKS | 4 | 2 | 2 | 0 |
| KGNZ | 1 | 1 | 1 | 0 |
| KGNZ | 2 | 0 | 1 | 0 |
| KTNZ | 1 | 1 | 1 | 0 |
| KTNZ | 3 | 2 | 1 | 0 |
| LIDK | 4 | 2 | 2 | 0 |
| MCOR | 5 | 4 | 1 | 0 |
| QBPR | 4 | 3 | | |
| SBIC | 3 | 2 | | |
| SCAZ | 4 | 2 | | |
| UPTD | 3 | 2 | 1 | 0 |
| WBTN | 6 | 3 | | |
| Total | 69 | 40 | 21 | 1 |

*Note:* Blank cells indicate no data, and, of the four omnivores (which are not listed on this table), none exceeded the weighted estimation of label in their food.

was assimilated by consumers. Rank analysis of the multipliers required for best model fits indicated that functional groups of primary consumers were not significantly different from each other (ANOVA, $P > 0.05$) and invertebrate predators had significantly lower multipliers (ANOVA, $P < 0.05$) than functional feeding groups that consume basal resources directly (primary consumers). Vertebrate predators had similarly low multipliers but were not included in the ANOVA due to their small sample size ($n = 5$ predators).

Several taxa modeled in this study are known to be omnivorous, eating more than one food source (see Eq. 1), so data were also analyzed by food source. The median multipliers were similar across primary food sources (Fig. 2B), however, within any single food source there was considerable variation. All food resources had minimum multiplier values of one and all but one had maximum values > 10. Animal material (i.e., prey for predators) was the exception, with multipliers that were significantly more constrained than the other food sources. Since most animals were assumed to feed from one food compartment, food-specific results did not vary substantially from the functional feeding group analyses.

## DISCUSSION

Our synthesis of 21 $^{15}N$ isotope tracer experiments provides clear evidence that primary consumers in streams assimilated food resources that differ from the material collected by standard methods used in stream food web studies, resulting in an apparent mismatch in
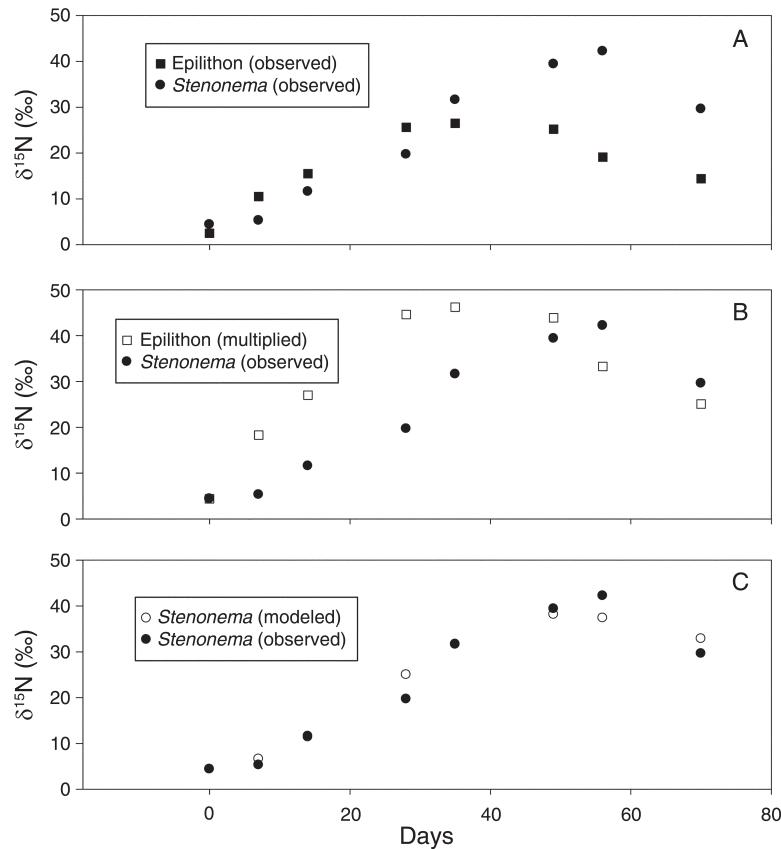
FIG. 1. Modeled and observed tracer $^{15}N$ label for epilithon and the grazer *Stenonema* spp. in a 42-day $^{15}N$ addition at Walker Branch, Tennessee, USA (WBTN). All observed data from Mulholland et al. 2000. (A) The observed tracer $^{15}N$ of epilithon and *Stenonema* demonstrated that the grazer became more labeled than its putative food source and that the label in the food reached plateau by day 25. (B) The model-estimated food label shows the $^{15}N$ of the corrected food label peaks before the grazer does (a multiplier has been used that corrects the food source for the proportion of available N). (C) The modeled tracer $^{15}N$ label in the grazer after correction of the epilithon pool and adjustment of uptake rates compared with the observed label in the field samples. In cases where the modeled value is not visible, it was very close to the observed value. Note that modeled values are represented by open symbols.

the isotopic labeling of consumers relative to their presumed food. The mismatch between food sources collected in these studies and those actually assimilated by consumers could be due to either selective ingestion or preferential assimilation of materials that are isotopically enriched relative to the sampled food matrix, and likely of higher nutritional quality. Animals can alter their diets to optimally forage for multiple elements leading to selective ingestion (Simpson et al. 2004, McNeely et al. 2009, Hawlena and Schmitz 2010). Less is known about N pools that are ingested but refractory to assimilation (as discussed subsequently). Most streams have at least some omnivorous animals (Gessner et al. 1999, Crowl et al. 2001). Omnivory and the prevalence of fine-particle feeding can make it challenging to connect resource availability, food ingestion as indicated by gut analyses, and nutrient cycling dynamics.

The resulting label-mismatch problem, often observed in stream isotope tracer experiments, highlights our inability to accurately sample and analyze food resourc-

es assimilated by consumers. This problem is not limited to isotope enrichment studies; sampling representative food sources also is essential for both natural abundance stable isotope studies and for many stoichiometric applications, although the consequences of inaccurate sampling of food resources may be less obvious in those studies. Our assessment of multiple $^{15}N$ tracer additions suggests that much of the uncertainty commonly observed in such studies may be a consequence of our inability to accurately quantify the isotopic signature of the basal food resources that are assimilated by consumers.

Fitting our model to synthetic data sets suggested that both turnover rates and multipliers can be accurately predicted as long as the food and the consumer pools both exhibit an increase and subsequent decrease in isotopic label over time, highlighting the need to conduct sampling both during and after the isotopic tracer addition. This requirement stems from the information content of each data set upon which we base our data-fitting approach. Information included in these experi-

ments includes whether the isotopic labels in the pools reach an equilibrium plateau or not; the timing of peak labeling of both food and consumers; and the magnitude of peak labeling. This information was clear for most primary consumers in our dataset, and the ability of our model to fit observed patterns in isotope labeling (e.g., Fig. 1) provides confidence that these multipliers accurately reflect selective assimilation in stream food webs.

Our findings pose a fundamental challenge for studies of food webs, as well as the stoichiometry of feeding relationships, because few studies have been able to isolate and quantify the specific component of basal food resources that consumers ingest and/or assimilate (for examples of studies that have, see Hamilton et al. 2001, 2005). Our data were obtained from streams, but we suspect that this issue is prevalent in systems where consumers forage in mixtures of fine particulate organic matter of varying nutritional quality, including suspended matter, soils, sediments, and biofilms.

Our analyses suggest that the challenge of tracing the flows of N in a stream food web exists for all types of food eaten by primary consumers. The basal food resources sampled in these streams are composed of a mixture of N that is actively cycling (e.g., algae, bacteria, fungi, and microfauna) and refractory (e.g., detrital organic compounds of various forms). Nearly all the primary consumers in these studies either selectively ingested or differentially assimilated more actively cycling fractions of the bulk food mixtures.

Carbon assimilation studies have documented several-fold greater assimilation efficiencies for omnivorous fish (*Campostoma*) and crayfish (*Orconectes*) fed animal tissue as opposed to leaf, algal, and detrital material (Evans-White et al. 2003). Less is known about organic N assimilation efficiencies but the pattern is expected to be similar. Organic N in food varies by source and digestibility. As detritus decomposes, there is an increase in the proportion of refractory organic N (e.g., humic compounds and chitin), that may increase total N content (if carbon is reduced more substantially), but reduce amino acid or protein content (Rice 1982). Amino acid content varies widely across food sources with ~10% amino acid or protein as a percentage of ash-free dry mass in detritus, ~23% for microalgae, and up to ~60% for macroinvertebrates (Bowen 1987). Protein-rich foods can be easily assimilated by animals (Gerking 1984). With respect to less-easily assimilated compounds, fungi can range from 2% to 21% chitin by dry mass (Blumenthal and Roseman 1957), and humic materials may contain up to 6% N (Wetzel 2001).

At higher trophic levels, isotopic signatures of prey and their predators showed less label mismatch. However, predators are expected to take longer to reach isotopic equilibrium with their food resources than primary consumers, and the duration of these $^{15}N$ tracer additions may not have been long enough for mismatches to become apparent (Hamilton et al. 2004).
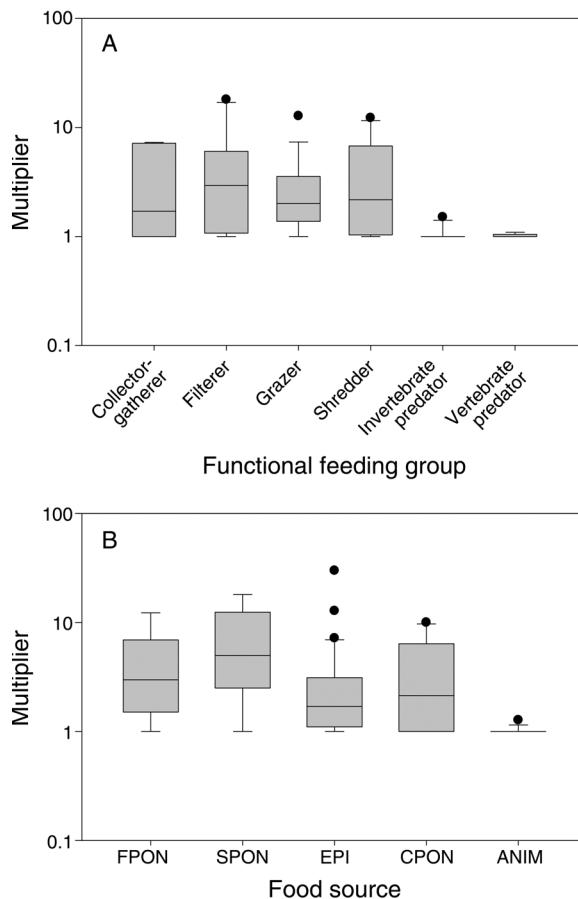


FIG. 2. Multipliers by (A) functional feeding group and (B) food source. Panel (A) shows the feeding groups collector-gatherers, filterers, grazers, shredders, invertebrate predators, and vertebrate predators. Vertebrate predators were not included in the ANOVA due to their small sample size ($n = 5$ predators). In panel (B), FPON is fine particulate organic N, SPON is suspended particulate organic N, EPI is epilithon, CPON is coarse particulate organic N, and ANIM is animal prey. The lower boundary of the boxes indicate the 25th percentile; the line within the box marks the median; and the upper boundary of the box indicates the 75th percentile. Error bars above and below the box indicate the 90th and 10th percentiles, respectively. Points outside those regions are plotted as circles. Invertebrate predators (see panel A) and animal food sources (see panel B) were significantly different from the other groups (ANOVA, $P < 0.05$).

Stated differently, there is less information content to inform our models when isotopic label does not reach equilibrium. Nevertheless, our data are consistent with the assumption that animal tissue is more readily assimilated than basal food resources consumed by primary consumers. It is not surprising that investigators are better able to sample predator food sources (i.e., other animals) than the dietary material of primary consumers (complex mixtures of organic matter).

Omnivores are particularly difficult to deal with in stable-isotope food web studies because they cannot be compared to a single food resource, and gut analyses do

not accurately reflect proportions of sources assimilated. For example, two coexisting shrimp species from Puerto Rican streams, *Atya* and *Xiphocaris*, have been considered to be a grazer and a shredder, respectively, based on field and laboratory observations of feeding behavior. However, stable isotope evidence indicates *Xiphocaris* is not exclusively a shredder because its tissue isotopic signature can be strongly influenced by algal biofilm in high-light streams (March and Pringle 2003). Our modeling of these species in the Puerto Rican LINX experiment (Quebrada Bisley; QBPR, Table 1) was consistent with *Atya* as a grazer, in that it exceeded the label observed in epilithon. However, labeling of *Xiphocaris* did not exceed that observed in leaf or algal material, suggesting it may be a shredder in that stream.

Our results indicate that a significant proportion of the N in stream primary uptake compartments is refractory and cycling at rates too slow to be detected using these approaches (sensu Newbold et al. 1983). It is likely that refractory or slowly cycling N pools are common where there is greater N limitation (i.e., higher C:N ratios) because these compartments tend to have low N turnover rates (Dodds et al. 2004). However, many factors likely influence the proportion of refractory N in materials, and C:N alone, as well as other abiotic factors measured in these tracer studies, did not significantly correlate with our multiplier values (data not shown).

The conclusions of this study generally agree with those from Hamilton et al. (2001), who used density-gradient centrifugation methods to separate biologically active materials that were isotopically enriched from more refractory fractions of the bulk food resource in a woodland stream in Michigan (ECMI, Table 1). Hamilton et al. (2001) found that the proportion of actively cycling N in basal food resources was variable; 23% was active N in epilithon, 1% in fine benthic organic matter, 5% in small pieces of decomposing wood, and 7% in decomposing leaves. Our estimates for the proportion of actively cycling N in basal food resources bracket those of Hamilton et al. (2001), but our median values were higher for most food sources.

Our findings have implications for isotopic tracer experiments, as well as approaches that use natural isotope abundance for characterizing food web dynamics. If tracer ^{15}N has reached long-term isotopic equilibrium in a food resource, then bulk collections should be a reasonable proxy for isotopic abundance in food sources. However, equilibration of refractory pools might take months or years to achieve. If inputs of ^{15}N to food resources are reasonably constant over time, the refractory and active N pools should approach similar isotopic values, with the exception of allochthonous materials, and this would be true for both isotopic tracer experiments and natural abundance studies. However, our results indicate that isotopic tracer additions of at least several months would be needed for refractory materials to reach equilibrium conditions; we had many

experiments where equilibrium was not reached in six weeks, and this was particularly pronounced in predators (e.g., Ashkenas et al. 2004, Hamilton et al. 2004). Larger consumers (e.g., fish and mussels), often have greater body mass and slower ^{15}N uptake rates than their prey, and as a result during a tracer addition they require more time to reach isotopic equilibrium relative to their prey.

Quantifying the size of the readily assimilated N pool in isotope tracer studies is required to measure the specific activity of the label and calculate N flux rates between food web compartments. We are aware of few studies, other than those analyzed here, that allow for this determination. We speculate that in lakes and the open ocean, the natural abundance of suspended particulate materials might more closely represent the isotopic composition of animal diets because most of the refractory N is in the dissolved organic N pool (Aluwihare et al. 2005). In soils, pulses of N that are available for uptake in primary food compartments (e.g., microbes associated with organic particles) could occur with precipitation and snowmelt events, and given the potentially high proportion of refractory materials (15–35%; Rovira and Vallejo 2002), isotopic composition of consumers could be far from equilibrium at most times. We suspect that streams and wetlands would be intermediate between lentic and terrestrial systems in this regard.

The mismatch we observed between N isotopic labeling of sampled food sources and primary consumers is also relevant to studies of ecological stoichiometry. Such studies typically rely on sampling consumers and food resources using similar techniques as reported here. These values are then used to quantify the magnitude of elemental ratio mismatch between food and consumer, estimate threshold elemental ratios, and examine the consequences of dietary mismatches for consumer growth (e.g., Frost and Elser 2002, Frost et al. 2005). Our results suggest that most food sources for primary consumers in streams contain a substantial portion of N that is not assimilated, and it is possible that similarly refractory pools of carbon (and perhaps even phosphorus) also occur in primary food sources. If so, then the problems identified in this study may be exaggerated in stoichiometric studies since the relative size of labile and refractory pools is likely to vary across elements in a manner that would be difficult to quantify. To make things more difficult, different elements may be preferentially assimilated from different diet components. For example, stream invertebrates collected from Australia and New Guinea apparently took more of their N from algae in the stream but more of their carbon from terrestrially derived plant materials (Bunn et al. 2013).

Methods of separating relatively refractory N from actively cycling N in samples of basal food sources exist, and our results suggest that isotopic studies of food webs could benefit from application of such approaches. One

PLATE 1. (Left) A damselfly nymph (Family Lestidae) in a mountain stream in Panama. Damselflies are important predators in streams and other aquatic habitats. (Right) Snails (Family Lymnaeidae) grazing on epilithic biofilms and associated filamentous green algae in the Teton Pines Waterway, an urban spring stream located in the Snake River basin, Wilson, Wyoming, USA. Photo credits: damselfly, Amanda T. Rugenski; snails, J. L. Tank.

approach previously discussed is to separate actively cycling biological material from other material using density gradient centrifugation in colloidal silica, which can yield a lighter fraction enriched in algae and/or bacteria (Hamilton et al. 2005). It might also be possible to select very rapidly growing materials to represent the food sources; Whiles et al. (2013) placed tiles in their study stream to provide a sample of rapidly accruing biofilm that more closely matched the maximum level of grazer labeling than did sampling of existing bulk epilithon. Bunn et al. (2013) used statistical correlations to assess the relative importance of food sources from field-derived data, but this requires results across a wide range of conditions. Finally, compound-specific (e.g., amino acid) isotope analyses hold considerable promise because they may be able to be optimized to reflect only actively cycling N (e.g., Evershed et al. 2007). Most separation techniques are more labor intensive and some are costly as well, but accurate characterization of food sources requires either physical separation of active food pools or modeling approaches similar to those described here.

Our study demonstrates the challenges associated with sampling and characterizing food resources for primary consumers (see Plate 1) whose presumed food sources are mixtures of actively cycling and refractory materials. We recommend that researchers consider ways to separate refractive from actively cycling materials, including those discussed in this paper. Incorporation of a multiplier to model relationships between food and consumers can account for the problem of a consumer having a higher $^{15}$N label than its presumed food source. Ecologists using isotopes as tracers in food webs, either through experimental isotope additions or natural abundance approaches, along with those conducting stoichiometric studies, should be cognizant of potential problems associated with sampling food sources that may not accurately represent what animals actually ingest and assimilate.

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