**Title:** The influence of temporal isotope heterogeneity and isotope incorporation rates on consumer trophic position estimation

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**1. Abstract (288)**

Nitrogen stable isotope ratios are frequently used to estimate trophic position of consumers. A key assumption in this application of stable isotope data is that the food webs in which consumers forage are in isotopic equlibrium with nitrogen resources. However, isotopic heterogeneity in the food web, which can arise from seasonal nutrient fluctuations, spatial variability in primary production, or variability in available prey, exists in most systems. Given resources are not immediately assimilated into the tissues of consumers, it is likely that the assumption of isotopic equilibrium is frequently violated in most systems. We tested the degree to which the violation of this assumption impacts consumer stable isotope ratios and trophic position estimates from those ratios. We constructed a compartment model to explore consumer isotope incorporation rates and their effect on trophic position calculations using bulk and compound specific stable isotope data from previous experimental and observational studies derived from a variety of aquatic systems. We also tested how different parameterizations of the stable isotope baseline in trophic position calculations can improve accuracy. We found trophic position estimates of higher trophic level consumers are less accurate than lower trophic level consumers when applying bulk stable isotope analysis (BSIA) with particulate organic matter as the stable isotope baseline in trophic position equations. Accuracy of consumer trophic position improved for tertiary consumers when applying a 90-day lag between the consumer stable isotope measurement compared to baseline measurements, but this was at the expense of decreased accuracy for lower trophic level consumers. Compound-specific stable isotope analysis (CSIA) of individual amino acids was more accurate in estimating trophic position for all consumers and systems compared to BSIA. Overall, our results show consideration of stable isotope heterogeneity and stable isotope incorporation rates of consumers is important for accurate trophic position estimates and should be carefully considered when designing stable isotope studies.

**2. Introduction**

In most ecosystems, seasonality is a key mediator of food web structure and function (McMeans et al. 2015). Nutrients, such as phosphorus, nitrogen, and silicon, can be seasonally limited in their availability which can determine the timing and amount of primary production (sensu., Moon and Carrick 2007, Kolzau et al. 2014, Søndergaard et al. 2017) in aquatic systems. Nutrient availability can be influenced by oceanographic conditions, due to upwelling (Barth et al. 2007, Ferreira et al. 2020), resources pulses (Benbow et al. 2020, Vespoor et al. 2011), or anthropogenic inputs (Khangaonkar et al. 2019, McCrackin et al. 2017), allowing for increased resource sequestration as primary producers are released from growth limiting constraints. In addition, the impact of nutrient limitation can propagate to the rest of the food web, as primary production can alter abundance in both adjacent and non-adjacent trophic levels (Ware and Thomson, 2005) by controlling the energy available for higher trophic level consumers.

Stable isotope analysis of nitrogen (15N/14N) serves as a useful tool for identifying trophic interactions and nutrient sources in food webs. The nitrogen stable isotope values (δ15N) of consumers within a food web are characterized by nitrogen availability, contributions of distinct nitrogen sources (i.e., anthropogenically fixed; Vander Zanden et al. 2005), and trophic position. Trophic position of a consumer is able to be calculated due to marked isotope enrichment between consumer and prey (typically 2 to 4 per mille, Deniro and Epstein 1981). However, these calculations also require, and are highly sensitive to, a characterization of the stable isotope baseline (stable isotope ratio of primary producers or inorganic nitrogen sources) within the food web, which can vary through space and time (Post 2002, Anderson and Cabana 2007, Possamai et al. 2021). There is no consistent methodology on measuring isotope baseline (Kjeldgaard et al. 2021, Possamai et al. 2021) although recommendations for best practices have been made (Post et al. 2002, Jardine et al. 2014, Possamai et al. 2021). Typically, the isotopic baseline is estimated from primary producers, particulate organic matter (POM), or primary consumers that were measured at the same time as the consumer of interest (Kjeldgaard et al. 2021). However, time average isotope baseline values or generic values that may not be from the same environment or time period are also utilized (i.e. Estrada et al. 2003, Ruiz-Cooley et al. 2006, Chiang et al. 2020) and fail to represent heterogeneity within and across systems.

The ecosystem heterogeneity created by seasonal fluctuations in both physical, chemical, and biological processes influence the isotopic values of primary producers and primary consumers (the isotope baseline) which vary both seasonally (Woodland et al. 2012) and spatially (Vokshoori et al. 2014). For example, seasonal variability in the nitrogen isotope values of seston, or phytoplankton are common in lake (Syväranta et al. 2006, Matthews and Mazumder 2007, Gu et al. 2009) estuarine (Rolff 2000) and marine systems (Wu et al. 1999, Vookshori et al. 2014). Such isotope variation has been shown to be transferred to higher trophic level consumers (Popp et al. 2007, Olson et al. 2010, Dale et al. 2011, Feddern et al. 2021). However, isotope incorporation into consumer tissues typically occurs at a slower rate than that of the prey, as the rate of isotope turnover increases with animal size (Thomas et al 2014, Vander Zanden et al. 2015) in addition to temperature, growth rate, and the type of tissue studied (Dalerum and Angerbjorn 2005). As a result of slower incorporation times, consumers can dampen short term temporal stable isotope heterogeneity relative to algae or particulate organic matter (Woodland et al. 2012). Nevertheless, the temporal mismatches created by differences in isotope incorporations rates between diet and consumer can affect the calculation of animal trophic position. In fact, a fundamental assumption of such calculations is that a consumer is in close equilibrium or ‘isotopic steady state’ with the dietary resources consumed (Deniro and Epstein 1976, Martinez del Rio et al. 2009, Phillips 2014) and the isotope baseline used to calculate trophic position. However, in nature such equilibria do not necessarily occur, and while such assumption violations are often acknowledged, they are rarely quantified.

A recent methodological advancement in stable isotope analysis, compound-specific stable isotope analysis (CSIA) of amino acid nitrogen (δ15N), provides researchers with a powerful new tool to estimate the stable isotope values of the base of the food web (Sherwood et al. 2011, McMahon et al. 2015, Feddern et al. 2021) which can be applied for trophic position calculations of a consumer (Popp 2007). A key advantage of estimating trophic position using specific δ15N amino acid values are the properties of source amino acids, which depict the stable isotope values of nitrogen resources of a system by showing little trophic enrichment with increasing trophic levels. In contrast, trophic amino acids track the apparent trophic position of the animal through trophic enrichment. This allows estimation of baseline values directly in the consumer organism of interest. Furthermore, the baseline value of the source amino acids represents an integrated dietary contribution relating directly to the physiology and thus isotope turnover of the consumer itself. That in principle results in a smaller temporal mismatch and standardization across a heterogenous environment between the source and trophic amino isotope values. However, incorporation rates of individual amino acids vary (Bradley et al. 2014, Downs et al. 2014; Figure 11) and whether source and trophic amino acids are incorporated at similar rates is at present not well studied. Downs et al. (2014) showed that the amino acid incorporation rates of glutamic acid (trophic) and phenylalanine (source), which are the most commonly applied amino acids for trophic position calculation due to their trophic enrichment factors, in shrimp appear similar (Figure 11). However, it is not yet known if this is a persistent pattern between glutamic acid and phenylalanine across animal groups, or tissue types as controlled feeding studies measuring turnover rates of individual amino acids are currently limited. There is evidence that turnover time of amino acids may also very with diet quality for some but not all amino acids (Chikaraishi et al. 2015). Furthermore, the same study found both faster and slower turnover rates for other trophic (aspartic acid, proline) and source amino acids (lysine, methionine), motivating an analysis of how temporal isotope integration in amino acids influence the accuracy of trophic position estimates.

Here we construct a framework to explore consumer isotope incorporation rates and their effect on trophic position calculations by conducting compartment model simulations using stable isotope data from previous experimental and observational studies. The objectives of this work are to:

1. Explore how potential mismatches in isotope turnover rates between food web baseline and consumers can influence trophic position calculations from bulk δ15N values among various trophic levels, tissue types, and aquatic systems.
2. Examine how variability of source amino acid and trophic amino acid turnover rates can influence trophic position calculations.
3. Consider how different trophic position calculations and isotope baseline sampling strategies for both BSIA and CSIA can improve accuracy in trophic position estimations that are the result of tissue turnover mismatches between the isotope baseline and consumers.

**3. Methods**

*3.1 Data compilation*

Data used for case studies was compiled from previous research measuring the stable isotope signature of nitrogen in particulate organic matter (POM) in both marine and freshwater systems and laboratory experiments measuring nitrogen stable isotope turnover rates in different taxa, tissues, and amino acids. These studies were used to obtain realistic estimates of the natural variation of δ15N at the base of the food web over the course of a year. We prioritized studies that 1) collected data at least monthly and 2) represented a range of aquatic systems (Table 1). Four diverse ecosystem studies were identified; a moderately eutrophic urban lake (Syväranta et al. 2008), a shallow subarctic lake in interior Alaska (Gu et al. 1994), a pristine oligotrophic lake (Mathews and Mazumder 2007) and a marine upwelling site near Vancouver Island (Wu et al 1999).

Experimental isotopic diet studies have been conducted to quantify animal tissue isotopic turnover rates, λ (% · day-1) which are frequently expressed as the isotopic half-life (ln(2)/ λ, days). Data for bulk nitrogen stable isotope turnover rates are available for a variety of taxa and tissues (i.e., Vander Zanden et al. 2015). For bulk tissue stable isotope model simulations, turnover rates were identified to construct six theoretical isotope scenarios for aquatic food webs that contain primary, secondary, and tertiary consumers and that could be associated with the baseline values for each of the four case studies (Table 1 & Figure 1).

For the bulk nitrogen stable isotope models, half-life data and baseline data were combined to create six theoretical food webs by matching consumers and their half-life values with isotope baseline data from an ecosystem in which they could reasonably forage. A marine food web was constructed from half-life values from amphipod, herring and great skua and freshwater food web was constructed from half-life values water strider, steelhead or small mouth bass, and black bear (Figure 1). These scenarios were used to make specific comparisons regarding stable isotope baseline and the half-life values of the consumers. The half-life value of the secondary consumer was increased between scenario a and b to represent different tissues (liver versus muscle), an increase in temperatures, or an increase of size, all of which can alter isotope incorporation rates. Comparison 2 decreased the half-life of the secondary consumer and increased the half-life of the tertiary consumer between scenario c and d (Figure 1). The objective of this comparison was to identify how dietary differences of the tertiary consumer can impact its stable isotope composition and how sampling different tissues of the tertiary consumer can impact trophic position estimates. Finally, comparison 3 examined how two isotope basslines with distinct shapes can impact trophic position estimates of the rest of the food web (Figure 1, Table 1).

For CSIA of amino acid nitrogen models, half-life data from two aquatic consumers (shrimp and tuna) were used to construct a single food web with four trophic amino acids and one source amino acid modelled for each consumer (Figure 2). The stable isotope values for each amino acid for both consumers were modelled for all four of the stable isotope baselines derived from particulate organic matter (Table 1). The stable isotope values for each amino acid were then compared for each baseline and both consumers to assess the performance of trophic amino acids in trophic position estimations.

*3.2 Tissue Turnover Modelling Structure*

The isotopic composition of consumers was modeled using a first order kinetics model as described by Martinez del Rio and Carleton (2012) and modified from Cerling et al. (2007). Many controlled feeding studies use an exponential fit model to estimate tissue turnover where isotope data is fit to:

**(4)** δt = αe-λt + *c*

where *δ* is the nitrogen stable isotope value of a consumer’s tissues that has been measured at different values of time *t* before and after the consumer has been switched to an isotopically distinct diet. The parameters *α*, *λ*, and *c* are each estimated empirically from a best fit of data, where *c* is the asymptotic value of the isotope composition of tissue after the diet switch once tissues have reached steady state. *α* represents the magnitude of isotopic change in tissues or the difference between the initial isotopic steady state before the diet switch and the final isotopic steady state after the diet switch. *λ* is a first order data-derived rate constant referred to as the turnover rate, which can be used to calculate the isotopic half-life:

**(5)** tα= ln(1-α)/λ

where *α* is 0.5. *t0.5* values are commonly reported in experimental studies and represent the amount of time (in days or hours) required for 50% equilibration with a new diet source, and thus were used to calculate λ in our model (Vander Zanden et al. 2015). *t0.95* values are also frequently reported and denote 95% equilibrium and are commonly accepted as the amount of time to reach isotopic steady state. Chemically, the first order rate constant is represented as:

**(6)** dN/dt = - λN

Where *N* is the number of atoms or molecules in the system, *t* is time (s) and *λ* is the rate constant (s-1). This assumes isotopic equilibration is a first order reaction given for a trace isotope where the concentration of the rare isotope (15N) is significantly less than that of the abundant (14N). Thus, the system can be closely approximated by changes in only the rare isotope. In the case of controlled feeding experiments the food source is treated as a reservoir where the isotopic value of the food supply is unaffected by consumption (Criss 1999). Equation 6 can then be integrated to yield:

**(7)** N= N0 e-λt

and can be linearized by:

**(8)** ln(N/N0) = - λt

For this model, we apply this approach but with a modified representation of equation 4 as presented in Martinez del Rio and Carleton (2012) with more biologically meaningful set of parameters:

**(9)** δxt = δx*∞* - (δx*∞* - δx*0*)e-λt

Where *x* represents a particular element (in this case nitrogen), *δx∞* is the asymptotic value representing steady state is reached with a new diet, *δx0* is the initial value of the tissue, and *λ* is the empirically derived rate constant as described above.

*3.3 Bulk Stable Isotope Model*

The objective of our work was to identify the degree to which tissue turnover and variability in stable isotope composition at the base of the food web resulted in deviations from the true trophic position (Figure 3) if the consumers and the isotope baseline were sampled simultaneously and consumers were assumed to be in equilibrium. The number of trophic transfers are a known component of the model structure and for all models simulations the true trophic level was 2 for the primary consumer, 3 for the secondary consumer, and 4 for the tertiary consumer. From observed stable isotope baseline data derived from particulate organic matter (POM) (Table 1) we modelled the stable isotope composition of primary, secondary and tertiary consumers associated with a particular food web scenario (Figure 1). δ15N of primary consumers was estimated using first order mixing of a well-mixed pool where:

**(11)** δ15NTL, t=0 = δ15NTL-1, t=1 + TEF

And

**(12)**  δ15NTL, t = (δ15NTL-1, t-1 + TEF) \* 1 – exp(λ)

And trophic position was calculated from estimated δ15N as:

**(13)** TP = δ15NTL- δ15NTL=1/TEF+1

Where *δ15NTL* is the δ15N value for a consumer at trophic level *TL*. *δ15NTL* where *TL* is 1 represents the *δ15N*of the base of the food web, which was derived from observational studies. The rate of tissue turnover for nitrogen isotopes is typically measured on the time scale of hours or days and thus daily values of the nitrogen isotope baseline is necessary to model isotopic incorporation by consumers. However, studies collect stable isotope data from POM and primary producers on weekly and monthly scales. Therefore, we linearly interpolated nitrogen stable isotope values between observations in order to approximate daily changes in the isotopic baseline (Table 1). *TEF* was assumed to be known and the canonical value of 3.4 per mille was applied. Trophic position was then calculated by applying equation 13. Finally, an effect size was estimated as the magnitude and duration the calculated TP deviated from the true TP (Figure 3):

**(14)** TPDev, TL = TP- TPTL

Where *TPTL* represents the true trophic position (*TL* = 2:4) of primary, secondary, and tertiary consumers. Duration of deviation was determined by summing the number of days *TPDev, TL* was ± 0.2 for each *TL* (Figure 3). This model assumed the stable isotope signature of the base of the food web, *TEF* and *tα*where known.

*3.4 Compound Specific Stable Isotope Model*

Diet switching studies that report the isotope turnover rates for individual amino acids are currently rare in stable isotope literature. For the CSIA model simulations we applied our modelling framework to two consumers with known isotope turnover rates for individual amino acids, bluefin tuna (*Thunnus orientalis*; Bradley et al. 2014) and Pacific white shrimp (*Litopenaeus vannamei*; Downs et al. 2014). For each consumer, four trophic amino acids (glutamic acid, alanine, proline, valine) and a single source amino were modelled (Figure 11). The canonical source amino acid for trophic position calculations, phenylalanine, was modelled for shrimp however a phenylalanine half-life value was not reported in Bradley et al. (2014) and lysine was used as the source amino acid for tuna. Tuna and shrimp were modelled as a single food web (Figure 2) for each of the four baseline case studies (Table 1) where shrimp was the secondary consumer and tuna was the tertiary consumer. Equation 12 was modified to model each of the 5 amino acids for each consumer:

**(15)**  δ15NTL, t, a = (δ15NTL-1, t-1, a + TEF) \* 1 – exp(λ, a)

as such, a different *λ* was distinct for each amino acid, *a* including the source amino acid. The trophic position equation was also modified to calculate the trophic position of shrimp and tuna from amino acids, with source amino acids instead of the measured baseline:

where, *δ15Ni* is the measured stable isotope value of a trophic amino acid *i* in a sample and *δ15No* is the stable isotope value of a source amino acid *o* in a sample. represents the total trophic enrichment that has occurred throughout the food web measurable from consumer tissues at a given trophic level (*TL*). *β (i-o)* is the difference of enrichment between a specific trophic amino acid *i* and source amino acid *o* for primary producers derived from laboratory experiments (Chikaraishi et al. 2007; Appendix S1: Table S2). represents the mean trophic enrichment factors for consumers, and is calculated from the mean difference between trophic amino acid *i* and source amino acid *o* across all consumers described in Nielsen et al. (2015). Equation 13 was applied to calculate trophic position deviation with TL=2 for shrimp and TL=3 for tuna.

*3.5 Sensitivity Analysis*

To further explore the degree how variability in tissue turnover time impacts trophic position estimates using bulk stable isotope analysis and CSIA, a sensitivity analysis was performed. The food web scenarios described in this study are useful for understanding variation in nitrogen stable isotope data and stable isotope derived trophic position estimates using data observed in the real world. However, tissue turnover times vary dramatically based on size, tissue, and growth rate of the consumer of interest (Figure 11). Similarly, the rate of stable isotope change at the base of the food web is highly dependent on the system and is subject to interannual variability driven by other abiotic factors. The trophic level of the consumer can also impact estimates if lower trophic level species are not in isotopic steady state with their resources

We simulated an isotope baseline that had a rate of isotope change that varied from 0 to 0.15 (per mille per day), represented 123 days, and represented a 1 to 6 per mille isotope change which was realistic based on previous studies (Table 1, Figure 4). A primary, secondary, and tertiary consumer were modelled for bulk stable isotope analysis (equation 11 - 14) and CSIA (equations 15 - 16). Half-life values ranged from 1 to 280 days and the model was run using the same simulated baseline for each of the 280 half-life values. To assess the sensitivity across half-life values, the mean and standard deviation of trophic position deviation was taken for each model run (equation 14) which represented the average deviation across the 123-day simulation. For the CSIA model, two analyses were run, one with a source amino acid half-life value of 130 days (representing the values for lysine in bluefin tuna reported by Bradley et al. 2014) and a second of 33 days (representing the values for phenylalanine in shrimp reported by Downs et al. 2014). The half-life value of the source amino acid was assumed to be known and the half-life value of the trophic amino acid was varied for each simulation. This method was repeated three times each for CSIA and BSIA, once only varying the half-life of the primary consumer, a second time varying primary and secondary consumer half-life, and finally varying the half-life values of all three trophic level consumers simultaneously.

*3.5 Comparison to recommended methodologies*

Guidance on BSIA baseline sampling using primary consumers as the stable isotope baseline rather than particulate organic matter or primary producers (Post et al. 2002), and sampling consumers of interest and the stable isotope baseline 90 days apart (Possamai et al. 2021). These two approaches can minimize baseline isotope variability or account for disconnect between the baseline and consumer due to tissue turnover. We assessed the utility of these approaches to improve trophic position accuracy across a variety of systems by applying them to our simulated food webs. To calculate trophic position using the primary consumer we applied:

**(17)** TP = δ15NTL- δ15NTL=2/TEF+2

Using the stable isotope values of the simulated primary consumer, *δ15NTL=2*. To calculate the trophic position using a 90 day delay we

**(18)** TP = δ15NTL, y - δ15NTL=1, z /TEF+2

Where *δ15NTL, y*was the isotope signature of consumers at days *y* 90, 270 and 360 and *δ15N TL=1, z* is the stable isotope value of the baseline at days, *z* which were 1, 90, 270, and 360. A third approach combining both of these methodologies was also applied to the model output and trophic position deviation was calculated using equation 14.

**4. Results**

The shape of the stable isotope baseline impacts the bulk stable isotope values and trophic position deviation for simulated primary, secondary, and tertiary consumers. The four stable isotope baselines (Table 1) showed a range of stable isotope variability over the course of a year (Figure 4). The oligotrophic lake (baseline 4, Figure 4F) had the greatest variability in the stable isotope values of particulate organic matter that ranged from 0 to over 10 per mille but was relatively stable until day 300. In contrast, the two other freshwater systems exhibited fluctuations within a 5 per mille range, but oscillated more frequently between values (Figure 4C – E) within that range. Of the two eutrophic lakes, the heavily polluted lake (baseline 3; Figure 4E) had the highest variability. The marine upwelling system showed baseline isotope values characteristic of seasonal nutrient fluctuations and spring phytoplankton blooms, nitrogen stable isotope values of POM were lowest in the spring and summer when upwelling is high, and higher in the winter when nutrients are more limiting (Figure 4A & B).

*4.2 Bulk Stable Isotope Model*

Our model showed that consumers are rarely in isotopic steady state with their available resources and the violation of this assumption resulted in deviations from true trophic position by as much as one trophic level (Table 2; Figure 5C & F). We define duration of deviation as the number of simulated days that a consumer has a trophic position deviation of greater than ± 0.2 trophic level (equivalent to ± 0.7 per mille deviation from steady state with resources). Primary consumers had at least 50 days duration of deviation (Table 2). The duration of deviation increased as you moved up the food web. For all scenarios, tertiary consumers had the longest duration of deviation, which was as high as 276 out of 365 days for scenario 3 (Table 2; Figure 4C). Based on comparison 1, the duration of deviation also increased for tertiary consumers when half-life of secondary consumers increased, despite the same half-life of tertiary consumers and same stable isotope baseline (Table 2; Figure 4A versus B). Therefore, duration of deviation was determined by the consumers half-life, in addition to the half-life of consumers lower food web that serve as resources for higher trophic levels.

Similarly, the magnitude of trophic position deviations depends on both the half-life of consumer of interest, and also the half-life of consumers lower in the food web. When a primary or secondary consumer is not in steady state with its resources, the resulting trophic position deviation propagates through the food web. In comparison 1, the trophic position deviation for the tertiary consumer was higher when half-life value of the secondary consumer (Figure 5A) was higher, despite the half-life value of the tertiary consumer being the same in both scenarios. Sampling a tissue of a tertiary consumer with extremely rapid turnover time (i.e., blood plasma Figure 5C) did not prevent trophic position deviation, despite the tertiary consumer being as close to isotopic steady state with its prey as possible. Sampling the blood plasma of a tertiary consumer that has prey with slow tissue turnover (i.e., adult steelhead muscle), results in substantial trophic position deviation ranging from 2.9 to 4.8 (*TLTrue* = 3, Figure 5C). This deviation is similar to sampling a tissue with a longer isotopic half-life from a tertiary consumer that eats prey with a faster half-life (i.e., juvenile small mouth bass muscle) (Figure 5D) which ranges from 3.1 to 4.8.

In addition to half-life value of the consumers in a food web, the shape of the stable isotope baseline (rate of isotopic change, inflection points, magnitude of change) also impacts the stable isotope values of the consumers, and as a result, the magnitude and duration of trophic position deviation. The eutrophic, urban lake (Syväranta et al. 2008) had an isotope baseline that demonstrated a slow rate of change that reached a high magnitude (Figure 4E), comparatively, the pristine oligotrophic lake showed frequent, small, rapid, fluctuations followed by a rapid increase of 10 per mille (Figure 4D). The eutrophic lake had a much longer duration of deviation for all consumers (Table 2) compared to the oligotrophic lake (Figure 5E & D). For almost the entire model simulation, the oligotrophic food web was within ± 0.2 trophic levels until day 300 when all consumers experience a high and rapid deviation from their true value (Figure 5D). In comparison, the consumers in the eutrophic lake were consistently underestimated but experienced a lower magnitude of deviation (Figure 5E).

*4.3 CSIA Model*

Magnitude of trophic position deviation and the duration of deviation was lower using CSIA compared to BSIA and varied across trophic amino acids. Baseline 3, the moderately eutrophic urban lake, had the highest magnitude of trophic position deviation of all stable isotope baselines for the CSIA model (Figure 7E & F). Of the tested trophic amino acids, proline had the greatest magnitude of trophic position deviation for both shrimp, the primary consumer (maximum deviation = 0.2), and tuna, the secondary consumer (maximum deviation = 0.4; Figure 7F). In comparison, the canonically used trophic amino acid for CSIA trophic position calculations, glutamic acid, only had a maximum trophic position deviation of 0.04 for shrimp and 0.2 for tuna for the same baseline, and lower magnitude of deviations for all other baselines (Figure 7E). Similarly, proline had the greatest duration of deviation, which was as high as 147 days for tuna in baseline 3 and as low as 32 days for shrimp in baseline 4 (Table 3). In contrast, glutamic acid had 0 days duration deviation for both shrimp and tuna for most baselines, with a notable exception for tuna in baseline 3, which had a 75-day duration of deviation (Table 3). All amino acids used in the CSIA model performed better in terms of the magnitude of trophic position deviation (Figure 7) compared to BSIA analysis (Figure 5). No amino acids for the primary and secondary consumers in the CSIA model exceeded a trophic position deviation of greater than 0.4 trophic levels and for most trophic amino acids it did not exceed ± 0.2 trophic levels, with glutamic acid and alanine preforming particularly well across consumers and baseline scenarios. In comparison, the secondary consumer in scenario 3 of the bulk stable isotope model had a magnitude of trophic position deviation of 0.8 trophic levels (Figure 5C) which was twice as high as the largest magnitude deviation for the CSIA model. Proline in the CSIA model performed similarly to the BSIA model in terms of duration of deviation (Tables 2 & 3), however it had a much lower magnitude of deviation (Figure 7). For the bulk stable isotope model, error always propagated up the food web and was greatest for higher level consumers in all scenarios. This was not necessarily the case for the CSIA model. In fact, for most baselines and amino acids, both shrimp and tuna performed similarly (Figure 7) despite tuna being modelled as a higher trophic level consumer (secondary) than shrimp (primary).

*4.4 Sensitivity analysis*

Sensitivity of trophic position deviation to consumer half-life increases with trophic level for bulk stable isotope analysis of nitrogen. For the bulk stable isotope model, sensitivity increased with primary consumer half-life (Figure 8B). However, the distribution of mean magnitude of trophic position deviation reached its greatest variability (highest standard deviation) at a half-life of approximately 100 days, and was similar at half-life values greater than 100 for primary consumers (Figure 8B) although the mean magnitude of deviation continued to decrease further from the true value. Mean magnitude of deviation of the secondary consumer was more sensitive to consumer half-life values than the primary consumer. Both the trophic position estimation and the variability in the estimation increased at a greater rate (Figure 8C). Mean magnitude trophic position deviation continued to decrease further from the true value as consumer half-life increased. Sensitivity of the mean magnitude of trophic position deviation of the tertiary consumer (Figure 8D) to half-life followed a similar pattern as the secondary consumer. Initially the deviation and variability increased at a higher rate than consumers lower in the food web, but eventually leveled off at a half-life of approximately 100 days, at which point the mean and standard deviation of the tertiary consumer was similar to that of the secondary consumer (Figure 8D).

Trophic position deviation for BSIAwas more sensitive to consumer half-life than CSIA. Sensitivity of trophic position deviation is minimized when the half-life of the trophic amino acid is equal to the half-life of the source amino acid, which was 130 days (Figure 9) and 33 days (Figure 10) for these model simulations. Mean trophic position deviation and its distribution decreases in magnitude initially before intersecting with the half-life of source amino acid. At half-life value greater than the source amino acid variability then increases slightly but does not deviate substantially from zero for any of the three trophic level consumers (Figure 9D). Unlike bulk stable isotope analysis, sensitivity to the consumer half-life only slightly magnifies up the food web, and only at low half-life values of the trophic amino acid measures (Figure 9) or at half-life values substantially greater than the source amino acid half-life when the source amino acid half-life is short (Figure 10). However, when the source amino acid half-life value is short (i.e., in the case of shrimp, Figure 11B) most trophic amino acids have short half-life values as well (less than 100 days). Therefore the greatest sensitivity of tropic position deviation is still at lower half-life values of trophic amino acids, even for short source amino acid half-life values.

*3.5 Comparison to recommended methodologies*

Alternative recommended methods to calculate trophic position from stable isotope data produced only moderate improvements for trophic position accuracy. The 90-day lag between the stable isotope baseline and tertiary consumers improved accuracy for trophic position, although estimates ranged ± 0.6 trophic levels for the eutrophic, arctic lake (Table 4A, Scenario 4). The range in trophic position estimates for secondary consumers were similar for the 90-day lag compared to a simultaneous sampling approach. In contrast the range in primary consumer trophic position was much greater with a 90-day lagged sampling strategy compared to a simultaneous strategy, with overestimates as high as two trophic levels in some systems (Table 4A). Using primary consumers as a stable isotope baseline improved trophic position deviation for all consumers in all systems (Figure 12) compared to the particulate organic isotope baseline model (Figure 5) although it still exceeded ± 0.2 for substantial amounts of time. Applying the 90-day lagged equation with the primary consumer as the stable isotope baseline further improved trophic position accuracy for the tertiary consumer (Table 4B) although accuracy for the secondary consumer was similar to the simultaneously measured model with the primary consumer as the stable isotope baseline (Figure 12).

**4. Discussion**

Temporal heterogeneity in the bulk nitrogen stable isotope baseline results in erroneous trophic position estimations when heterogeneity and tissue turnover are not accounted for. Based on our results, this error can even occur when the isotope baseline is able to be sampled continuously, and simultaneously with the consumer and is considered known. Both the shape of the heterogeneity of the stable isotope baseline and the trophic level of the consumers are important factors in the magnitude and duration of trophic position deviation for a given aquatic food web. Notably, for BSIA, trophic position deviation propagates up the food web with greater error in estimation for consumers feeding at higher trophic levels. Using CSIA to sample consumers provides the best approach for minimizing trophic position deviation due to temporal heterogeneity and tissue turnover rates however the relative rate of turnover between the source amino acid and the trophic amino acid used to calculate trophic position should be considered. When CSIA is not feasible, using primary consumers as the stable isotope baseline or sampling the consumer 90 days after sampling the baseline can provide moderate improvement to trophic position deviation of high trophic level consumers, but does not eliminate it entirely and the improvement comes with decreased accuracy for lower trophic level consumers.

The duration of trophic position deviation is substantial for all of the modelled consumers using BSIA. However, no consumer had a trophic position deviation of greater than ± 0.2 for the entire modelled time period. In fact, for at least 25% of the model time for all consumers in all food webs trophic deviation was less than ± 0.2 trophic positions. Based on this result, there are substantial time periods within each food web when trophic deviation can be minimized simply based on sampling time. For most modelled food webs, days 0 - 100 (January 1 – April 10) and 200 - 250 (July 19 – September 7) produced accurate trophic position estimations for secondary and tertiary consumers (Figure 5 A-F), although the moderately eutrophic, urban lake (scenario 5; Figure 5E) was an exception. Carefully considered sampling time could help improve the accuracy of trophic position estimation of consumers within heterogenous environments. However, this approach may not be applicable to all research questions, as trophic position measurements during the spring or autumn may be of particular interest. The applicability of this strategy across systems also warrants additional research as the food web baselines modelled in this study represent temperate systems.

Notably, errors associated with turnover and heterogeneity propagate up food webs and are more pronounced and occur for a longer duration (Table 2) in higher trophic level consumers when using BSIA to estimate trophic position (Figure 5). The sensitivity of trophic position to stable isotope half-life also propagates up the food web (Figure 8), meaning, higher trophic level consumers experience more trophic position deviation at higher half-life values than their lower trophic level counterparts. This is a result of tertiary consumers being even further from isotopic steady state with the sampled isotope baseline, in this case POM, compared to lower trophic level consumers. Cabana and Rasmussen (1996) observed that δ15N values of phytoplankton is ten times more variable than primary consumers. Due to the short life span of primary producers, they only represent a short-term measure of the isotope baseline (Vizzini and Mazzola 2003) and thus by sampling primary consumers isotopic variability isotope heterogeneity is dampened by being time averaged over the rate of tissue turnover. Our results agree with this, while trophic deviation is higher in secondary and tertiary consumers, variability in the magnitude of trophic position deviation is dampened relative to the stable isotope values observed lover in the food web (Figure 4). This indicates that when consumers tissues are sampled is less impactful for the overall magnitude of trophic position deviation than when and how the baseline is sampled and modifications to stable isotope baseline sampling is the most fruitful approach to minimizing trophic position deviation (compared to adjusting consumer sampling).

Previous research has provided guidance to improve accuracy of trophic position estimation from bulk stable isotope data. Our analysis utilized stable isotope data of particulate organic matter because it is prevalent and longer-term data was available for a greater variety of systems. It also allowed us to manipulate the tissue turnover time of all of the consumers in the modelled food web. However, Kjeldgaard et al. (2021) found that there were 10 methods to quantify stable isotope baseline in the literature, of which particulate organic matter was only one. Guidance on stable isotope sampling strategy has included increasing sample size (Kjeldgaard et al. 2021), using primary consumers as the stable isotope baseline rather than particulate organic matter or primary producers (Post et al. 2002), and sampling consumers of interest and the stable isotope baseline 90 days apart (Possamai et al. 2021). With our model, we explored these options and found that applying a 90-day lag or using the primary consumer as the stable isotope baseline offered only moderate improvement of the accuracy in trophic position estimation (Table 4; Figure 12). Accuracy in trophic position estimation improved the most for tertiary consumers for both methods but was worse for primary consumers when applying the 90-day delay. This result indicates the isotopic steady-state assumption is more accurate for higher trophic level consumers when applying a 90-day sampling delay and using primary consumers as the stable isotope baseline. When applying both of the sampling strategies at once, the trophic position deviation of tertiary consumers was only ± 0.3 trophic levels for most systems (Table 4B). Using a 90-day lag between the consumer of interest and isotope baseline, using primary consumers as the stable isotope baseline, or both, could be a useful strategy for minimizing error in trophic position estimation from stable isotope heterogeneity and tissue turnover time for tertiary consumers or higher but will decrease the accuracy of lower trophic level consumers. This strategy could be further improved upon by applying a different time lag for primary consumers that is less than 90 days and aligns more closely with their isotopic turnover rates. This strategy would be particularly useful for studies where large sample size or CSIA is impractical or cost prohibitive. It is important to note that in order to utilize primary consumers as the isotopic baseline, the trophic level of the primary consumers must be known with certainty, or it could introduce another component of error into the trophic position estimates. This can be particularly challenging when employing a method such as plankton tows, which frequently include mixture of planktonic taxa (i.e., microbial heterotrophs, detritus, microzooplankton) in different life stages, and as a result can impact trophic position estimates of consumers (McCarthy et al. 2007, Hannides et al. 2013). In addition, omnivory is common in zooplankton (Atkinson 1996, Castellani et al. 2008) making it challenging to know their trophic level absolutely if they are used as the stable isotope baseline for trophic position calculations of other consumers. Using sessile filter feeders (i.e. mussels, barnacles) when available in a system would be the best practice for using consumers as suggested by other research (Vander Zanden and Rasmussen 1999, 2001, Post 2002, Mancinelli et al. 2013).

CSIA substantially reduces the magnitude and duration of trophic position deviation relative to BSIA. Some trophic amino acids performed better than others, specifically, proline had the highest magnitude and duration of trophic position deviation compared to glutamic acid and alanine which had the lowest. Based on our sensitivity analysis, trophic position deviation is less sensitive to longer trophic amino acid half-life values (Figure 9 & 10). Calculating trophic position using a source amino acid with a longer half-life (Figure 9) also reduced sensitivity of trophic position deviation to trophic amino acid half-life values compared to a shorter half-life of the source amino acid (Figure 10). CSIA allows researchers to measure the stable isotope value of multiple amino acids from a single sample. Phenylalanine and glutamic acid have become the canonical source and trophic amino acids applied in CSIA because phenylamine demonstrates the lowest trophic enrichment while glutamic acid has the highest (McClelleand and Montoya 2002, Chikaraishi et al. 2007, 2009, Hannides et al. 2009). Recent studies have shown using multiple amino acids can improve trophic position estimates (Nielsen et al. 2015, Feddern et al. in prep) but there is little guidance in the literature regarding which amino acids are best and why. Best practices in CSIA should focus on utilizing source - trophic amino acid pairs where the amino acids have similar half-life values. When researchers are choosing between pairs, it is best to select source amino acids with longer half-life values, and generally, selecting a trophic amino acid that has a longer half-life than the selected source amino acid will produce the best results. Based on our results, glutamic acid and alanine are the best trophic amino acids to use ad are suitable to be paired with either lysine or phenylalanine as source amino acids.

While our results show CSIA reduces error in trophic position estimation relative to BSIA, CSIA is can be cost prohibitive and half-life values of individual amino acids are not well studied across taxa and tissues. Studies examining nitrogen stable isotope half-life values of individual amino acids are limited, and the field of CSIA would greatly benefit from more half-life values reported in the literature. Our results show lysine, phenylalanine, glutamic acid, and alanine perform well based on half-life values of tuna white muscle and shrimp tail muscle. Similarly, Feddern et al. in prep found that using both alanine and glutamic acid paired with phenylalanine from harbor seal bone offered the most improvement to model certainty compared to using either amino acid alone or other trophic amino acids. This indicates that the best CSIA amino acid pairs may be fairly consistent across taxa and tissues, but additional studies of tissue turnover rates would verify this result, particularly in animals that utilize urea-excretion (Germain et al. 2013) and in tissues commonly used for historical CSIA studies such as bone or coral (Misarti et al. 2009, Feddern et al. 2021, Sherwood et al. 2005, 2008, 2011).

Here we focused on seasonal, temporal heterogeneity, but other types of stable isotope heterogeneity exist within aquatic systems and impact trophic position calculations. Spatial heterogeneity is common in stable isotope baselines (Lorrain et al. 2015) and changes with latitude (Lorrain et al. 2015, Vokshoori et al. 2015) and depth (Wu et al. 1999, Sigman et al. 2009, Peters et al. 2012) within ocean basins. As a result, mobile consumers are frequently integrating distinct stable isotope baselines, that may not reflect the baseline where they were caught for sampling. Stable isotope values of primary producers also vary based on taxa in both freshwater (Vourio 2006) and marine habitats (Ramirez et al. 2021, Lorrain et al. 2015, Sigman et al. 2009). On smaller scales in freshwater lakes, habitat availability and population abundance can also influence isotopically distinct basal resource use of aquatic consumers (Stiling et al. 2021). Changes in diet that impact resource use can result in changes in the stable isotope baseline that is assimilated by a consumer of interest. Similar considerations for tissue turnover times and its implications for trophic position estimates should be made for mobile consumers in spatially heterogenous systems as well as temporally heterogenous systems.

This study examined temporal isotope heterogeneity, tissue turnover times and their impact of estimating trophic position from stable isotope data. While we focused on the assumption of isotopic steady state between consumers and their prey, this is not the only assumption for calculating stable isotope values that impacts the interpretation of stable isotope data. Trophic enrichment factors are often assumed to be equal for consumers but is known to be vary based on taxa, tissues, excretion pathway, and diet (Deniro and Epstein 1981, Vanderklift and Ponsard 2003, Caut et al. 2008, Blanke et al. 2015). We did not model the effect of variable trophic enrichment factors, though this value is known to influence trophic position calculations and resource estimates (Bond and Diamond 2011). Variability in trophic enrichment factors and baseline heterogeneity can act both synergistically and antagonistically on trophic position estimates. The scope of this study was to understand error associated with heterogeneity and tissue turnover specifically. While our model is limited in the number of parameters that vary, it fulfills this crucial knowledge gap.

**Conclusions**

Stable isotope heterogeneity and stable isotope turnover of consumer tissues impact the accuracy of trophic position estimates. Based on our results CSIA reduces trophic position deviation more than any of the tested trophic position equations for BSIA. Using primary consumers as the stable isotope baseline or applying a 90-day lag between baseline and consumer sampling can improve accuracy of trophic position estimation for tertiary consumers or higher, although it does not eliminate it. Thus, stable isotope heterogeneity and its potential effects on trophic position estimates should be considered when interpreting stable isotope values. Our results agree with the recommendations made by Possamai et al (2021), when possible, trophic position studies should use CSIA for at least a subset of samples. Researchers applying stable isotope data to calculate consumer trophic position should carefully consider heterogeneity in baseline isotope values expected in their system, isotope turnover rates for their consumers of interest, and isotope turnover rates of the resources of their consumers of interest. We caution researchers against a one size fits all approach for improving trophic position estimation, and it may be beneficial to apply different approaches for different consumers in the same system.

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