



# Shifting baselines: Integrating ecological and isotopic time lags improves trophic position estimates in aquatic consumers

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**ABSTRACT:** Nitrogen stable isotope ratios ( $\delta^{15}\text{N}$ ) are routinely used to estimate consumer trophic positions (TPs). This method is very sensitive to the chosen isotopic baseline, which should incorporate all temporal isotopic variability occurring in the base of the food web sustaining the consumers. However, there is still no consensus on the different methods to represent time intervals for isotopic assimilation. It is uncertain if researchers should compute a single baseline obtained by averaging  $\delta^{15}\text{N}$  source values across all time periods, multiple baselines based on  $\delta^{15}\text{N}$  source values collected concomitantly with consumers, or consider a time lag between sources and consumers. Aiming to provide empirical evidence to help select the best option, we designed 4 methods using different temporal lags between consumers and producers based on a 9 yr time series of carbon and nitrogen stable isotopes in an estuary. The 4 tested baseline methods considered the  $\delta^{15}\text{N}$  average of producers sampled in all years (Global); the same season as consumers (Concomitantly); the season before the collection of consumers (Delayed); and the previous season as well as the same season of consumer sampling (Interpolation). Comparison of results with an expected model (based on stomach content data) showed that the Delayed method is the most appropriate. We conclude that time lags in the incorporation of stable isotope values between sources and consumers must be considered in baseline  $\delta^{15}\text{N}$  calculations to improve TP estimates and provide more reliable modeling results. Additionally, we provide other recommendations to improve sampling designs when using stable isotopes in TP estimations.

**KEY WORDS:** Aquatic ecology · Isotopic baseline · Isotopic turnover · Nitrogen isotope · Stable isotopes · Trophic level

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## 1. INTRODUCTION

Stable isotope analysis (SIA) is widely used in trophic ecology studies, allowing descriptions of carbon and nitrogen flow within ecosystems (Hoeinghaus et al. 2011, Claudino et al. 2013, 2015, Garcia et al. 2016, Domingos & Lana 2017), contributions of allochthonous material to estuarine food chains (França et al. 2015, Nelson et al. 2015, Garcia et al. 2017, Morais et al. 2017, Garcia et al. 2019a), and to investigate the variability in consumer trophic

positions (TPs) and in food-chain length (Post et al. 2000, Hoeinghaus et al. 2008, Saigo et al. 2015, Ishikawa et al. 2017, Ruiz-Cooley et al. 2017, Possamai et al. 2021). The most frequently used stable isotopes are carbon ( $^{13}\text{C}/^{12}\text{C}$ ) and nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ); carbon isotope ratios (expressed as  $\delta^{13}\text{C}$ ) are useful to evaluate the origin of organic matter sustaining consumers, and nitrogen isotope ratios ( $\delta^{15}\text{N}$ ) are used to estimate TPs (Fry 2006).

Nitrogen stable isotope ratios have been used to estimate consumer TP due to marked isotopic frac-

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tionation (usually ranging from 2 to 4‰) with each trophic interaction (Vander Zanden & Rasmussen 2001, Post 2002, Vanderklift & Ponsard 2003). However, this method of TP estimation is very sensitive to the chosen isotopic baseline, which may vary in time and space (Post 2002, Anderson & Cabana 2007). The isotopic baseline is based on  $\delta^{15}\text{N}$  values of primary food sources sustaining the consumer of interest and is used as a reference point when computing TP (Post 2002). Hence, a key step before estimating TP of a consumer using SIA is the establishment of a relevant isotopic baseline. For instance, an appropriate isotopic baseline should incorporate all temporal isotopic variability occurring in the base of the food web sustaining the consumer (Vander Zanden & Rasmussen 2001, Post 2002), but there is no consensus on the different methods to represent time intervals for isotopic assimilation when generating an isotopic baseline (Phillips et al. 2014).

Usually, the isotopic baseline is estimated using  $\delta^{15}\text{N}$  values of representative producers or primary consumers from the study system collected at the same time as samples of the consumers of interest. Prior studies have suggested using relatively sessile primary consumers such as filter-feeding or grazing molluscs, sponges or resident fishes to estimate the isotopic baseline in aquatic systems, as these organisms incorporate the isotopic variability from the producers they feed upon in their tissues (Vander Zanden & Rasmussen 1999, 2001, Post 2002, Mancinelli et al. 2013). Representative producers (e.g. microalgae) fueling aquatic communities are typically short-lived compared to primary consumers, and the isotopic variability of producers may not be reflected in higher trophic levels (TLs) due to these differences in lifespan. However, using a primary consumer as an isotopic baseline may not be feasible when these are rare or unavailable throughout the year. In such cases, primary producers, such as phytoplankton and algae, and other potential primary food sources such as organic detritus are commonly used as isotopic baselines (Bastos et al. 2017, Villamarín et al. 2018, Potapov et al. 2019).

Differences between isotopic turnover in different groups of organisms are another issue to be considered in studies applying SIA (Vander Zanden & Rasmussen 2001, Hussey et al. 2014, Phillips et al. 2014). Turnover rates can vary among organisms and individuals in relation to body size, metabolic rate and ontogeny (Fry & Arnold 1982, Post 2002, Phillips et al. 2014). Thus, the time from consump-

tion to isotopic assimilation in consumer tissues must be considered. In any case, the chosen baseline should integrate isotopic variability at a time scale compatible with that of the secondary consumer of interest (Post 2002). However, there is still no consensus on how to integrate variability in primary sources across time scales to achieve more appropriate and representative isotopic baselines. For instance, there are no established criteria on how to choose between different methods to compute the isotopic baseline when sampling across several time periods (e.g. months, seasons). It is uncertain if the computation should be a single baseline obtained by averaging  $\delta^{15}\text{N}$  source values across all time periods, multiple baselines based on  $\delta^{15}\text{N}$  source values collected concomitantly with consumers, or whether a time lag between sources and consumers should be used. These various approaches can be found in the literature (e.g. Hoeninghaus et al. 2008, Svanbäck et al. 2015, Bastos et al. 2017, Villamarín et al. 2018, Possamai et al. 2020), but there is no comparative study evaluating the sensitivity of TP estimation to these different methods to compute isotopic baselines.

Due to the difficulty in establishing an adequate baseline across spatial and temporal gradients, logistical sampling issues, or lack of awareness of fundamental assumptions, many studies have used generic baselines (i.e. baselines sampled in a different environment and/or period than when the consumers were collected). Such generic isotopic baselines do not consider appropriate time lags in the isotopic turnover of sources and consumers (e.g. Estrada et al. 2003, Ruiz-Cooley et al. 2006, Chiang et al. 2020). This generates isotopic mismatches and is not a robust use of stable isotope techniques (Phillips et al. 2014), which may yield spurious TP estimates. Considering the aforementioned concerns and shortcomings of establishing appropriate isotopic baselines, especially regarding the issue of different time intervals between source and consumer sampling, we designed 4 approaches to generate isotopic baselines that reveal the sensitivity of TP estimates to different methods of isotopic baseline computation. Based on a 9 yr isotope dataset of sources and consumers, we designed these methods considering different temporal lags between consumers and producers and considering the approaches commonly used in aquatic ecology research. These different methods allowed us to visualize the importance of incorporating isotopic turnover to reduce error in TP estimates.

## 2. MATERIALS AND METHODS

### 2.1. Sampling and laboratory processing

We used the isotopic dataset from the Brazilian Long-Term Ecological Research (B-LTER) program in Patos Lagoon Estuary. Samples of estuarine producers and consumers were obtained seasonally from 2010 to 2018 in a shallow embayment of the estuary (<1.5 m; 32° 1' 23.8" S, 52° 7' 53.6" W). Seasonal sampling takes into account the results of experimental studies with estuarine fishes in the region showing that carbon and nitrogen isotopic turnovers in their muscle tissues reflect food assimilated up to 2 to 3 mo prior (Mont'Alverne et al. 2016, Oliveira et al. 2017).

The main estuarine producers of this estuary are saltmarshes (including plants with C<sub>3</sub> and C<sub>4</sub> photosynthetic pathways), macroalgae, seagrass, phytoplankton and microphytobenthos. We sampled this broad range of producers to encompass the range of baselines possible for this environment. However, as prior studies at Patos Lagoon Estuary had shown that the assimilation of saltmarsh C<sub>3</sub> plants (e.g. *Scirpus maritimus*) by consumers was negligible (Claudino et al. 2013, Lanari et al. 2018), we did not use data from C<sub>3</sub> saltmarsh plants in the analyses. Saltmarsh C<sub>4</sub> plants (*Spartina alterniflora* and *S. densiflora*), macroalgae (*Rizochlonium* sp. and *Ulva* sp.) and seagrass (*Ruppia maritima*) were collected by hand using scissors. Suspended particulate organic matter (SPOM) was used as a proxy for phytoplankton (Post et al. 2000) and was obtained by filtering 0.25–1.00 l of water onto a pre-combusted (450°C for 4 h) Whatman glass fiber filter (0.75 µm). Sedimentary organic matter (SOM, a mixture of microphytobenthos and detritus; Phillips et al. 2014) was obtained by removing the top 2 cm from the surface of the sediment using a plastic core (10 cm diameter). Aquatic consumers (fishes, blue crabs, and shrimps) were sampled by beach seine with 13 mm mesh in the wings and 5 mm in the center of the net. Terrestrial crabs near the edge of the saltmarsh were collected by hand (see Table 1 for species list).

In the laboratory, producer samples were rinsed with distilled water, placed in sterile Petri dishes and dried in an oven at 60°C for 48 h. For consumers, the dorsal muscle of fish and crabs was removed, whereas for shrimps, all muscle was used after removing the gut. Consumer samples were also rinsed with distilled water and dried as indicated for producer samples. Dried samples were ground to a fine powder with a mortar and pestle, weighed to 10<sup>-3</sup> g,

pressed into ultra-pure tin capsules (Costech) and sent to a specialized laboratory for analysis of carbon and nitrogen isotope ratios. Stable isotopes are reported as parts per thousand (‰) difference from a corresponding standard:  $\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 10^3$ , where R = <sup>13</sup>C/<sup>12</sup>C or <sup>15</sup>N/<sup>14</sup>N. Standards were Vienna PeeDee Belemnite (VPDB) for carbon, and nitrogen in air, calibrated using certified reference materials. Certified reference materials and internal laboratory standards (animal and plant) of known carbon and nitrogen composition were analyzed with each sequence. Variation in values from repeated measures of lab standards provide an estimate of instrument precision. SD of values for lab standards was 0.07‰ for δ<sup>13</sup>C and 0.06‰ for δ<sup>15</sup>N for animal samples (n = 102), and 0.15‰ for δ<sup>13</sup>C and 0.37‰ for δ<sup>15</sup>N for plant samples (n = 102). Samples were analyzed at 2 different laboratories over the course of the study; samples from 2010–2015 were analyzed at the Analytical Chemistry Laboratory at the University of Georgia, and samples from 2016–2018 were analyzed at the Stable Isotope Ecology Laboratory of D. J. Hoeinghaus at the University of North Texas. We analyzed a subset of random samples in both laboratories to check for consistency in isotopic determination, and the test revealed low between-laboratory SD and no statistically significant differences for nitrogen (SD = 0.08‰, t = 0.899, df = 9, p = 0.391) or carbon (SD = 0.03‰, t = 0.240, df = 9, p = 0.815).

### 2.2. Evaluating different isotopic baseline computations

In order to evaluate the sensitivity of TP estimates to different approaches of generating isotopic baselines regarding time intervals between sources and consumers, we employed 4 distinct methods of computation: (1) an overall average (±1 SD) of the δ<sup>15</sup>N values of all primary food sources collected between 2010 and 2018 (denoted as 'Global'), (2) average (±1 SD) δ<sup>15</sup>N values of primary food sources collected concomitantly with consumers in the same season ('Concomitantly'), (3) average (±1 SD) δ<sup>15</sup>N values of primary food sources collected in the prior season before the collection of consumers ('Delayed'), and (4) average (±1 SD) δ<sup>15</sup>N values of primary food sources collected in the prior and in the same season where consumers were sampled ('Interpolation'). The Global and Concomitantly methods were evaluated because they are very common methods used by stable isotope ecologists in trophic studies (e.g.

Hoeinghaus et al. 2008, Svanbäck et al. 2015, Bastos et al. 2017, Possamai et al. 2020). The Delayed method reflects the lag in isotopic turnover in consumer tissues (e.g. Mont'Alverne et al. 2016, Oliveira et al. 2017), and the Interpolation method was considered as an alternative to Concomitantly and Delayed that might improve TP estimates for a diverse assemblage by incorporating the variability of organisms with both faster and slower isotopic turnover rates. These approaches allowed us to evaluate isotopic baselines encompassing distinct time windows (e.g. from years to different seasonal intervals) of isotopic variability in the primary food sources.

For each method (excluding Global), we evaluated the differences in average values of  $\delta^{15}\text{N}$  among producers in each season using the Tukey HSD test with  $\alpha = 0.05$  (Tables S1 & S2 in the Supplement at [www.int-res.com/articles/suppl/m666p019\\_supp.pdf](http://www.int-res.com/articles/suppl/m666p019_supp.pdf)). When the test distinguished 2 different groups with distinct average  $\delta^{15}\text{N}$  values, we used these groups as 2 different isotopic baselines in our models to estimate TPs. The primary food sources for each season and the isotopic baselines chosen for each method can be found in Tables S1 & S2.

### 2.3. TP estimation

TP estimation was performed separately in each season for each baseline estimation method using the 'tRophicPosition' package in R (Quezada-Romegialli et al. 2018). This new method constitutes a better tool to estimate consumer TP than prior methods because it uses a Bayesian approach that incorporates individual variability and propagating sampling error of trophic discrimination, isotopic baselines and consumer values, and posterior estimates of parameters (Quezada-Romegialli et al. 2018). The TP of each species was modeled using the Markov chain Monte Carlo (MCMC) method with 10 000 interactions and 10 000 adaptive samples in JAGS 4.3.0 for both models of 1 or 2 baselines.

Isotopic fractionation is affected by differences in the metabolism of plants and animals; therefore, it is necessary to include this variation in the TP estimates (Vander Zanden & Fetzer 2007, Hussey et al. 2014). Nitrogen in animals is more homogeneous than in plants, since it originates mainly from proteins. In plants, nitrogen is assimilated from the environment, where sources may be highly variable, and its uptake depends on assimilation efficiency and nitrogen availability (Lanari et al. 2021). In this

sense, the fractionation in herbivores (HBV) (i.e. nitrogen from plants to HBV) is more variable than in carnivores (Vander Zanden & Rasmussen 2001). Following Bastos et al. (2017), the trophic discrimination factors used were  $\delta^{13}\text{C} = 0.54 \pm 0.53$  and  $\delta^{15}\text{N} = 3.02 \pm 0.47$  for omnivores (OMN), zooplanktivores (ZPL) and zoobenthivores (ZBV);  $\delta^{13}\text{C} = 0.54 \pm 0.53$  and  $\delta^{15}\text{N} = 4.78 \pm 1.30$  for HBV and detritivores (DTV); and  $\delta^{13}\text{C} = 0.82 \pm 0.47$  and  $\delta^{15}\text{N} = 1.77 \pm 0.30$  for piscivores (PSV).

### 2.4. Assessing isotopic baseline performance

To establish a criterion to choose the best method to generate the isotopic baseline, we first classified consumers into the following trophic guilds based on stomach content data obtained in the literature (Possamai et al. 2018 for fishes; Collins 1999, Oliveira et al. 2006, Abreu et al. 2007, Bemvenuti & Colling 2010 for crustaceans): DTV, HBV, OMN, ZPL, ZBV and PSV. Next, we ranked the TL of each consumer from lowest to highest using FishBase (Froese & Pauly 2019) and data in the literature (Bemvenuti & Colling 2010, Rodrigues et al. 2014, Mancinelli et al. 2016) (Table 1). TL was used instead of TP because it is the variable available in FishBase and is comparable with TPs estimated using stable isotopes (Mancinelli et al. 2013). Trophic guilds were then ordered according to the expected increasing TPs from the base to the top of the food chain (1 for DTV, 2 for HBV, 3 for OMN, 4 for ZPL, 5 for ZBV and 6 for PSV), and a linear model called 'Expected model' was constructed with this prediction using the TP average and TP median of each guild. The average and median TPs for each guild were also used to model the results of TPs estimated from consumer data from Patos Lagoon Estuary using each baseline method. Average and median values were chosen because they summarize the intrinsic ecological variability of the species comprising the guilds (e.g. the TP of OMN can range from near the 2<sup>nd</sup> to the 4<sup>th</sup> TL) while also integrating temporal variability. As we are aiming to discern the most appropriate method to estimate increasing TP, integrating this variability can reduce the differences among each guild and improve model fit. TPs of each guild calculated using the baseline estimation methods (Global, Concomitantly, Delayed, and Interpolation) were compared with the Expected model using a Tukey HSD post hoc test following a significant ANOVA with  $\alpha = 0.05$ . Assumptions were assessed using the Shapiro-Wilk test for normality and the

Table 1. Trophic level (TL) estimates from FishBase (Froese & Pauly 2019) and published literature used as an expected model to define the best baseline method. All species sampled in Patos Lagoon Estuary between 2010 and 2018. TG: trophic guild; DTV: detritivores; HBV: herbivores; OMN: omnivores; ZPL: zooplanktivores; ZBV: zoobenthivores; PSV: piscivores

TG	Species	Describing authority	TL	Reference
DTV	<i>Cyphocharax voga</i>	(Hensel, 1869)	2.00	Froese & Pauly (2019)
DTV	<i>Mugil brevirostris</i>	(Ribeiro, 1915)	2.00	Froese & Pauly (2019)
DTV	<i>Mugil curema</i>	Valenciennes, 1836	2.00	Froese & Pauly (2019)
DTV	<i>Mugil liza</i>	Valenciennes, 1836	2.00	Froese & Pauly (2019)
DTV	<i>Mugil</i> sp.	Linnaeus, 1758	2.00	Froese & Pauly (2019)
HBV	Clupeidae		2.00	Froese & Pauly (2019)
HBV	<i>Neohelice granulata</i>	(Dana, 1851)	2.00	Bemvenuti & Colling (2010)
HBV	<i>Platanichthys platana</i>	(Regan, 1917)	3.00	Froese & Pauly (2019)
OMN	<i>Palaemon argentinus</i>	(Nobili, 1901)	2.00	Collins (1999)
OMN	<i>Astyanax eigenmanniorum</i>	Cope, 1894	2.80	Froese & Pauly (2019)
OMN	<i>Jenynsia multidentata</i>	Jenyns, 1842	2.90	Froese & Pauly (2019)
OMN	<i>Penaeus paulensis</i>	(Pérez Farfante, 1967)	3.00	Abreu et al. (2007)
ZPL	<i>Atherinella brasiliensis</i>	(Quoy & Gaimard, 1824)	3.20	Froese & Pauly (2019)
ZPL	<i>Brevoortia pectinata</i>	(Jenyns, 1842)	3.40	Froese & Pauly (2019)
ZPL	<i>Lycengraulis grossidens</i>	(Spix & Agassiz, 1829)	3.70	Froese & Pauly (2019)
ZPL	<i>Odontesthes argentinensis</i>	(Valenciennes, 1835)	3.80	Froese & Pauly (2019)
ZPL	<i>Pomatomus saltatrix</i>	(Linnaeus, 1766)	4.50	Froese & Pauly (2019)
ZBV	<i>Cyrtograpsus angulatus</i>	Dana, 1851	3.00	Bemvenuti & Colling (2010)
ZBV	<i>Micropogonias furnieri</i>	(Desmarest, 1823)	3.10	Froese & Pauly (2019)
ZBV	<i>Syngnathus folletti</i>	Herald, 1942	3.30	Froese & Pauly (2019)
ZBV	<i>Ctenogobius shufeldti</i>	(Jordan & Eigenmann, 1887)	3.40	Froese & Pauly (2019)
ZBV	<i>Citharichthys spilopterus</i>	(Günther 1862)	3.60	Froese & Pauly (2019)
ZBV	<i>Callinectes sapidus</i>	Rathbun, 1896	3.73	Oliveira et al. (2006)
PSV	<i>Oligosarcus jeninsii</i>	(Günther, 1864)	4.00	Froese & Pauly (2019)
PSV	<i>Oligosarcus robustus</i>	(Menezes, 1969)	4.20	Froese & Pauly (2019)

Bartlett test for homoscedasticity. All analyses were performed in R 4.0.0. (R Core Team 2020).

### 3. RESULTS

The Global baseline was estimated to be  $\delta^{15}\text{N} = 7.13 \pm 0.63$ . Most periods showed differences in producer  $\delta^{15}\text{N}$  values, requiring >1 baseline to be determined for the same environment (Tables S1 & S2). When >1 baseline was required, the distinction of baseline values was mainly due to differences between the planktonic vs. benthic trophic pathways (Table 2). However, this pattern was weaker when analyzing the producer data using the Interpolation method (i.e. grouping data from 2 different seasons in the analysis) (Tables 3 & S2).

The best fit model of ordered guild TL based on FishBase and literature data (Expected model) was  $\text{TL} = 0.426x + 1.534$ , with  $r^2 = 0.96$ , calculated using average TL values for each guild (Expected model 2 in Fig. 1). The best fit models of ordered guild TP estimates calculated using consumer data from Patos Lagoon Estuary and each baseline method were for the Delayed and the Global methods ( $r^2 = 0.99$  and

0.62, respectively; Table 4, Fig. 2). Guild TP estimates calculated using each baseline estimation model were significantly different from the expected values, except for estimates calculated using the Delayed baseline model (Table 5).

### 4. DISCUSSION

The choice of baselines is one of the most critical issues when computing TPs using stable isotope data (Post 2002, Hussey et al. 2014). This is because  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values of an organism may vary among ecosystems and through time. Moreover, long-lived migrating organisms, like some fishes, can incorporate the isotopic variation of producers and detritus in different ecosystems (Post 2002, Possamai et al. 2020) during different phases of their life cycle, which makes it difficult to define a unique baseline for these consumers.

In aquatic ecosystems, the variation in  $\delta^{15}\text{N}$  values of producers and hence consumers is higher than in terrestrial systems (Casey & Post 2011), due to both autochthonous and allochthonous sources of dissolved inorganic nitrogen (DIN) entering aquatic sys-

Table 2. Baselines utilized for trophic position estimates in the Concomitantly and Delayed methods. b1: baseline 1; b2: baseline 2. Values are  $\pm$ SD. For the producers that comprised each baseline, see Table S1 in the Supplement. Gaps indicate only 1 baseline found

Season	b1	$\delta^{15}\text{N}$ b1 (‰)	b2	$\delta^{15}\text{N}$ b2 (‰)
2010 summer	Benthic	6.98 $\pm$ 0.00	Planktonic	3.06 $\pm$ 2.57
2010 autumn	Benthic	6.08 $\pm$ 1.29	Planktonic	2.09 $\pm$ 1.76
2010 winter	Benthic	6.45 $\pm$ 1.04		
2010 spring	Benthic	6.85 $\pm$ 0.84	Benthic	8.98 $\pm$ 0.98
2011 summer	Benthic	5.37 $\pm$ 0.71	Benthic + planktonic	6.86 $\pm$ 0.68
2011 autumn	Benthic	8.47 $\pm$ 1.03	Planktonic	3.84 $\pm$ 0.00
2011 winter	Benthic + planktonic	6.16 $\pm$ 1.94		
2012 autumn	Benthic + planktonic	13.09 $\pm$ 6.75		
2012 winter	Benthic + planktonic	8.85 $\pm$ 1.85		
2012 spring	Benthic	9.51 $\pm$ 0.70	Benthic	6.02 $\pm$ 0.29
2013 summer	Benthic	11.13 $\pm$ 0.37	Benthic	6.52 $\pm$ 0.94
2013 autumn	Benthic + planktonic	5.17 $\pm$ 0.60		
2013 winter	Benthic + planktonic	5.91 $\pm$ 1.45		
2013 spring	Benthic	5.33 $\pm$ 1.87	Planktonic	1.00 $\pm$ 1.12
2014 summer	Benthic	14.52 $\pm$ 4.18	Planktonic	18.25 $\pm$ 1.73
2014 autumn	Benthic + planktonic	5.24 $\pm$ 2.02		
2014 spring	Benthic	13.01 $\pm$ 1.94	Benthic + planktonic	8.66 $\pm$ 0.56
2015 winter	Benthic + planktonic	9.07 $\pm$ 0.98	Benthic	4.70 $\pm$ 0.34
2015 spring	Benthic	6.30 $\pm$ 0.27	Benthic	7.98 $\pm$ 0.16
2016 summer	Benthic	6.97 $\pm$ 0.66	Planktonic	3.84 $\pm$ 0.84
2016 autumn	Benthic + planktonic	4.70 $\pm$ 1.88		
2016 winter	Benthic	3.32 $\pm$ 0.64	Benthic + planktonic	7.17 $\pm$ 0.64
2016 spring	Benthic	7.36 $\pm$ 0.65	Planktonic	4.27 $\pm$ 0.21
2017 summer	Benthic	7.97 $\pm$ 0.89	Planktonic	5.45 $\pm$ 0.62
2017 autumn	Benthic	6.25 $\pm$ 1.27	Benthic + planktonic	9.44 $\pm$ 0.83
2017 winter	Benthic + planktonic	8.29 $\pm$ 0.92		
2017 spring	Benthic	12.52 $\pm$ 0.56	Benthic + planktonic	6.26 $\pm$ 0.64
2018 summer	Benthic + planktonic	9.09 $\pm$ 4.66		
2018 autumn	Benthic	7.82 $\pm$ 1.14	Planktonic	12.68 $\pm$ 2.89

tems. In estuaries, for example, the allochthonous pool of DIN can be provided by rivers (natural pool, as well as from anthropogenic activities such as agriculture and wastewater), by the marine system, or by remineralization of organic matter from terrestrial plants and other autochthonous sources. Each of these processes brings different values of  $\delta^{15}\text{N}$ , as fixed nitrogen (e.g. by *Cyanobacteria*) has depleted values ( $-1 \pm 1\%$ ), marine sediments have enriched values

(0–6‰) (Brandes & Devol 2002), and anthropogenic activities result in even further enriched values (+6‰ above background  $\delta^{15}\text{N}$ ) (Bedard-Haughn et al. 2003). This variability is reflected in the isotopic composition of producers and is dynamic in time, resulting in a wide range of  $\delta^{15}\text{N}$  values found in estuarine producers (Casey & Post 2011, Mancinelli et al. 2013, Garcia et al. 2019b, Possamai et al. 2020, Lanari et al. 2021). In this sense, the temporal variability of  $\delta^{15}\text{N}$  must be taken into account when planning studies using stable isotope data. In the case of studies encompassing multiple time periods or long-term monitoring programs, we encourage seasonal sampling of sources and consumers to enable the use of different isotopic time lags in order to improve estimates of trophic structure. A caveat about the seasonal sampling of SPOM and macroalgae should be considered, as these sources show wide temporal and spatial variability, and thus more frequent sampling of these primary producers should be implemented.

Here, we used estuarine producers as  $\delta^{15}\text{N}$  baselines for TP estimation of estuarine consumers. It is worth noting that the same locality (an embayment within the estuary, in our study case) can support one or more baselines. Depending on the sampling period, different producers can exhibit similar  $\delta^{15}\text{N}$  values and represent a single baseline for a particular sampling station, while in another sampling period, the same producer  $\delta^{15}\text{N}$  values may differ enough to be considered different baselines. Thus, sampling multiple different organisms to establish the baseline of a sampling station is crucial

(Casey & Post 2011, Phillips et al. 2014) in order to better establish the maximum range of  $\delta^{15}\text{N}$  values and avoid isotopic mismatches (e.g. estimating TP of pelagic consumers with baselines based on benthic sources). At the same time, interspecific and intraspecific variability must be considered (Phillips et al. 2014). For example, organisms in the pelagic food web can incorporate energy from benthic food web pathways, making it important to know the feeding

Table 3. Baselines utilized for trophic position estimates in the Interpolation method. b1: baseline 1; b2: baseline 2. Values are  $\pm$ SD. For the producers that comprised each baseline, see Table S2 in the Supplement. Gaps indicate only 1 baseline found

Season	b1	$\delta^{15}\text{N}$ b1 (‰)	b2	$\delta^{15}\text{N}$ b2 (‰)
2010 summer	Benthic	6.98 $\pm$ 0.00	Planktonic	3.06 $\pm$ 2.57
2010 summer/autumn	Benthic	6.17 $\pm$ 1.23	Planktonic	1.81 $\pm$ 1.50
2010 autumn/winter	Benthic	6.13 $\pm$ 1.15	Planktonic	2.08 $\pm$ 1.75
2010 winter/spring	Benthic	7.24 $\pm$ 1.19	Benthic	5.94 $\pm$ 0.93
2010 spring/2011 summer	Benthic + planktonic	6.58 $\pm$ 0.95	Benthic	8.30 $\pm$ 1.19
2011 summer/autumn	Benthic + planktonic	6.55 $\pm$ 1.28		
2011 autumn/winter	Benthic + planktonic	6.27 $\pm$ 2.03		
2011 winter/2012 autumn	Benthic + planktonic	7.54 $\pm$ 4.45		
2012 autumn/winter	Benthic + planktonic	10.39 $\pm$ 4.79		
2012 winter/spring	Benthic + planktonic	7.80 $\pm$ 1.96		
2012 spring/2013 summer	Benthic	10.32 $\pm$ 0.98	Benthic	5.84 $\pm$ 0.36
2013 summer/autumn	Benthic	8.39 $\pm$ 2.76	Benthic	5.16 $\pm$ 0.46
2013 autumn/winter	Benthic	5.62 $\pm$ 1.25		
2013 winter/spring	Benthic	5.64 $\pm$ 1.68	Planktonic	0.99 $\pm$ 1.11
2013 spring/2014 summer	Benthic + planktonic	10.61 $\pm$ 6.59		
2014 summer/autumn	Benthic + planktonic	10.27 $\pm$ 5.98		
2014 autumn/spring	Benthic	11.02 $\pm$ 3.60	Benthic + planktonic	5.91 $\pm$ 2.03
2014 spring/2015 winter	Benthic	4.70 $\pm$ 0.34	Benthic + planktonic	8.79 $\pm$ 0.82
2015 winter/spring	Benthic + planktonic	8.74 $\pm$ 0.96	Benthic	5.10 $\pm$ 0.76
2015 spring/2016 summer	Benthic + planktonic	5.25 $\pm$ 1.42	Benthic	7.74 $\pm$ 0.27
2016 summer/autumn	Benthic + planktonic	5.18 $\pm$ 1.86		
2016 autumn/winter	Benthic + planktonic	5.68 $\pm$ 1.94		
2016 winter/spring	Benthic + planktonic	4.95 $\pm$ 1.44	Benthic	7.41 $\pm$ 0.49
2016 spring/2017 summer	Benthic + planktonic	5.57 $\pm$ 1.20	Benthic	7.66 $\pm$ 0.83
2017 summer/autumn	Benthic + planktonic	7.14 $\pm$ 1.60		
2017 autumn/winter	Benthic + planktonic	7.87 $\pm$ 1.49		
2017 winter/spring	Benthic	10.22 $\pm$ 2.63	Benthic + planktonic	7.18 $\pm$ 1.18
2017 spring/2018 summer	Benthic	13.85 $\pm$ 3.48	Benthic + planktonic	6.81 $\pm$ 2.22
2018 summer/autumn	Benthic + planktonic	10.18 $\pm$ 3.71	Benthic + planktonic	6.53 $\pm$ 3.00

habits of the studied species before field-sampling campaigns for collecting samples for SIA (Mancinelli et al. 2013, Phillips et al. 2014). In general, it is important to sample all of the possible basal sources in the environment to better estimate TPs as well as the relative contributions of sources to consumers in mixing models.

In addition to spatial and temporal variation in isotope ratios, differences in isotopic turnover rates of consumers and producers should be considered in isotopic analyses of food web structure (Vander Zanden & Rasmussen 2001, Hussey et al. 2014, Lanari et al. 2021). Smaller organisms with fast growth rates show quicker isotopic incorporation of food in their tissues than larger, slower-growth organisms (Fry & Arnold 1982). The choice of isotopic baselines should consider the potential isotopic mismatch due to such differences. In our study, consumers (especially fishes) have much longer lifespans than most primary producers (e.g. phytoplankton) and they have differences in growth rates, metabolism and ontogeny, which influence isotopic assimilation rate (Fry &

Arnold 1982, Phillips et al. 2014). This likely explains why the Delayed baseline estimation method gave the best results in our study. The time lag used in the Delayed method was 3 mo, which approximates the isotopic turnover time expected for food assimilation and incorporation in tissues of small fishes (Heady & Moore 2013, Mont'Alverne et al. 2016, Oliveira et al. 2017). This turnover time could be longer for macrocrustaceans, estimated between 4 and 6 mo (Suring & Wing 2009, deVries et al. 2015). In contrast, the isotopic baseline estimated using the Concomitantly method, which has been commonly used in trophic studies employing SIA (e.g. Hoenighaus et al. 2008, Mancinelli et al. 2013, Saigo et al. 2015, Mazumder et al. 2017, Possamai et al. 2020), did not render good fits, likely because it failed to account for isotopic turnover between consumers and their assimilated food. The Global baseline estimation method is also commonly used (Anderson & Cabana 2007, Svanbäck et al. 2015, Bastos et al. 2017) and yielded a poor fit, although it was better than the Concomitantly method. Our findings reinforce the need to

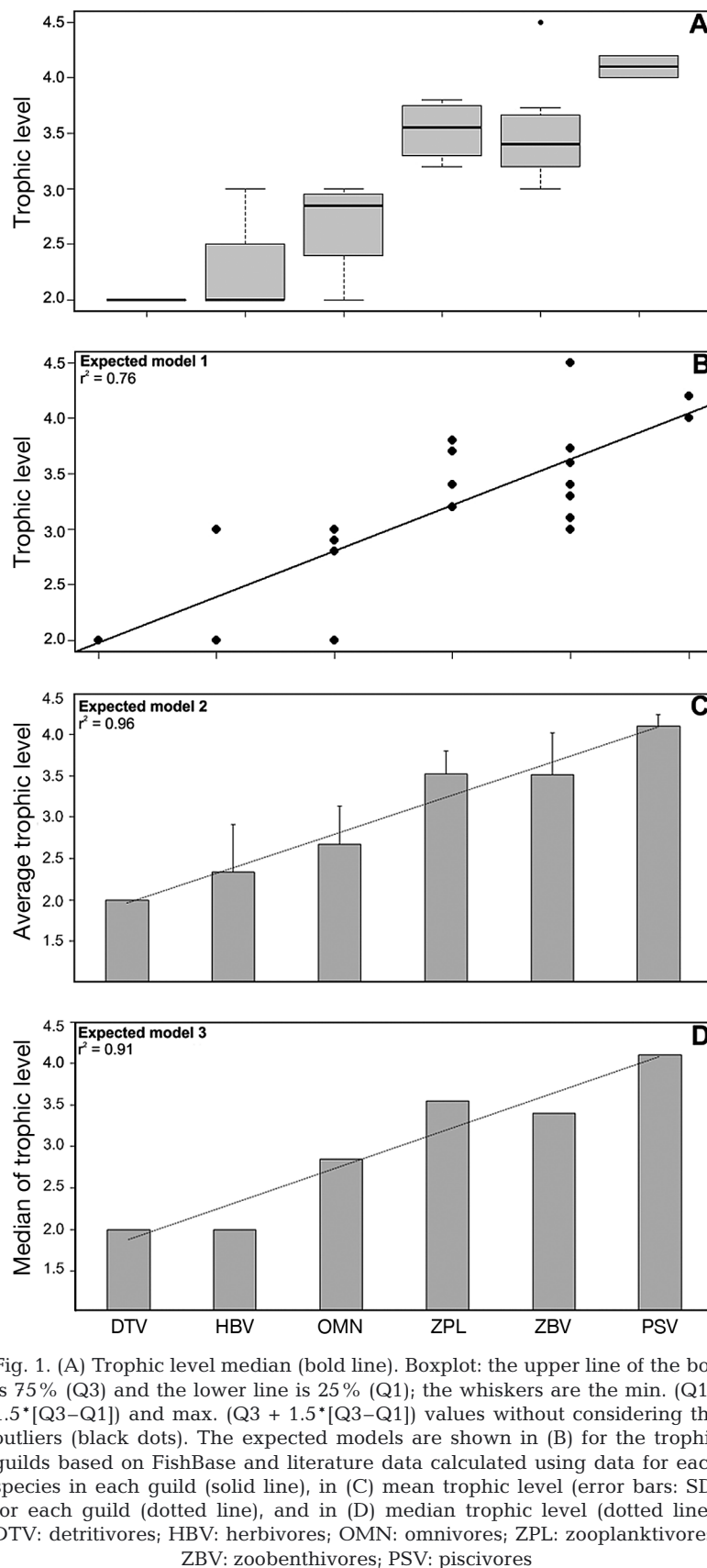


Fig. 1. (A) Trophic level median (bold line). Boxplot: the upper line of the box is 75% (Q3) and the lower line is 25% (Q1); the whiskers are the min. ( $Q1 - 1.5 \cdot [Q3 - Q1]$ ) and max. ( $Q3 + 1.5 \cdot [Q3 - Q1]$ ) values without considering the outliers (black dots). The expected models are shown in (B) for the trophic guilds based on FishBase and literature data calculated using data for each species in each guild (solid line), in (C) mean trophic level (error bars: SD) for each guild (dotted line), and in (D) median trophic level (dotted line). DTV: detritivores; HBV: herbivores; OMN: omnivores; ZPL: zooplanktivores; ZBV: zoobenthivores; PSV: piscivores

have an appropriate sampling design of primary food sources to match the isotopic composition of the consumers, before computing their TPs (Post 2002, Phillips et al. 2014, Lanari et al. 2021). It is important to evaluate the lifespan and isotopic turnover of the consumers before deciding on the frequency and the adequate time intervals to isotopically match possible delays between food sources and consumers.

Primary consumers are often recommended as indicators of baseline values because they incorporate the isotopic variability of the producers they feed upon and have similar isotopic turnover rates in comparison with other consumers in the food web (Vander Zanden & Rasmussen 1999, 2001, Post 2002, Mancinelli et al. 2013). Here, we used  $\delta^{15}\text{N}$  values of producers as baselines to estimate consumer TPs. We sampled all of the producers available in this estuarine system, collected replicates for each source, and tested the  $\delta^{15}\text{N}$  values to better aggregate sources into different baselines for each sampling event. Although producers can have highly variable isotopic values, that variation can be used to actually improve estimates of trophic interactions and food web structure if the dataset sufficiently captures important components of spatial and temporal variation and the correct baseline estimation method is used. Moreover, estuarine ecosystems are highly dynamic, and in our study system in particular, there were no abundant and/or frequent benthic primary consumers that could be collected in all sampling events during the 9 yr of field campaigns. Zooplankton were consistently present and could feasibly be used to estimate baselines; however, the majority of the fish species in our study system are zoobenthivorous (Possamai et al. 2018, A. C. G. Mai & B. Possamai unpubl. data), so using just a planktonic baseline does not align with stable isotope best practices (Mancinelli et al. 2013, Phillips et al. 2014). Previous studies and per-



Table 4. Linear models to estimate guild trophic levels (TLs) and trophic positions (TPs) based on different methods of baseline estimation. Trophic guilds represented by detritivores (DTV), herbivores (HBV), omnivores (OMN), zooplanktivores (ZPL), zoo-benthivores (ZBV) and piscivores (PSV).  $x$ : trophic guild order (1 for DTV, 2 for HBV, 3 for OMN, 4 for ZPL, 5 for ZBV and 6 for PSV)

Method	Model	DTV	HBV	OMN	ZPL	ZBV	PSV
Expected	$TL = 0.426x + 1.534$	1.960	2.386	2.812	3.238	3.664	4.090
Global	$TP = 0.129x + 2.886$	3.015	3.144	3.273	3.402	3.531	3.660
Concomitantly	$TP = 0.008x + 2.712$	2.720	2.728	2.736	2.744	3.009	3.108
Delayed	$TP = 0.099x + 2.514$	2.613	2.712	2.811	2.910	2.752	2.760
Interpolation	$TP = 0.036x + 2.619$	2.655	2.691	2.727	2.763	2.799	2.835

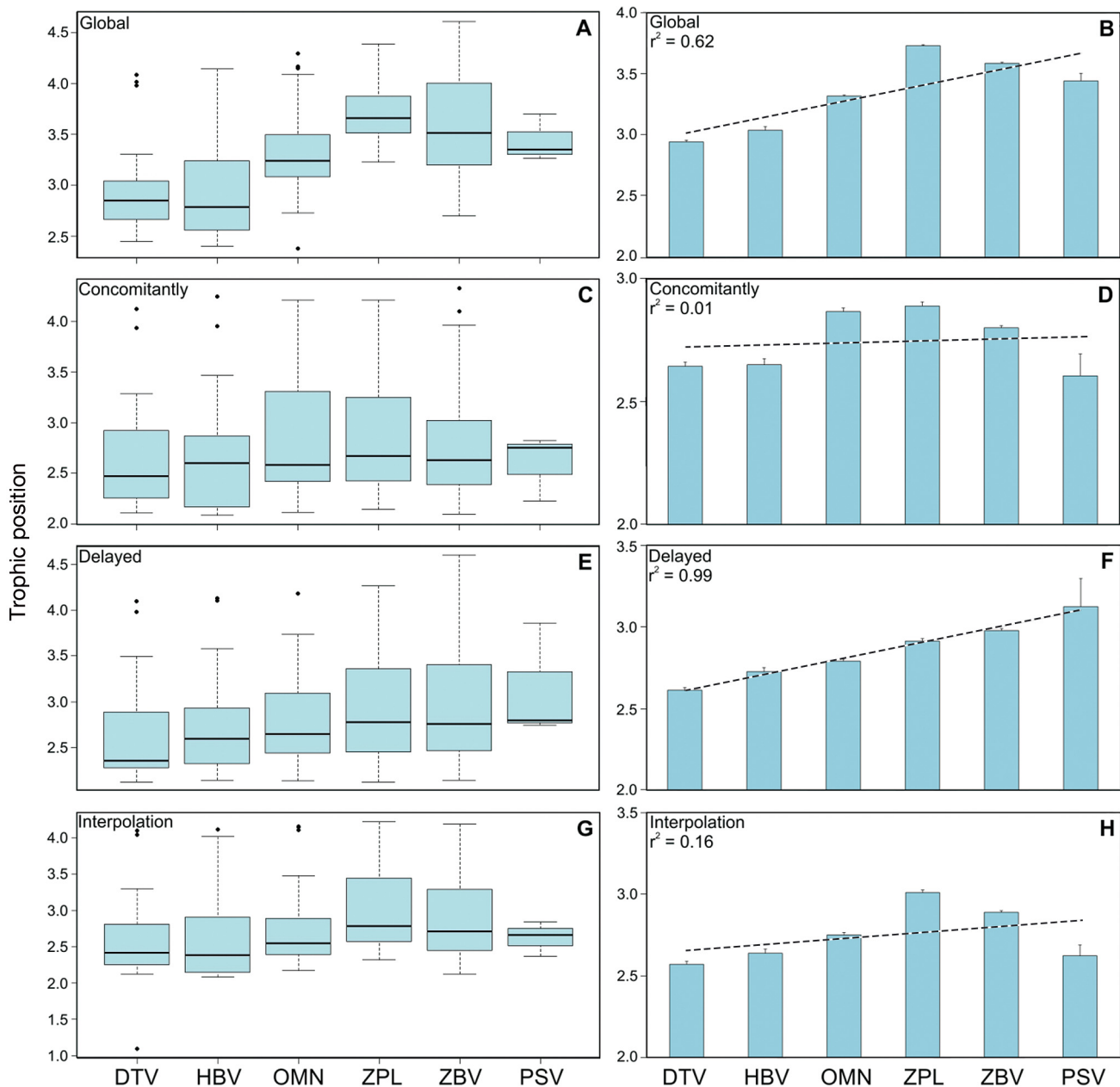


Fig. 2. Using the (A,B) Global, (C,D) Concomitantly, (E,F) Delayed, and (G,H) Interpolation methods: (A,C,E,G) trophic position (TP) median for each estuarine trophic guild based on consumer data from Patos Lagoon Estuary (2010–2018) calculated using the different baseline estimation methods, and (B,D,F,H) linear models (dashed lines) assessing fit with increasing trophic position for the ordered trophic guilds (bars are TP mean  $\pm$  SD). See Fig. 1 for abbreviations and boxplot limits

Table 5. ANOVA comparing expected trophic position (TP) and estimated TPs from all baseline estimation methods (Global, Concomitantly, Delayed, and Interpolation). Post hoc results are for pairwise comparisons between expected TP values and TP estimates calculated using each baseline method, with non-significance at  $\alpha = 0.05$  indicating close fit between expected values and TPs estimated from the associated baseline method. Upper and lower indicate the 95% CI

Variable	F	df	p
Method	14.88	4	<0.001
Trophic guild	67.82	1	<0.001
Method $\times$ Trophic guild	19.37	4	<0.001
<b>Pairwise comparison, Tukey HSD test</b>	lower	upper	p
Expected–Global	0.039	0.588	0.020
Expected–Concomitantly	0.011	0.560	0.038
Expected–Delayed	–0.011	0.437	0.412
Expected–Interpolation	–0.553	–0.004	0.045

spectives have provided a number of recommendations for quantifying isotopic baselines and consumer TPs (i.e. Vander Zanden & Rasmussen 1999, 2001, Post 2002, Mancinelli et al. 2013), and those recommendations should be considered and used whenever possible. However, for systems similar to Patos Lagoon Estuary that lack primary consumers that are abundant and/or available year-round and reliably reflect a trophic pathway of interest (e.g. benthic or pelagic), our approach using robust temporal sampling of primary consumers can be a good alternative for estimating baseline  $\delta^{15}\text{N}$  values for quantification of consumer TPs.

Combined with robust estimation of baseline  $\delta^{15}\text{N}$  values, improved understanding of factors affecting trophic enrichment and novel methods for estimating consumer TP contribute to refined ability to quantify food web attributes with stable isotope data. As previously mentioned, trophic enrichment factors (TEFs) of nitrogen and carbon can vary greatly among organisms due to differences in diet, physiology and metabolism (Vander Zanden & Rasmussen 2001, Manetta et al. 2003, Vander Zanden & Fetzer 2007, Hussey et al. 2014, Bastos et al. 2017). The increase in experimental studies estimating TEFs (Suring & Wing 2009, Heady & Moore 2013, deVries et al. 2015, Mont'Alverne et al. 2016, Oliveira et al. 2017) is helping to improve TP estimation, as well as source apportionment estimates using mixing models. Another notable improvement for estimating TP is the tRophic-Position package used in this study (Quezada-Romegialli et al. 2018). tRophicPosition incorporates Bayesian modeling of consumer TP and can accommodate 2 baselines by using a mixing model with carbon and nitrogen isotope ratios to differentiate

source pools when baseline heterogeneity is present. This is a significant advance over the common approach of estimating consumer TP by taking the difference in  $\delta^{15}\text{N}$  between the top consumer (i.e. highest observed value of  $\delta^{15}\text{N}$ ) and a single baseline and dividing by the chosen TEF without accounting for error in baseline and consumer values, TEF or baseline heterogeneity.

In conclusion, our findings demonstrate that incorporating isotopic time lags can improve estimation of baseline  $\delta^{15}\text{N}$  values and subsequently estimates of consumer TP. Our long-term dataset is well suited to assess the performance of various methods to

estimate baseline  $\delta^{15}\text{N}$ , but the frequency of sampling was actually intended to capture seasonal variation in trophic structure and was not specifically planned with isotopic time lags in mind. This highlights an important implication of our results — future studies should directly incorporate isotopic time lags in the study design before conducting field sampling campaigns, ideally based on knowledge of diets and isotopic turnover rates of the target species. Such foresight will avoid consumer–source isotopic mismatches in mixing models and TP estimates, and when combined with other advances such as experimentally determined TEFs and novel modeling approaches that incorporate variation in parameter estimates and baseline heterogeneity, should provide more robust estimates of food web structure.

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