

Isotopic trophic discrimination factors ($\Delta^{13}\text{C}$, $\Delta^{15}\text{N}$) and their determinants in a subtropical macroinvertebrate food chain

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ABSTRACT

Isotopic trophic discrimination factors ($\Delta^{13}\text{C}$, $\Delta^{15}\text{N}$) and their determinants in a subtropical macroinvertebrate food chain.

The accurate estimation of trophic discrimination factors (TDFs) for stable carbon and nitrogen isotopes ($\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$) requires studies for each particular species or taxonomic group, because the use of general TDFs values for all animals obtained from the literature represents an important bias in isotopic modelling. Values for $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ were estimated in an aquatic food chain (periphyton-Chironomini-Perithemis sp.), in a subtropical region of South America, under experimental conditions using two approaches: i) Traditional arithmetic equation and ii) Bayesian inference from mixing models. The effect of diet quality on TDF variability was also evaluated. We report values for $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ for Chironomini when feeding on periphyton ($1.12 \pm 1.31\text{‰}$ and $0.92 \pm 1.94\text{‰}$ for C and N, respectively using the arithmetic equation) and for Perithemis sp. when feeding on Chironomini ($0.65 \pm 1.52\text{‰}$ and $0.90 \pm 1.08\text{‰}$ for C and N, respectively, according to the arithmetic equation). We obtained similar results when using Bayesian inference. We did not find effects of diet quality on $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ values; although we highlighted that, unexpectedly, the taxonomic composition of periphyton strongly affected the isotopic values of C and N. These reported values improve the accuracy of isotopic modeling for subtropical aquatic macroinvertebrates in future food web research.

KEY WORDS: trophic enrichment; isotope fractionation; diet-consumer isotope variation.

RESUMEN

Factores de discriminación trófica isotópica ($\Delta^{13}\text{C}$, $\Delta^{15}\text{N}$) y sus determinantes en una cadena alimenticia de macroinvertebrados subtropicales.

La estimación precisa de los factores de discriminación trófica (TDFs) para isótopos estables de carbono y nitrógeno ($\Delta^{13}\text{C}$ y $\Delta^{15}\text{N}$) requiere estudios para cada especie o grupo taxonómico en particular; porque el uso de valores generales de TDFs para todos los animales obtenidos de la literatura, representa un sesgo importante en el modelado isotópico. Se estimaron valores de $\Delta^{13}\text{C}$ y $\Delta^{15}\text{N}$ en una cadena alimentaria acuática (perifiton-Chironomini-Perithemis sp.), en una región subtropical de América del Sur, bajo condiciones experimentales utilizando dos enfoques: i) Ecuación aritmética tradicional e ii) Inferencia bayesiana a partir de modelos de mezcla. También se evaluó el efecto de la calidad de la

dieta sobre la variabilidad de los TDFs. Reportamos valores de $\Delta^{13}\text{C}$ y $\Delta^{15}\text{N}$ para Chironomini cuando se alimentan de perifiton ($1.12 \pm 1.31\text{‰}$ y $0.92 \pm 1.94\text{‰}$ para C y N, respectivamente, usando la ecuación aritmética) y para Perithemis sp. cuando se alimentan de Chironomini ($0.65 \pm 1.52\text{‰}$ y $0.90 \pm 1.08\text{‰}$ para C y N, respectivamente, de acuerdo con la ecuación aritmética). Obtuvimos resultados similares cuando usamos inferencia bayesiana. No encontramos efectos de la calidad de la dieta en los valores de $\Delta^{13}\text{C}$ y $\Delta^{15}\text{N}$; aunque destacamos que, inesperadamente, la composición taxonómica del perifiton afectó fuertemente los valores isotópicos de C y N. Estos valores reportados mejoran la precisión del modelado isotópico para macroinvertebrados acuáticos subtropicales en futuras investigaciones sobre redes tróficas.

PALABRAS CLAVE: Enriquecimiento trófico; fraccionamiento isotópico; variación isotópica dieta-consumidor.

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INTRODUCTION

Trophic interactions can be quantified using Stable Isotope Analysis (SIA) of Carbon (C) and Nitrogen (N) (Post, 2002; Carreon-Martinez, 2010; Boecklen et al., 2011; Layman et al., 2012). In this sense, C and N isotopes are excellent trophic indicators (Gu et al., 1996), whereby C isotopes can be used to trace the origin of the dietary carbon source assimilated by consumers (DeNiro & Epstein, 1978; Fry & Sherr, 1984; Gearing, 1991), while N isotopes are especially useful for determining consumer trophic level (Hesslein et al., 1991; Post, 2002). The main premise of SIA is that the carbon and nitrogen assimilated in the body tissues of any organism, originate in the prey they have ingested throughout their lives (Schoeller, 1999). In particular, SIA makes use of natural differences in the abundance of isotopes ^{13}C and ^{12}C , and ^{15}N and ^{14}N , between the tissues of consumers and that of their resources (DeNiro & Epstein, 1978). These differences are mainly attributed to selective ^{13}C uptake during digestion by the consumer via preferential ^{12}C loss during respiration, as well as preferential ^{14}N loss during excretion (Olive et al., 2003). As such, the tissues of consumers usually become enriched in the heavier isotopes (^{13}C and ^{15}N) relative to isotope ratios in their diet (Wada et al., 1991; Martínez del Río et al., 2009). This predictable change in stable isotope ratios of C ($^{13}\text{C}/^{12}\text{C}$) and N ($^{15}\text{N}/^{14}\text{N}$) relative to a standard (denoted as $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values) between the consumer and its prey is known as trophic discrimination factor (TDF, denoted by Δ) (DeNiro & Epstein, 1981; Gannes et al., 1997; Fry, 2006). TDFs can be estimated simply as $\Delta = \delta_{\text{consumer}} - \delta_{\text{diet}}$ (Vanderklift & Ponsard, 2003; Martínez del Río

et al., 2009; Scharnweber et al., 2021).

Isotopic mixing models allow for the quantification of the contribution of each of the dietary resources assimilated into consumer tissues (Phillips, 2001; Phillips & Gregg, 2003; Fry, 2006; Phillips et al., 2014), adopting Bayesian statistical approaches that incorporate the uncertainty associated with TDFs (Bond & Diamond, 2011; Parnell et al., 2013). To obtain a precise estimation of the contribution of a certain food item to a given consumer, and in addition to knowing the isotopic values of the consumer and their sources, it is essential to have information on the TDF. This is because different preys have different isotopic compositions and are metabolized differently after ingestion by the consumer. The TDFs of different organisms have been traditionally determined by experiments under controlled conditions where known diets are given to the consumer (e.g. Bearhop et al., 2002; Kelly et al., 2012; Kurle et al., 2013). As these experiments are often expensive and time consuming, TDFs have currently been established for relatively few species (e.g. Dalerum & Angerbjörn, 2005; Robbins et al., 2005; Crawford et al., 2008; Kelly et al., 2012), which has led to the use of estimated fractionation values for taxonomically related species or generic values from different groups of organisms (see reviews by Post, 2002; McCutchan et al., 2003). Alternatively, researchers have also used observed differences in isotopic values of consumers and prey collected in the field to directly establish TDFs (e.g. Bunn et al., 2013; Stephens et al., 2022), particularly in scenarios in which TDF is meant to be described at a broader level including several potential preys regularly included in diet (e.g. Bunn et al., 2013). In this sense, a recent meta-analysis supported the use of

field-derived estimates as they proved to be useful estimates to fill gaps in the knowledge of TDFs, where information on isotope ratios in natural diets and their specific fractionation by consumers are absent (Stephens et al., 2022). Nevertheless, recent studies highlight that experimental trial with an unrealistic diet can severely bias TDF estimates, which strongly depends upon diet type. Furthermore, natural diets should be considered when selecting appropriate TDF values to model food resource contribution to consumer biomass based on isotopic data (Stephens et al., 2022, 2023).

According to the literature, $\delta^{13}\text{C}$ changes minimally between trophic transfers, registering a mean enrichment of approximately 0‰–1‰ per trophic level (Rounick & Winterbourn, 1986; McCutchan et al., 2003). Alternatively, $\delta^{15}\text{N}$ generally increases on average between 3‰–4‰ for each trophic transfer (Minagawa & Wada, 1984; Vander Zanden & Rasmussen, 2001; Post, 2002). Although the use of these mean fractionation values are employed in many trophic ecology studies (e.g. De Niro & Epstein, 1978, 1981; Vander Zanden & Rasmussen, 2001; Post, 2002), their use can lead to imprecise conclusions, particularly given that TDFs have been shown to vary among species and ecosystem types (e.g. aquatic vs. terrestrial) (McCutchan et al., 2003; Dubois et al., 2007; Caut et al., 2009; Stephens et al., 2022, 2023). In addition, the vast majority of TDFs reported to date, including those used as reference values; originate from studies of species from temperate regions, with limited consideration of species from other climate regions, including tropical and subtropical regions (e.g. de Carvalho et al., 2015; Wang et al., 2020). This lack of information could potentially affect the interpretation of food web functioning, as conclusions drawn from mixing model analyses are highly dependent on the precision of these values (Phillips et al., 2014; Stephens et al., 2022).

In addition to the high variability in TDFs among species (e.g. Macko et al., 1982; Minagawa & Wada, 1984; Dubois et al., 2007; Caut et al., 2009), there are multiple factors that can cause intra-specific variability in TDF values. These include the type of tissue analyzed (Tieszen et al., 1983), efficiency in resource assimilation by the

consumer (Gannes et al., 1997), and the quantity and quality of the resource consumed (Hobson & Clark, 1993; Brauns et al., 2018). Regarding the latter, the C:N ratio of food sources has been used as a measure of resource quality, and significant relationships have been established between this variable and the TDFs of C and N in different organisms (Adams & Sterner, 2000; Vanderklift & Ponsard, 2003; Brauns et al., 2018). More recently, the relationships between TDFs and the quality of consumed resources have been integrated into a broader stoichiometric framework (Brauns et al., 2018). Ecological stoichiometric studies analyze organisms in terms of their elemental composition, particularly in terms of C and N (Elser et al., 1996; Sterner & Elser, 2002). For example, the relative abundance of C with respect to N has been associated with varying growth rates of different organisms. In general, limiting elements will transfer into the food web with high efficiency. Hence, when a consumer has a diet with a high C:N ratio, N is used with a high efficiency, while a good part of the C is not assimilated or is either excreted or respired (Cross et al., 2003; Elser & Hessen, 2005).

Stoichiometry predicts that diet quality is adjusted according to consumer requirements as optimal growth is only attainable when the quality of the resource is within the range of C:N of the consumer. This concept is called consumer-resource imbalance (Sterner & Elser, 2002). As previously mentioned, isotopic discrimination depends on the metabolic processes of organisms, and thus the stoichiometric ratios of C:N could be determinant factors in the variation of TDFs of C and N between the consumers and their prey. Specifically, the C:N ratio of the resource (and therefore the C:N imbalance between the consumer and food sources) is an influential factor in the variation of $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ for aquatic invertebrates (Brauns et al., 2018). This emphasizes the importance of including stoichiometric information into experimental studies that aim to estimate TDFs for C and N stable isotopes with greater certainty.

In this study, we estimate the values of $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ through a mesocosm experiment using a simple, but realistic, aquatic trophic chain from a subtropical region of South America. This chain incorporates the energy transfer from periphyton

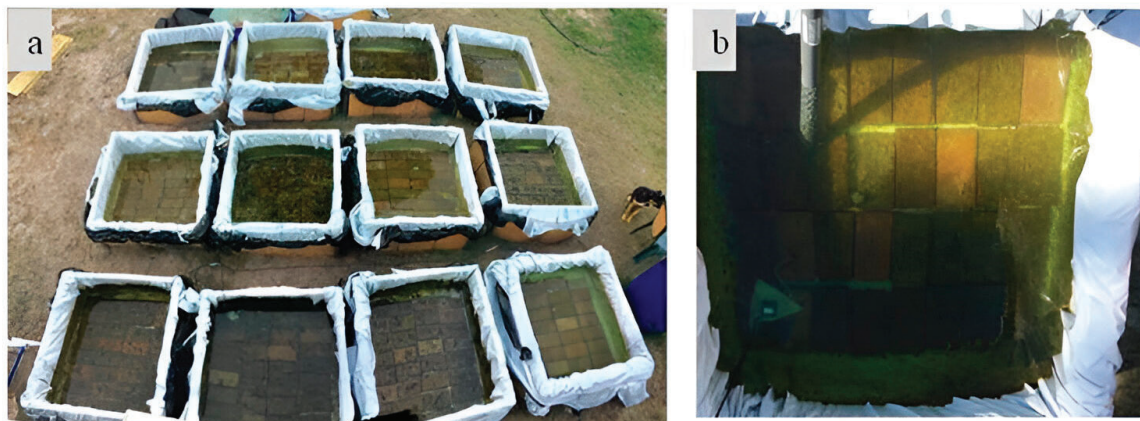


Figure 1 **a.** Experimental design: 12 mesocosm systems with 32 bricks placed along the bottom, functioning as artificial substrates for the colonization of periphyton and invertebrates. **b.** Mesocosm colonized by periphyton and Chironomidae during the recording of *in situ* physicochemical data. **a.** *Diseño experimental: 12 mesocosmos con 32 ladrillos en el fondo, funcionando como sustratos artificiales para la colonización de perifiton e invertebrados.* **b.** *Mesocosmo colonizado por perifiton y Chironomidae durante el registro de datos físicoquímicos in situ.*

to chironomids (Chironomini tribe; primary consumers) and from chironomids to odonates (Libellulidae, *Perithemis* sp.; secondary consumers). Our overall aim was to provide inputs to improve the accuracy of isotopic modelling for these aquatic subtropical consumers in future research. We use two approaches to estimate TDFs: (i) arithmetic equation, and (ii) Bayesian inference as a complementary and novel method (Parnell & Inger, 2016; Healy et al., 2018). In addition, we tested whether food quality was a predictor of $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ among these organisms, hypothesizing that resource and consumer tissue C:N ratios, as well as stoichiometric imbalances between consumers and resources and the taxonomic composition of periphyton (biomass of diatoms, cyanophytes, and chlorophytes) are influential factors in the variation of TDF values in the consumers.

MATERIAL AND METHODS

Experimental design

The experiment was conducted during the late summer-autumn of 2018 in the Department of Canelones, Uruguay ($34^{\circ}45'95''\text{S}$, $55^{\circ}50'90''\text{W}$), from March 5th to April 13th, and consisted of the installation of 12 mesocosm systems (size: 1.0 m long, 1.0 m wide x 0.5 m high) of 350 liters each, placing 32 bricks (0.086 m^2 in area) inside,

at the bottom of each system (Fig. 1). The bricks acted as artificial substrates for colonization by periphyton and macroinvertebrates. The mesocosms were filled with water extracted from a eutrophic subtropical lake, Kennedy Lake, Canelones Department ($34^{\circ}50'38''\text{S}$, $56^{\circ}0'19''\text{W}$). The region where water was collected is characterized as a humid subtropical climate according to the Köppen-Geiger climate classification system (Cfa; Beck et al., 2018) with an average annual temperature of 17°C and an accumulated annual precipitation of 1163 mm/y (INUMET, period 1991-2020, Department of Canelones, Uruguay). During the experiment, aerators were used to provide oxygen and recirculate the water. From the onset of the experiment, a qualitative sampling of artificial substrates was performed every 5 days. The initial sampling confirmed that no macroinvertebrates were present at the beginning of the experiment. By one week after the start date, on March 20th, chironomids were observed in all mesocosms; by March 30th small-sized odonates appeared and consistently increased in size and abundance as the experiment advanced. By April 13th odonates were observed frequently consuming chironomids and a full food chain was established in the mesocosms. Regardless of the origin of macroinvertebrates, as either eggs in the lake water or as eggs oviposited in the mesocosms by adults), all the biomass of insect larvae (chirono-

mids and odonates) and periphyton was originated in enclosed conditions and carbon and nitrogen in consumer tissues was coming from their prey and its diets. This experimental setup allowed us to determine TDFs for these two links: periphyton-chironomids and chironomids-odonates.

Stable Isotope sample collection and preparation

To obtain samples for analysis of C and N isotopes of algae and macroinvertebrates, three bricks were randomly selected from each of the mesocosms approximately seven weeks after the start date on April 13th. All macroinvertebrates found were collected, and periphyton samples were obtained by scraping the entire surface of each selected substrate. Physicochemical parameters of the water were measured with a YSI V6600 multiparameter probe and samples were taken for nutrient analysis (total phosphorus and nitrogen). Periphyton biomass was also measured (selecting three bricks at random from each mesocosm), using a fluorometer (Benthos Torch, bbe-Moldaenke), which quantifies the total biomass of diatoms, cyanophytes and chlorophytes.

Samples were kept frozen (-20°C) until their subsequent analysis in the laboratory. The macroinvertebrates collected were identified to the minimum possible taxonomic level, using a binocular stereomicroscope and taxonomic keys (Domínguez & Fernández, 2009). In addition to chironomids and odonates, other macroinvertebrates were identified: Ephyridae (Diptera) and Baetidae (Ephemeroptera), which altogether recorded relative abundances of < 5% within each sampled mesocosm. As these two groups were exclusively found in only two mesocosms, they were considered negligible for this study. At least one bulk sample of 1-5 individuals of each macroinvertebrate taxa was taken from each mesocosm system, such that each mesocosm served as a replicate. All samples were rinsed with distilled water to carefully remove debris from the field collection container and prepared following the standardized procedures described by Levin & Currin (2012). Subsequently, the samples were oven-dried (for 48 h at 60°C), weighed (0.5-1.5 mg for invertebrates, 3-5 mg for periphyton), placed in tin capsules and shipped for isotope analysis by a continuous flow isotope ratio mass spectrometer (IR-MS) at UC Davis, California,

USA. The natural abundance of stable C and N heavy and light isotopes ($^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$) was estimated in relation to the proportions of these isotopes in a standard. Nitrogen in atmospheric air served as standard for $\delta^{15}\text{N}$ and Pee Dee Belemnite as standard for $\delta^{13}\text{C}$ measurements. Isotopic values were expressed as $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ (‰) = $(R_{\text{sample}} \times R_{\text{standard}}) / R_{\text{standard}} \times 1000$. These data were used for all subsequent analyses.

Data analysis

The collected chironomids were identified as individuals of the tribe Chironomini (Chironomidae, Diptera), while the odonates belonged to the genus *Perithemis* sp. (Libellulidae, Odonata). These organisms and the periphyton were analyzed considering 11 mesocosms (since in one of them not enough samples were collected). According to previous studies, “dragonflies” are predatory insects (or secondary consumers) that preferentially consume dipteran larvae (Corbet, 1999, Ocon et al., 2013), consistent with our visual observations of dragonfly larvae feeding on these chironomids in the mesocosms. Chironomidae larvae, particularly those belonging to the subfamily Chironominae, have been described as primary consumers, feeding on organic matter, specifically periphyton (Galizzi et al., 2012; Henriques-Oliveira et al., 2012). As a high percentage of lipids in the samples can distort $\delta^{13}\text{C}$ values relative to that expected by trophic discrimination (McConnaughey & McRoy, 1979; Post, 2002; Logan et al., 2008), a lipid correction was applied prior to statistical analyses to the $\delta^{13}\text{C}$ values of macroinvertebrate samples with C:N ratios > 3.5. For this correction, we used the equation proposed by Post et al. (2007) for aquatic animals: $\Delta^{13}\text{C} = -3.32 + 0.99 \times \text{C:N}$.

The relationships *Perithemis* sp.-Chironomini-periphyton were first corroborated through the construction of a biplot of $\delta^{15}\text{N}$ vs. $\delta^{13}\text{C}$ between each consumer and their diet. Subsequently, the relationships between mean $\delta^{13}\text{C}_{\text{consumer}}$ - mean $\delta^{13}\text{C}_{\text{diet}}$ and mean $\delta^{15}\text{N}_{\text{consumer}}$ - mean $\delta^{15}\text{N}_{\text{diet}}$ were tested using Linear Models (LM, $\alpha=0.05$), using the isotopic value of the consumer as the response variable and the isotopic value of the

diet as the predictor.

The TDF (Δ) between the selected organisms were first estimated by the arithmetic equation previously used by several authors (e.g. Vanderklift & Ponsard, 2003; Martínez del Río et al., 2009; Scharnweber et al., 2021) for both C and N:

$$\Delta^{13}\text{C} = \delta^{13}\text{C}_{\text{consumer}} - \delta^{13}\text{C}_{\text{diet}}$$

$$\Delta^{15}\text{N} = \delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{diet}}$$

For these estimations, we used the mean value per mesocosm for $\delta^{13}\text{C}$ y $\delta^{15}\text{N}$ in each case.

In addition, the “*simmr*” package for stable isotope mixing models in R was used as a complementary method for the estimation of the TDFs (Govan et al., 2023). This package is a useful and novel tool to estimate isotope fractionation in feeding studies under controlled conditions, where the consumer receives a known diet (Parnell & Inger 2016). The $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of each consumer and the known dietary proportions for each organism (a single food item in each case in this study) were provided, and the median and 95% credibility intervals for the TDFs for chironomids and odonates were obtained using the “*simmr_mcmc_tdf*” function. In order to evaluate the performance of both TDF estimation approaches, we also tested for correlations between arithmetic equation-derived TDFs and the Bayesian estimate of mean TDF using mean values from each mesocosm (LM, $\alpha=0.05$). We expected that a good fit in the correlation would validate the similarity of both approaches.

To evaluate if the quality of the diet was a determinant factor for variation of the TDFs, linear models (LM, $\alpha=0.05$) were adjusted to evaluate the relationship between $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ (estimated using the arithmetic equation) and the C:N ratio of the resource (i.e. diet), the C:N ratio of the consumer, and the stoichiometric imbalance of C:N between the consumer and the resource ($\text{C:N}_{\text{consumer}} - \text{C:N}_{\text{diet}}$), following Brauns et al. (2018). The influence of the taxonomic composition of periphyton on estimated TDFs was also evaluated using LMs. These LMs ($\alpha=0.05$) were adjusted by using TDF values as the response variables and the average biomass of diatoms,

cyanophytes and chlorophytes per mesocosm as the predictors. This was based on the fact that, for example, diatoms have a high proportion of essential fatty acids, higher than those of blue-green algae, making them nutritionally superior (Hayashi et al., 1986), potentially affecting trophic discrimination of consumers. Additionally, the effect of periphyton taxonomical composition over its isotopic values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) was tested through the adjustment of linear models. According to previous studies, periphyton isotopic values in freshwater ecosystems show high variability (e.g. McLeod & Barton, 1988; SyvÄRanta et al., 2006; Camillieri & Ozersky, 2019), which could be related to the relative biomass of the different algal groups comprising the periphyton and could ultimately translate into changes in trophic discrimination.

Prior to fitting each model, the goodness of fit of normal and lognormal distributions for each response variable was tested, using the functions “*fitdist*” and “*gofstat*” from the package “*fitdistrplus*” (Delignette-Muller & Dutang, 2015). Visual inspection of the residual plots in the “*ggplot2*” package (Wickham, 2016) was performed to verify the assumptions of each model (Zuur et al., 2010). Both the construction of the biplot and the adjustment of the models were carried out in R software (R core team, 2019).

RESULTS

The biplot of $\delta^{15}\text{N}$ vs. $\delta^{13}\text{C}$ showed a slight but consistent increase in both mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ from basal resources (represented by periphyton) to the primary consumer (Chironomini tribe of chironomids) and to the secondary consumer (odonates of the *Perithemis* genus), displaying, nonetheless, large variability (Fig. 2). This pattern was corroborated by the positive relationships established for both mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in each mesocosm between consumer and resource (LM, $p<0.05$, Table 3), indicating that increasing isotopic values of the resource resulted in a consequent increase in the values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of the consumer (Fig. 3).

The mean values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for periphyton varied among mesocosms (Supplementary material, Appendix 1, available at <https://>

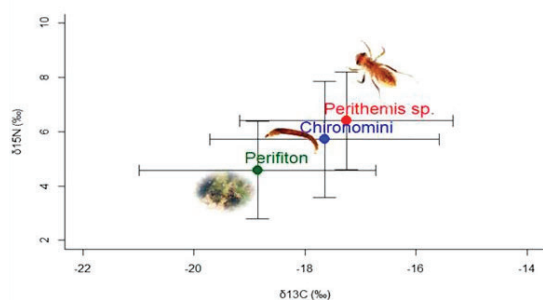


Figure 2. Isotopic values of carbon and nitrogen in the simple trophic chain: *Perithemis* sp.-Chironomini-periphyton. Points indicate mean values and bars the standard deviation. *Valores isotópicos de carbono y nitrógeno en la cadena trófica simple: Perithemis sp.-Chironomini-perifiton. Los puntos indican los valores medios y las barras la desviación estándar.*

www.limnetica.net/en/limnetica), presenting a range from -21.13‰ to -15.81‰ for $\delta^{13}\text{C}$ with a mean of -17.88 ± 4.53 (Table 1) and from 2.21‰ to 6.37‰ for $\delta^{15}\text{N}$ with a mean of 4.58 ± 2.26 (Table 1). Chironomini exhibited a range of $\delta^{13}\text{C}$ values from -21.61‰ to -14.19‰ , while a range of 1.14‰ to 8.16‰ was obtained for $\delta^{15}\text{N}$ (means -19.60 ± 3.56 and 5.94 ± 2.30 , for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively, Table 1). Finally, *Perithemis* sp. also presented considerable variation in the isotopic values among mesocosms, where by the range for $\delta^{13}\text{C}$ was -19.44‰ to -15.55‰ , and for $\delta^{15}\text{N}$ 3.81‰ to 8.12‰ (means of -17.25 ± 1.91 for $\delta^{13}\text{C}$ and 6.40 ± 1.80 for $\delta^{15}\text{N}$).

TDF estimates yielded different values depending on the method used. For Chironomini, the arithmetic equation showed that $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ values were $1.12 \pm 1.31\text{‰}$ and $0.92 \pm 1.94\text{‰}$ on average, respectively. Alternatively, Bayesian inference estimated average values of $1.30 \pm 0.89\text{‰}$ and $1.00 \pm 0.96\text{‰}$ for $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$, respectively. Regarding the trophic discrimination factor for *Perithemis* sp. the arithmetic equation showed a mean value for $\Delta^{13}\text{C}$ of $0.65 \pm 1.52\text{‰}$ and a mean

value for $\Delta^{15}\text{N}$ of $0.90 \pm 1.08\text{‰}$; Bayesian inference, however, estimated values of $\Delta^{13}\text{C} = 0.72 \pm 0.75\text{‰}$ and $\Delta^{15}\text{N} = 0.84 \pm 0.72\text{‰}$ for these same organisms (Table 2). Notably, results from both Bayesian inference and arithmetic equation correlated well, for both periphyton-chironomids (LM, $p=0.002$, $R^2=0.64$ and $p=0.002$, $R^2=0.76$ for $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$, respectively) and chironomids-odonates (LM, $p=0.004$, $R^2=0.67$ and $p=0.001$, $R^2=0.77$ for $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$, respectively) (Supplementary material, Appendix 2, available at <https://www.limnetica.net/en/limnetica>).

The C:N ratio for each organism varied among mesocosms, particularly for periphyton, which varied on average between 8.2 and 15.06. The average C:N ratio ranged between 4.14 and 5.35 for Chironomini, and between 3.68 and 4.74 for *Perithemis* sp. (see Supplementary material, Appendix 1). The stoichiometric C:N imbalance between Chironomini and periphyton varied between -3.29 and -10.41 , while for *Perithemis* sp. and Chironomini it varied between -1.65 and 0.14 . Neither the C:N ratio of each consumer, nor the C:N ratio of the diet, nor the C:N imbalances were determinants of the variation of $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ (LM, $p>0.05$; Supplementary material, Appendix 3, available at <https://www.limnetica.net/en/limnetica>).

Biomass of the main algae groups comprising the periphyton community was also variable among mesocosms (Supplementary material, Appendix 4, available at <https://www.limnetica.net/en/limnetica>). Particularly, the biomass of diatoms and chlorophytes ranged between 0.10 and $3.98 \mu\text{g}/\text{cm}^2$ and 0.16 and $3.88 \mu\text{g}/\text{cm}^2$, respectively. The biomass of cyanophytes ranged from 0.05 and $1.86 \mu\text{g}/\text{cm}^2$. The biomass of diatom algae and cyanophytes were determinants of the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (LM, $p<0.05$; see Table 3) such that when the biomass of these two groups

Table 1. Isotopic values of C and N for the study organisms. Means and standard deviation (sd) are presented considering all mesocosms. In addition, mean values of C:N and the number of samples analyzed in each case are also shown. *Valores isotópicos de C y N para los organismos de estudio. Se presentan las medias y la desviación estándar (sd) considerando todos los mesocosmos. Además, también se muestran los valores medios de C:N y el número de muestras analizadas en cada caso.*

Taxa	$\delta^{13}\text{C}$ (‰) (mean \pm sd)	$\delta^{15}\text{N}$ (‰) (mean \pm sd)	C:N (mean \pm sd)	n
Periphyton	-17.88 ± 4.53	4.58 ± 2.26	11.20 ± 3.22	37
Chironomini	-19.60 ± 3.56	5.94 ± 2.30	4.72 ± 0.35	19
<i>Perithemis</i> sp.	-17.25 ± 1.91	6.40 ± 1.80	4.31 ± 3.13	26

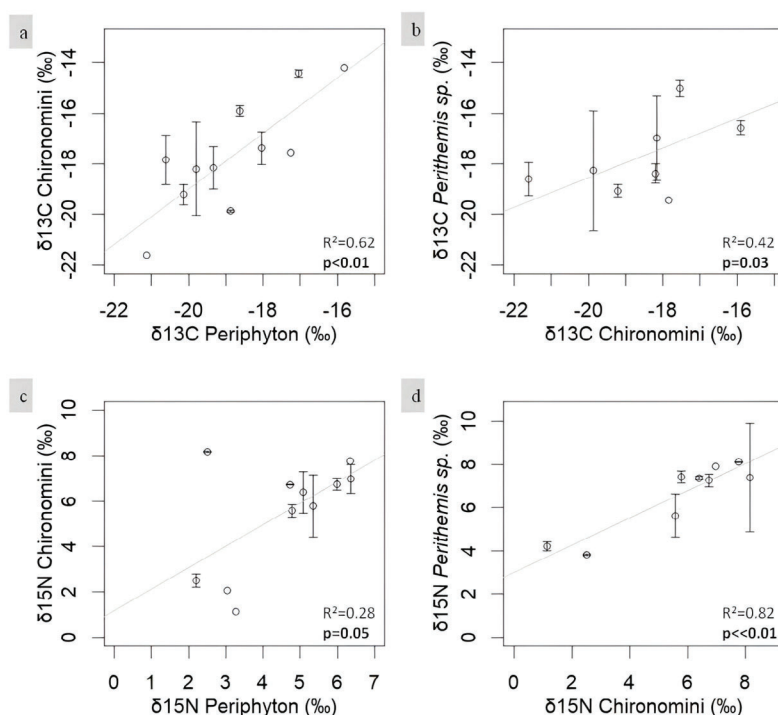


Figure 3 a-d. Relationship between the mean isotopic values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of consumers and their diet in each mesocosm. Significant relationships ($p < 0.05$) are indicated in bold, and the fit of each model (R^2) is also provided. Points indicate mean values and bars the standard deviation per mesocosm. *Relación entre los valores isotópicos medios ($\delta^{13}\text{C}$ y $\delta^{15}\text{N}$) de los consumidores y su dieta en cada mesocosmo. Las relaciones significativas ($p < 0.05$) se indican en negrita y también se proporciona el ajuste de cada modelo (R^2). Los puntos indican los valores medios y las barras la desviación estándar por mesocosmo.*

Table 2. Trophic discrimination factors ($\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$) estimated from two approaches (arithmetic equation and Bayesian inference). Means, standard deviation (sd) and 95% confidence interval (95% CI) for each case are reported. *Factores de discriminación trófica ($\Delta^{13}\text{C}$ y $\Delta^{15}\text{N}$) estimados a partir de dos enfoques (ecuación aritmética e inferencia bayesiana). Se informan las medias, la desviación estándar (sd, por sus siglas en inglés) y el intervalo de confianza del 95% (CI 95%) para cada caso.*

Method			$\Delta^{13}\text{C}$ (‰)			$\Delta^{15}\text{N}$ (‰)		
	Consumer	Diet	Mean	sd	95% CI	Mean	sd	95% CI
Arithmetic equation	Chironomini	Periphyton	1.12	1.31	-	0.92	1.94	-
	<i>Perithemis sp.</i>	Chironomini	0.65	1.52	-	0.90	1.08	-
Bayesian inference	Chironomini	Periphyton	1.30	0.89	0.16-1.79	1.00	0.96	0.28-2.15
	<i>Perithemis sp.</i>	Chironomini	0.72	0.75	0.13-1.54	0.84	0.72	0.22-1.65

of algae increased, $\delta^{13}\text{C}$ decreased and $\delta^{15}\text{N}$ increased (Fig. 4; Table 3). However, there was no relationship between the algal group biomass and the estimated TDFs (Supplementary material, Appendix 3).

DISCUSSION

The use of stable C and N isotopes as indicators

in trophic ecology research is currently booming with new analytical tools published every year (e.g. Healy *et al.*, 2018, Heikkinen *et al.*, 2022, Stephens *et al.*, 2022; Johnson *et al.*, 2023). However, for most methodological approaches to isotope analysis – such as those that evaluate the contribution of basal resources to consumer tissues through mixing models – baseline information on the isotopic trophic discrimination for

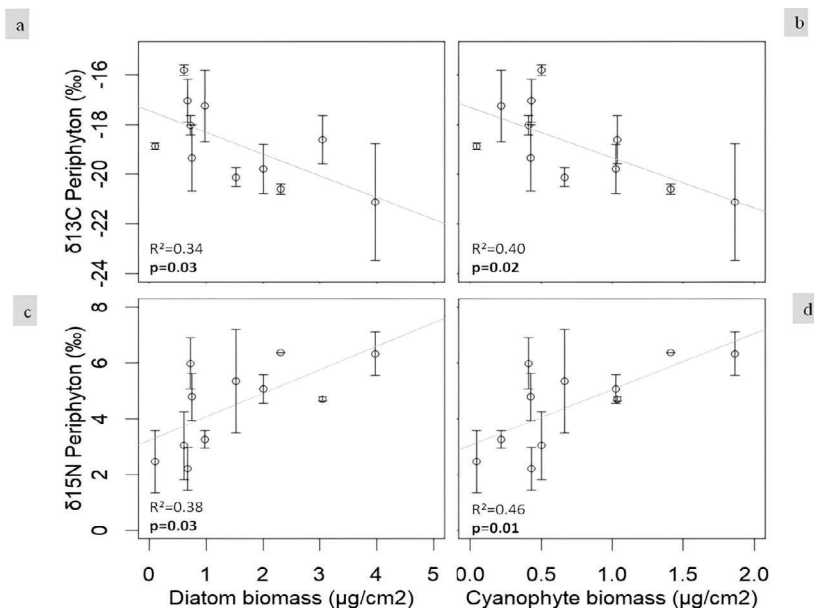


Figure 4 a-d. Significant relationships ($p < 0.05$) between the mean isotopic values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of periphyton and biomass of diatom and cyanophyte algae per mesocosm. The p value and R^2 are indicated in each case. Points indicate mean values and bars the standard deviation per mesocosm. *Relaciones significativas ($p < 0.05$) entre los valores isotópicos medios ($\delta^{13}\text{C}$ y $\delta^{15}\text{N}$) del perifiton y la biomasa de diatomeas y algas cianófitas por mesocosmo. En cada caso se indican el valor de p y R^2 . Los puntos indican los valores medios y las barras la desviación estándar por mesocosmo.*

Table 3. Description of adjusted linear models ($\alpha = 0.05$) that indicate a significant relationship between the variables. The values reported correspond to: the response variable, the predictors, the degrees of freedom, the F and p value and R^2 . All response variables were fitted to a normal distribution. Only the values of $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ estimated using the traditional arithmetic equation were considered. *Descripción de modelos lineales ajustados ($\alpha = 0.05$) que indican una relación significativa entre las variables. Los valores reportados corresponden a: la variable respuesta, los predictores, los grados de libertad, el valor F y p y R^2 . Todas las variables de respuesta se ajustaron a una distribución normal. Sólo se consideraron los valores de $\Delta^{13}\text{C}$ y $\Delta^{15}\text{N}$ estimados mediante la ecuación aritmética tradicional.*

Response variable	Predictor variable	df	F value	p value	R^2
$\delta^{13}\text{C}$ Chironomini	$\delta^{13}\text{C}$ Periphyton	9	16.97	<0.01	0.62
$\delta^{13}\text{C}$ <i>Perithemis</i> sp.	$\delta^{13}\text{C}$ Chironomini	7	6.81	0.03	0.42
$\delta^{15}\text{N}$ Chironomini	$\delta^{15}\text{N}$ Periphyton	9	4.93	0.05	0.28
$\delta^{15}\text{N}$ <i>Perithemis</i> sp.	$\delta^{15}\text{N}$ Chironomini	7	38.52	<<0.01	0.82
$\delta^{13}\text{C}$ Periphyton	Diatom biomass	9	6.17	0.03	0.34
$\delta^{13}\text{C}$ Periphyton	Cyanophyte biomass	9	7.55	0.02	0.40
$\delta^{15}\text{N}$ Periphyton	Diatom biomass	9	6.84	0.03	0.38
$\delta^{15}\text{N}$ Periphyton	Cyanophyte biomass	9	9.64	0.01	0.46

study organisms is required, representing a bottleneck in advancing accurate isotopic modelling (e.g. Stephens et al., 2023). The sustained use of “universal” values for $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$, usually obtained from reviews, do not consider the important nuances that these TDFs can present.

This work provides new knowledge about TDFs for subtropical aquatic organisms showing remarkable differences in comparison to the mean

trophic discrimination values frequently used when no other information is available (Stephens et al., 2023). This reinforces the importance of estimating these factors experimentally, avoiding the potential bias of using values available in the literature. In addition, the variability in TDFs values was explored, evaluating whether the quality of the resources consumed and the consumer's requirements were important determinants of the

variation in $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$. We did not find a significant effect of these factors on $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$, contrasting what has been found in other studies (e.g. Brauns *et al.*, 2018). Although not a main focus of this study, the relationship found between the isotopic values of periphyton ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) and diatom and cyanophyte biomass was compelling, as it represents the first evidence, to our knowledge, that the taxonomic composition of benthic algae in periphyton can affect isotopic values, even though this may not translate into important differences in trophic discrimination.

Estimation of trophic discrimination factors

TDFs were estimated in this study using two approaches, the arithmetic equation and an analysis based on Bayesian inference. Although many experimental studies use arithmetic equations to calculate TDFs, using Bayesian tools is currently considered as a valuable alternative (e.g. Stephens *et al.*, 2022). In this sense, the *simmr* package was found to be particularly useful for feeding studies where the consumer is fed a known diet, and the discrimination factor is then used in a stable isotope mixing model analysis. For this, the *simmr* tool only requires values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for each consumer and the known dietary proportions for each organism (Parnell & Inger, 2016; Govan *et al.*, 2023). Here, both the arithmetic equation and the Bayesian inference tools were compared, highlighting a significant relationship between the outputs of both methods and also demonstrating that values estimated by both methods change proportionally. Nonetheless, despite similar overall mean values, the Bayesian inference method obtained a narrower dispersion of the $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ values among individual mesocosms, compared to the dispersion obtained by the arithmetic equation. Furthermore, in no case did Bayesian inference report negative values, as produced by the arithmetic equation in some mesocosms. This indicates that Bayesian inference is potentially better at mitigating data with extreme outliers compared to the simple arithmetic equation, particularly when considering smaller sample size. Therefore, Bayesian inference could be used as a complement or potentially a substitute to the arithmetic equation, considering its ability to es-

timate TDFs in a simple and quick manner. To the best of our knowledge, to date, no published study has used the *simmr* package to estimate these factors and compare the performance of Bayesian approaches with traditional arithmetic equation as done here. Therefore, our study provides a precedent that incorporates Bayesian tool, encouraging its application in future research and providing probabilistic results (incorporating uncertainty in the model estimates) that provide relevant and novel knowledge that can later be used as input data for studies that aim to estimate isotopic niche metrics.

Many isotopic studies, upon the lack of group-specific trophic discrimination values, rely on general values or values belonging to other taxonomic groups when estimating either trophic position of a species or the contribution of different resources to consumer biomass. For example, studies that have modelled macroinvertebrate food webs, such as González-Bergonzoni *et al.* (2018) or Demars *et al.* (2021), used TDF values proposed by Post *et al.* (2002) and by McCutchan *et al.* (2003), respectively. In fact, most stable isotope studies used a general TDF for vertebrates that averages data from diverse taxa, ecosystems and tissue types (as recently reviewed by Stephens *et al.*, 2023). This represents an important limitation in the precision of many isotopic models and, to date, several studies have shown differences in the TDFs among species or taxonomic groups (and among organisms of the same species), according to diet type, ecosystem, and tissue type analyzed, among several other factors (Stephens *et al.*, 2022, 2023). With the recent identification of this limitation, alternatives to find most appropriate TDFs have been proposed. For example, the *SIDER* package uses a phylogenetic regression model based on a pre-existing data set of TDFs from bird and mammals to impute (estimate) a TDF for a particular avian or mammalian consumer, considering different characteristics such as tissue type, basic ecology, and position in the phylogenetic tree (Healy *et al.*, 2018). While this implement provides a novel approach, and permits the addition of data from other taxonomic groups (i.e. invertebrate TDFs as determined here) to widen its applicability, it also involves inaccuracies because fractionation data from re-

lated species is used instead of estimating the particular fractionation of each element for the target organism (Stephens et al., 2023). New evidence from TDF across diverse and largely isotopically unexplored taxonomic groups (as those reported here) represents an essential contribution towards the enhancement of global-use isotopic tools such as SIDER. This again indicates the need for more field and experimental studies that report TDFs for as many species and taxa as possible, as this is still a bottleneck for improving the analysis of energy fluxes in ecosystems and trophic niches of species when using stable isotope approaches. For large vertebrates, for which experimental approaches are challenging, the combination of field studies that aim to determine their diet with the adjustment of isotopic models is advised to estimate TDFs (Johnson et al., 2023). Nonetheless, for small aquatic organisms, such as the macroinvertebrates used in this study, experimental laboratory studies (under realistic feeding conditions) remain the best available methodology.

This study represents the first report of the isotopic trophic discrimination factors for the *Perithemis* sp., and Chironomini. However, previous studies have used taxonomically similar organisms to those treated here. For example, Brauns et al. (2018) estimated TDFs for *Erythemis* sp. (Odonata, Libellulidae) collected in Muggelsee Lake, Berlin, and fed on Chironomidae for 40 days under controlled conditions. These authors reported average $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ values of 0.8‰ and 0.0‰, respectively (SD not provided), which are slightly different to those estimated here ($\Delta^{13}\text{C}$: $0.65 \pm 1.52\text{‰}$; $\Delta^{15}\text{N}$: $0.90 \pm 1.08\text{‰}$, according to the arithmetic equation). Alternatively, Goodkoop et al. (2006) estimated, also through laboratory experiments, the isotopic trophic discrimination of ^{15}N for *Chironomus riparius* (Chironomini tribe) that fed on *Spirulina* (cyanophyte algae), finding a $\Delta^{15}\text{N}$ of $1.1 \pm 0.42\text{‰}$, similar to the value found in this study ($0.92 \pm 1.94\text{‰}$). It is important to highlight the large variation (sd) reported in our data, as well as in previous studies, implying that the estimated values are particularly similar to those reported for other organisms in the literature. The relatively low number of samples used in this study and in others, could represent a bias that results

in large variation around the estimates. In this respect, Bayesian inference of TDFs in our study proved to reduce such variability under low n . We thus encourage further experimental studies to use Bayesian inference-obtained TDF to improve accuracy and model performance when using isotopic tools such as Bayesian mixing models to infer consumer diets from resources and their TDFs.

Determinants of the variation of TDFs

TDFs are known to vary widely, even within a species when individuals are fed on different diet types or under contrasting physiological conditions (e.g. Adams & Sterner, 2000; Wolf et al., 2015; Canseco et al., 2021). A few studies have evaluated C:N ratios (a proxy of dietary quality) as potential predictors of trophic discrimination. For example, Adams & Sterner (2000) found a positive relationship between the $\Delta^{15}\text{N}$ between *Daphnia magna* and its food source (*Scenedesmus acutus*, green algae) and the C:N of the resource. These authors also note that the values of $\delta^{15}\text{N}$ and $\Delta^{15}\text{N}$ are directly influenced by the quality of the food consumed. Vanderklift & Ponsard (2003) described a similar pattern through a meta-analysis that collected information on $\Delta^{15}\text{N}$ values for different organisms and the C:N of the resource consumed. In their study, resources with lower C:N (i.e. better quality) corresponded to lower $\Delta^{15}\text{N}$ values between the consumer and their diet. This could be related to nutritional stress, whereby if the intake of available nitrogen decreases, organisms resort to using internal reserves of this nutrient (Adams & Sterner, 2000). Although this pattern has also been found in other studies (e.g. Hobson & Clark, 1992; Hobson et al., 1993), support for the generality of this relationship and its meaning remains unclear. Evidence is still largely ambiguous with some studies finding the opposite pattern (lower ^{15}N discrimination in consumer tissues when experiencing nutritional stress) (Oelbermann & Scheu, 2002). In Other studies, as in this study, no effect of diet quality on TDFs was found (e.g. Schmidt et al., 1999). For carbon discrimination factors, there is scarce evidence relating TDFs to diet quality, except for Brauns et al., (2018). They reported that the C:N ratio

of resources and the C:N imbalance between the consumer and food sources are influential factors in the variation of $\Delta^{13}\text{C}$ for aquatic invertebrates. Here, no significant relationship between C:N of resources and consumers and TDFs of C and N was found for either of the two studied macroinvertebrates. However, we emphasize the importance of reporting data on the quality of the nutrients measured by C:N stoichiometry to expand the necessary database for future analyses where these relationships can be evaluated at broader scales, i.e. as a meta-analysis.

This study did not find a relationship between the biomass of different benthic algae groups comprising periphyton and the TDFs estimated for the Chironomini tribe when feeding on this resource. However, a strong relationship was found between the biomass of diatoms and cyanophytes and values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. This relationship denotes that the taxonomic composition of periphyton be considered in isotopic trophic studies. Although the relationships between algae taxonomical composition and their isotopic values were not a main focus, these relationships have not been previously reported, to our knowledge. Most of the studies that have focused on understanding the variability in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of periphyton are based on field experiments in lotic environments that evaluate the effect of water velocity (e.g. MacLeod & Barton, 1998; Finlay *et al.*, 1999; Singer *et al.*, 2005; Hill & Middleton, 2006), light intensity (e.g. MacLeod & Barton, 1998; Rasmussen & Trudeau, 2007), temperature (e.g. Trochine *et al.*, 2017), nutrient concentration (e.g. Trochine *et al.*, 2017; Camillieri & Ozersky, 2019), and other factors. However, to date, few studies have considered algal composition as a potential determinant of its isotopic values. Some authors report a positive relationship between periphyton $\delta^{13}\text{C}$ and chlorophyll *a* concentration, finding no significant effects of this factor on $\delta^{15}\text{N}$ (Hill & Middleton, 2006; Rasmussen & Trudeau, 2007). On the other hand, Ishikawa *et al.* (2012) found that the presence of cyanobacteria (cyanophyte algae) as the dominant group was strongly and positively related to $\delta^{13}\text{C}$ in low velocity systems. Although these studies suggest that algal community can explain the high variability of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in periphyton, this relationship be-

tween algae biomass and isotopic values has not been experimentally demonstrated. In this sense, the relationships between algal taxonomic composition (measured as the biomass of each group of algae) and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ of the periphyton reported here represent a very important finding. Furthermore, although this relationship did not translate into an effect on $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ values for chironomids, we recommend this variable as a proxy for quality (as well as C:N ratios) in future experimental studies that aim to estimate TDFs.

We conclude that the trophic discrimination factors along a simple macroinvertebrate food chain for subtropical South American organisms reported here represent an input to replace missing data for similar taxa and study regions in isotopic modeling studies. Furthermore, this analysis adds evidence regarding possible relationships between food quality and trophic discrimination factors. Although we did not find an effect of food quality on these factors, this is an issue that is far from being resolved at a larger scale.

AUTHOR CONTRIBUTIONS

I.S.: Methodology, Data curation, Formal analysis, Writing- Original draft preparation. **D.N.:** Investigation, Methodology, Writing- Reviewing and Editing. **E.B.:** Investigation, Methodology, Writing- Reviewing and Editing. **A.D'A.:** Investigation, Methodology, Writing- Reviewing and Editing. **F.T.M.:** Investigation, Methodology, Writing- Reviewing and Editing. **N.V.:** Investigation, Methodology, Writing- Reviewing and Editing. **I.G.B.:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing- Reviewing and Editing

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