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# Using MixSIAR to quantify mixed contributions of primary producers from amino acid $\delta^{15}N$ of marine consumers

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ABSTRACT

Estimations of the trophic position and the food web nitrogen baseline from compound-specific isotope analysis of individual amino acids (CSIA-AA) are challenged when the diet of consumer organisms relies on different proportions of vascular and non-vascular primary producers. Here we propose a method to infer such proportions using mixing models and the $\delta^{15}N$ CSIA-AA values from marine herbivores. Combining published and new data, we first characterized CSIA-AA values in phytoplankton, macroalgae and vascular plants, and determined their characteristic β values (i.e. the isotopic difference between trophic and source AA). Then, we applied MixSIAR Bayesian isotope mixing models to investigate the transfer of these isotopic signals to marine herbivores (molluscs, green turtles, zooplankton and fish), and their utility to quantify autotrophic sources. We demonstrated that primary producer groups have distinct $\delta^{15}N_{AA}$ fingerprints that can be tracked into their primary consumers, thus offering a rapid solution to quantify resource utilization and estimate $\beta_{mix}$ values in mixed-sourced environments.

## 1. Introduction

Accurate knowledge of trophic interactions is critical to elucidate the structure and function of ecological communities, understand energy flows, or quantify resource utilization within ecosystems (Chikaraishi et al., 2014; Nielsen et al., 2015; Zhang et al., 2019). Recent efforts are directed to improve trophic position (TP) estimates in consumers using compound-specific isotope analysis in amino acids (CSIA-AA), a powerful tool to simultaneously estimate TPs of organisms and nitrogen (N) sources (baseline) from the isotopic abundance values of AA ($\delta^{15}N_{AA}$) in the same sample (McMahon and McCarthy 2016; Ohkouchi et al., 2017). This method is based in the differential isotopic fractionation of N in individual AA during trophic transfer. This fractionation has led to their categorization into "trophic" (e.g. Glx = glutamic acid (Glu) + glutamine (Gln)), as these AA are generally determined as a mixture) and "source" (e.g. Phe), according to their relatively high and low isotopic enrichment, respectively (e.g. Popp et al., 2007; McMahon and McCarthy 2016). Most calculations of TP from CSIA-AA use $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$ values (e.g. Chikaraishi et al., 2009):

$$TP_{Glx-Phe} = \frac{(\delta^{15}N_{Glx} - \delta^{15}N_{Phe} - \beta_{Glx-Phe})}{TDF_{Glx-Phe}} + 1 \quad (1)$$

were $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$ are the isotopic values for Glx and Phe in the consumer, $\beta_{Glx\text{-}Phe}$ is the difference between $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$ in primary producers, and $TDF_{Glx\text{-}Phe}$ is the isotopic enrichment or trophic discrimination factor between diet and consumer in Glx relative to Phe.

The accuracy of these estimates critically depends on the consistency of both, $\beta_{Glx\text{-}Phe}$ and $TDF_{Glx\text{-}Phe}$ values which, in turn, vary across consumer taxa and ecosystems (Nielsen et al., 2015; McMahon and McCarthy 2016; Ramirez et al., 2021). However, more attention has been paid to the importance of $TDF_{Glx\text{-}Phe}$ than of $\beta_{Glx\text{-}Phe}$ in TP estimates. Because $\beta_{Glx\text{-}Phe}$ exhibits significant differences between vascular and non-vascular primary producers (Ramirez et al., 2021), using a single species- or group-specific $\beta_{Glx\text{-}Phe}$ value cannot estimate reliable TPs of consumers in environments where N originates from a mixture of vascular and non-vascular sources, thus requiring a pooled value (multi-$\beta_{Glx\text{-}Phe}$ or $\beta_{mix}$). This limitation was noted when studying ecosystems with multiple primary producer sources such as estuaries, mangrove swamps, freshwater or seagrass ecosystems, where the use of a single $\beta_{Glx\text{-}Phe}$ value significantly under- or overestimated consumer TPs (e.g. Vander Zanden et al., 2013, Choi et al., 2017; Ishikawa et al., 2018; Zhang et al., 2019). Hence, the potential of CSIA-AA to evaluate TPs in these environments will depend on the existence of a proper methodology for quantifying the contribution of each autotrophic source to the

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consumer and thus estimating accurate $\beta_{mix}$ values. Sampling primary producers along with consumers would be the best option for constraining β values and TP uncertainty (Vander Zanden et al., 2013; Zhang et al., 2019). However, this procedure can be challenging or even impossible in many environments. For example, marine microalgae have very short life spans and can be difficult to isolate (Popp et al., 2007; Bowes and Thorp 2015). Alternatively, estimating the contribution of primary producer groups from gut content analysis of primary consumers may be unfeasible for small-bodied groups, and biased towards non-digestible remnants (Dalsgaard et al., 2003). Moreover, this latter method provides a qualitative identification of the most recent dietary intake, but cannot provide space or time-integrated measures of the resources consumed. Even when isotopic assessment of the basal resources is possible, it may not reliably represent consumer dietary incorporation due to the preferential assimilation of some compounds (Post, 2002).

Prior work has shown that $\delta^{15}N_{AA}$ values provide information of both biosynthetic origin and mode of N acquisition in primary producers (McCarthy et al., 2013), and could be used to infer the baseline of N in food webs. In addition, the $\delta^{15}N_{AA}$ composition in consumer tissues represents a time-integrated dietary proxy, holding information of the sources the organism has been feeding on, and hence, of the isotopic composition of N sources assimilated by the primary producers (Ohkouchi et al., 2017). Thus, analysing $\delta^{15}N_{AA}$ of primary consumers may overcome some of the constraints for estimating autotrophic source contributions. Because primary consumers link primary producers and higher-level consumers, and the $\delta^{15}N_{AA}$ fingerprints are likely to be diluted with each trophic step, eventually disappearing in top consumers (Larsen et al., 2020), studying primary consumers is a very good approach to investigate the transmission of $\delta^{15}N_{AA}$ fingerprints to the food web.

Stable isotope analysis, including a combination of $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ values in bulk tissues (e.g. Moore and Semmens 2008; Moreno et al., 2010), $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$ (e.g. Naito et al., 2016), or $\delta^{13}C$ values in essential AA (e.g. Larsen et al., 2013, 2020; Arthur et al., 2014; Jarman et al., 2017; Wall et al., 2021), has been widely used as a quantitative approach in ecological studies to estimate the composition of consumer diets, mainly using linear (e.g. IsoSource) or Bayesian (e.g. SIAR, MixSIR and FRUITS) mixing models (Phillips and Gregg 2003; Moore and Semmens 2008; Parnell et al., 2010; Fernandes et al., 2014). More recently, MixSIAR integrated a set of parameterizations in a Bayesian framework improving the error structure of its predecessors SIAR and MixSIR (Stock et al., 2018). Moreover, the accuracy of Bayesian mixing models to resolve expected diets improves when the number of tracers is increased, such as essential AA in $\delta^{13}C$ fingerprinting (e.g. Larsen et al., 2013), or fatty acids (e.g. Galloway et al., 2015). In this way, MixSIAR has been applied in quantitative diet estimates using e.g. fatty acids (Guerrero and Rogers 2020), or a combination of bulk $\delta^{15}N$ and $\delta^{13}C$ (Kadye et al., 2020). However, to date, MixSIAR has never been employed to infer the contribution of basal sources to primary consumers using multiple $\delta^{15}N_{AA}$, which may also provide more robust results than traditional models based on two or three tracers. However, because dietary $\delta^{15}N_{AA}$ patterns are not strictly conservative during trophic transfer, but undergo a certain fractionation depending on both the AA and the consumer (Nielsen et al., 2015; McMahon and McCarthy 2016), the selection of appropriate TDFs is crucial for a correct interpretation of mixing model outputs (Phillips et al., 2014; Kadye et al., 2020).

Therefore, the first goal of this paper is to test whether the $\delta^{15}N_{AA}$ fingerprints are passed from primary producers to primary consumers (i.e. herbivores) by applying MixSIAR models to four case studies from diverse marine habitats. For this purpose, we first analysed specific $\delta^{15}N_{AA}$ patterns in the major primary producer groups (i.e. phytoplankton, macroalgae and vascular plants), extending previous knowledge on β variability (Ramirez et al., 2021). Then, using MixSIAR, we showed that the $\delta^{15}N_{AA}$ values from these consumers could be used as quantitative tracers of autotrophic N sources. Finally, from MixSIAR results, we estimated $\beta_{mix}$ values, and analysed the influence of TDF in source predictions, and of TDF and β values on TP calculation.

## 2. Material and methods

### 2.1. Data collection

Nitrogen isotopic values in AA of aquatic and terrestrial primary producers (including phytoplankton, macroalgae, and vascular plants) from around the globe were compiled from the published literature. A total of 387 records, from 50 articles published between 1991 and 2022 were selected (Supplementary Table S1). It should be noted that, although most of these studies were included in a recent meta-analysis of β values in primary producers (Ramirez et al., 2021), $\delta^{15}N_{AA}$ data were used here to meet different objectives, first to assess the potential of $\delta^{15}N_{AA}$ fingerprints to discriminate autotrophic sources and, second, to explore their ability to reconstruct primary source contributions from primary consumers.

Additional data (>550 measurements) were obtained from 53 marine primary producer samples, including different species of macroalgae, seagrasses, and salt marsh macrophytes collected at various spatial and temporal scales for the purpose of this study, together with the cultivated cyanobacteria *Arthrospira platensis* (Supplementary Methods, Table S1).

### 2.2. Compound-specific isotope analysis in amino acids (CSIA-AA)

Amino acid-specific stable N isotope composition was determined in the new samples, as described in McCarthy et al. (2013) and Mompeán et al. (2016), for 7 trophic AA (alanine [Ala], aspartic acid + asparagine [Asx], glutamic acid + glutamine [Glx], isoleucine [Ile], leucine [Leu], proline [Pro], and valine [Val]), and 6 source AA (glycine [Gly], lysine [Lys], phenylalanine [Phe], serine [Ser], methionine [Met], and threonine [Thr]). To ensure precision and accuracy of $\delta^{15}N$ measurements, each sample was derivatized with an accompanying internal standard with known $\delta^{15}N$ values (L-Norleucine) (McCarthy et al., 2013). Reproducibility (± standard error) associated with isotopic analysis of individual AA was <1‰. Analyses were conducted at the Servicio de Análisis Instrumental from the University of A Coruña (Spain). Details of the analysis are provided in Supplementary Methods.

### 2.3. Statistical analysis

#### 2.3.1. $\delta^{15}N$ patterns of individual amino acids and estimation of $\beta$ values

Non-metric multidimensional scaling (nMDS) was used to explore groupings between the major groups of primary producers based on the patterns of similarity in $\delta^{15}N$ values of individual AA (Ala, Glx, Leu, Pro, Val, and Gly). These AA (along with Phe) were consistently measured across the taxa evaluated and $\delta^{15}N$ values were available in >50% of the records. The nMDS analysis was based on the Bray-Curtis dissimilarity matrix of $\delta^{15}N_{AA}$ normalized to Phe ($\delta^{15}N_{AA\text{-}Phe}$), scaled up to the lowest value (so that all the values became positive), and discarding the minimum number of missing values. Further multivariate analysis (including ANOSIM, PERMANOVA, and SIMPER tests) was conducted to validate groupings outlined by nMDS. For comparative purposes, autotrophic groups discrimination was also examined by linear discriminant function analysis (LDA) (Supplementary Methods).

From the data compiled (n = 440), we used all possible combinations of one trophic (Trp) and one source (Src) AA to calculate the $\beta_{Trp\text{-}Src}$ values ($\beta_{Trp\text{-}Src} = \delta^{15}N_{Trp} - \delta^{15}N_{Src}$). These AA (see AA in Table 1) were selected because their isotopic values were previously reported in the available literature and could be obtained in the present study for the majority of taxa. Additional details on the nMDS, $\beta_{Trp\text{-}Src}$ values calculation and complementary tests are provided in Supplementary Methods.

**Table 1**
Mean ± SD of $\beta_{Trp\text{-}Src}$ values (‰) calculated for different combinations of "trophic" and "source" amino acids (AA) in significantly different groups of primary producers ($F_{3,268} = 189.890$, $p < 0.001$, PERMANOVA). Sample sizes: n = 52 for phytoplankton (phyto), n = 62 for macroalgae (macro), n = 326 for vascular plants (vas). Background shading stresses the $\beta_{Trp\text{-}Phe}$ values used in MixSIAR as input data for primary producer sources. For AA abbreviations see description in section 2.

| | Group | Phyto | Macro | Vas | Phyto | Macro | Vas | Phyto | Macro | Vas | Phyto | Macro | Vas | Phyto | Macro | Vas | Phyto | Macro | Vas |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Source AA* | | | | | | | | | | | | | | | | | |
| | | **Gly** | | | **Lys** | | | **Phe** | | | **Ser** | | | **Met** | | | **Thr** | | |
| *Trophic AA* | **Ala** | 4.1 ± 4.2 | 6.1 ± 2.2 | 5.8 ± 5.2 | 5.8 ± 4.9 | 3.0 ± 3.8 | 1.9 ± 1.3 | 2.8 ± 2.6 | 1.4 ± 2.3 | -7.6 ± 2.5 | 7.0 ± 2.9 | 6.8 ± 3.3 | 5.1 ± 3.1 | 2.6 ± 1.7 | 3.6 ± 2.4 | 6.8 ± 5.2 | 1.2 ± 3.4 | -1.6 ± 5.3 | 4.9 ± 3.5 |
| | **Asx** | 1.8 ± 3.6 | 7.1 ± 2.7 | 6.4 ± 5.0 | 4.9 ± 3.4 | 4.6 ± 3.4 | 3.2 ± 1.2 | 1.9 ± 1.8 | 1.6 ± 2.6 | -6.2 ± 2.8 | 5.2 ± 2.6 | 7.6 ± 3.3 | 5.2 ± 2.7 | 7.3 ± 0.0 | 3.0 ± 1.1 | 4.1 ± 2.0 | 0.3 ± 2.2 | 0.8 ± 4.8 | 4.7 ± 3.2 |
| | **Glx** | 3.6 ± 5.1 | 7.7 ± 2.3 | 7.1 ± 5.1 | 4.4 ± 3.3 | 5.6 ± 3.8 | 2.8 ± 1.6 | 3.1 ± 1.9 | 3.1 ± 1.6 | -6.7 ± 3.5 | 6.7 ± 3.3 | 9.1 ± 2.6 | 6.9 ± 3.0 | 4.8 ± 1.8 | 5.0 ± 1.4 | 7.5 ± 5.2 | -0.1 ± 2.9 | 1.8 ± 4.3 | 5.5 ± 4.3 |
| | **Ile** | 1.7 ± 4.9 | 4.3 ± 2.7 | 5.3 ± 5.4 | 3.0 ± 2.6 | 0.9 ± 4.3 | -0.1 ± 1.8 | 1.3 ± 2.6 | -0.1 ± 2.7 | -9.0 ± 2.8 | 4.6 ± 4.1 | 6.0 ± 2.9 | 5.4 ± 2.5 | 4.5 ± 1.8 | 1.4 ± 3.5 | 3.9 ± 3.0 | -1.8 ± 3.3 | -3.3 ± 4.1 | 3.3 ± 4.5 |
| | **Leu** | 0.7 ± 3.7 | 4.5 ± 1.9 | 3.5 ± 5.2 | 2.1 ± 2.3 | 1.8 ± 3.5 | -1.0 ± 1.3 | 0.6 ± 2.7 | 0.0 ± 1.7 | -9.6 ± 2.6 | 4.6 ± 3.0 | 5.6 ± 2.8 | 3.9 ± 2.5 | 4.2 ± 1.4 | 1.8 ± 2.2 | 2.9 ± 4.2 | -2.0 ± 2.8 | -2.1 ± 5.4 | 1.4 ± 3.7 |
| | **Pro** | 3.9 ± 4.9 | 5.7 ± 3.3 | 9.2 ± 5.7 | 4.2 ± 3.1 | 2.6 ± 2.2 | 4.8 ± 2.6 | 2.6 ± 2.1 | 1.5 ± 2.6 | -3.9 ± 2.7 | 6.6 ± 3.7 | 6.5 ± 4.0 | 9.1 ± 3.8 | 3.1 ± 3.3 | 4.1 ± 1.9 | 9.8 ± 6.2 | 0.9 ± 2.8 | -1.4 ± 4.6 | 7.2 ± 5.1 |
| | **Val** | 4.7 ± 5.1 | 6.9 ± 2.4 | 6.6 ± 5.1 | 5.9 ± 2.5 | 4.2 ± 3.4 | 2.3 ± 1.2 | 4.1 ± 2.0 | 2.3 ± 2.1 | -6.8 ± 2.6 | 8.0 ± 3.0 | 8.3 ± 2.6 | 6.8 ± 2.9 | 5.2 ± 1.2 | 3.9 ± 2.8 | 6.7 ± 3.6 | 0.6 ± 2.9 | 0.2 ± 3.4 | 5.0 ± 3.9 |

#### 2.3.2. Isotope mixing models applied to four case studies

We selected four groups of marine primary consumers from different aquatic ecosystems (littoral, coastal, pelagic, deep-sea and reefs) as case studies to examine whether their $\delta^{15}N_{AA}$ patterns can estimate the relative contribution of different primary producer sources to their dietary composition. Bayesian isotope mixing models were fitted using the package "MixSIAR" (Stock and Semmens 2016; Stock et al., 2018) from R-3.4.0 (R Development Core Team 2008). MixSIAR models iteratively assess potential combinations of the possible sources to select those combinations best reflecting the consumer $\delta^{15}N_{AA}$ profiles.

In case study 1, we selected 3 examples of molluscs (M) representative of single-source primary consumers. These molluscs primarily feed on phytoplankton (M1), on brown macroalgae (M2), or on terrigenous woody materials (M3). In case study 2, we selected 3 examples of herbivorous green turtles (T) with diets composed predominately of seagrasses (T1), macroalgae and seagrasses (T2), or a mixed diet of macroalgae/phytoplankton-derived nutrients and seagrasses (T3). In case study 3, we selected 3 groups of zooplankton (Z1, Z2, and Z3), with a diet mainly based on phytoplankton. Finally, in case study 4, we selected 2 examples of herbivorous teleost fish (F) from coastal reef environments: butterfish, having a narrow diet mainly consisting of macroalgae (F1), and parrotfish, mainly grazing on benthic algae, either macro or microalgae (F2). Ethics approval was not required for this study because data from the animals selected as case studies were obtained from the published literature, not involving animal manipulation. A brief description of the case studies and their diets is provided hereafter (see Tables S2 and S3).

MixSIAR models were created using multiple isotopic tracers (Ala, Glx, Ile, Leu, Pro, Val, and Gly). When isotopic values of any of these AA in the consumer were not provided in the original publication, or was not possible to calculate specific TDFs for certain AA, a model with 5 or 6 tracers was computed instead. Isotopic values were normalized to Phe ($\delta^{15}N_{AA\text{-}Phe}$). For running the MixSIAR models, we used the Isopod Example applied to fatty acids (Galloway et al., 2014; Stock and Semmens 2016). The R script of this example is available in (mixsiar.dir, "/example_scripts/mixsiar_script_isopod.R"). MixSIAR models were run using 3 parallel chains and $30^5$ iterations, and model convergence was checked using the diagnostic tests and plots available in MixSIAR (Geweke, 1991; Gelman et al., 2014). Models were run with uninformative Bayesian priors on source proportion contributions ($\alpha = 1, 1, 1$) and no random effects. The results of MixSIAR are shown as the median (50% quartile) and the 5%–95% Bayesian credible intervals of diet proportions (BCI). Datasets including data inputs for the models and the results from MixSIAR are available in Supplementary Appendix (Supplementary Material). Additionally, LDA plots including consumer $\delta^{15}N_{AA\text{-}Phe}$ enabled to visualize the performance of MixSIAR models in each case study (Fig. S1).

#### 2.3.3. Influence of trophic discrimination factors in source prediction and trophic position estimates

To test the sensitivity to the TDFs employed in the Bayesian isotope mixing models, we compared the model outputs obtained using literature-based TDFs ($TDF_A$) from the meta-analysis of McMahon and McCarthy (2016) as common values to all consumers in the case studies, or empirically-derived TDFs specific for each group of consumers ($TDF_B$). The latter were obtained from studies with controlled or quasi-controlled diets (e.g. Vander Zanden et al., 2013; Yamanaka et al., 2015). TDFs were incorporated in the models as mean ± SD (see Supplementary Appendix in Supplementary Material).

#### 2.3.4. Trophic position estimates and $\beta_{mix}$ values

The TP of consumers in the case studies was estimated using equation (1). Calculations used either specific $\beta_{Glx\text{-}Phe}$ values estimated for each primary producer source (Table 1), assuming a single primary producer contribution (i.e. $TP_{phyto}$, $TP_{macro}$, or $TP_{vas}$ for phytoplankton, macroalgae and vascular plants, respectively), or a $\beta_{mix}$ value (equation (2)), based on the mass proportions in the diet, and on the specific $\beta_{Glx\text{-}Phe}$ values of these 3 sources, assuming a mixed primary producer-source contribution (i.e. $TP_{mix}$).

$$\beta_{mix} = (\beta_{phyto} \times C_{phyto}) + (\beta_{macro} \times C_{macro}) + (\beta_{vas} \times C_{vas}) \quad (2)$$

Here, $\beta_{phyto}$, $\beta_{macro}$ and $\beta_{vas}$ correspond to the mean $\beta_{Glx\text{-}Phe}$ values estimated for phytoplankton, macroalgae, and vascular plants, respectively, while $C_{phyto}$, $C_{macro}$ and $C_{vas}$ correspond to the median N contributions of phytoplankton, macroalgae and vascular plants respectively to the consumers' diet, estimated by MixSIAR ($C_{phyto} + C_{macro} + C_{vas} = 1$). The TPs were calculated using combinations of different $\beta_{Glx\text{-}Phe}$ values and TDFs.

## 3. Results

### 3.1. $\delta^{15}N_{AA\text{-}Phe}$ fingerprints and $\beta$ estimates

The nMDS analysis revealed 4 distinct clusters: phytoplankton, macroalgae, aquatic, and terrestrial vascular plants, suggesting unique fingerprints for major groups of primary producers (Fig. 1). The first nMDS component clearly separated non-vascular from vascular autotrophs. The second component separated phytoplankton from macroalgae but did not discriminate between the two groups of vascular plants, as some overlap between the 95% confidence ellipses is observed. The AA fingerprints were significantly different among groups (Global R statistic = 0.727, $p < 0.001$, ANOSIM), with groups differences accounting for 68% of the variation in distance ($F_{3,268} = 189.890$, $p < 0.001$, PERMANOVA, Table S4). All pairwise comparisons were significant ($F_{1,80} = 65.247$ for macroalgae vs. phytoplankton, $F_{1,51} = 34.346$ for macroalgae vs. vascular aquatic plants, $F_{1,231} = 169.718$ for macroalgae vs. vascular terrestrial plants, $F_{1,36} = 50.499$ for phytoplankton vs. vascular aquatic plants, and $F_{1,216} = 466.584$ for phytoplankton vs.

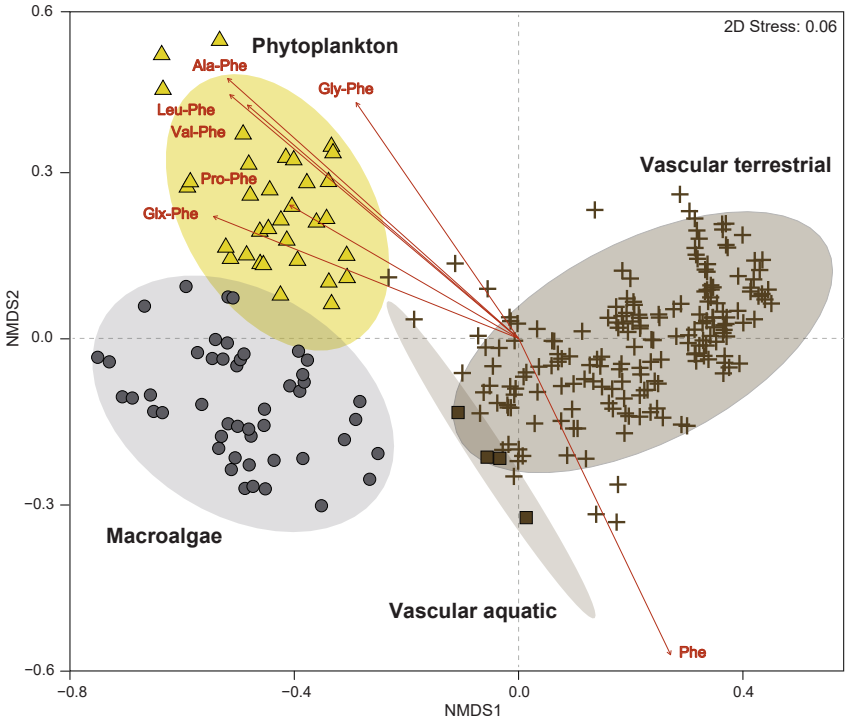


**Fig. 1.** Non-metric multidimensional scaling (nMDS) ordination illustrating differences among major groups of primary producers based on nitrogen isotopic values of the amino acids (AA) Ala, Glx, Leu, Pro, Val and Gly normalized to Phe ($\delta^{15}N_{AA\text{-}Phe}$, ‰). Ellipses represent the 95% confidence intervals of each group. Vectors indicate the direction (increase) and strength (length) of the variables that were significantly correlated ($p < 0.001$) with the nMDS scores. Sample sizes: n = 33 for phytoplankton, n = 48 for macroalgae, n = 4 for vascular aquatic and n = 184 for vascular terrestrial plants. For AA abbreviations see description in section 2. Triangles: phytoplankton; circles: macroalgae; crosses: vascular terrestrial plants; squares: vascular aquatic plants.

vascular terrestrial plants, $p < 0.01$ in all cases, PERMANOVA) except for aquatic vs. terrestrial plants ($F_{1,187} = 4.431$, $p = 0.180$, PERMANOVA) (Table S5). SIMPER analysis identified four AA (Glx, Pro, Val, and Gly) as the main contributors (>70%) to the differences observed (21% overall average dissimilarity). This group separation in the nMDS was further supported by one-way ANOVA and Tukey HSD tests showing significant differences on mean $\delta^{15}N_{AA\text{-}Phe}$ values among groups for all AA ($p < 0.001$ in all cases, Table S6), particularly between non-vascular and vascular plant groups, and between phytoplankton and macroalgae for Ala-Phe, Val-Phe and Gly-Phe.

Vectors of the $\delta^{15}N_{AA\text{-}Phe}$ were significantly correlated with the nMDS scores (negative correlations with the first axis and positive correlations with the second axis), indicating that values increase towards non-vascular autotrophs. Vector lengths ($r^2$ between 0.37 and 0.81, $p < 0.001$ for all AA) demonstrated that all AA contributed to the two first ordination components. However, $\delta^{15}N_{Phe}$ values used for AA normalization were positively and negatively correlated with the first and second axis respectively ($r^2 = 0.74$), indicating its increase towards vascular plants. Vectors of Ala-Phe, Leu-Phe and Val-Phe were practically overlapping and of similar magnitude, slightly diverging from Glx-Phe and Pro-Phe. In turn, Gly-Phe was slightly separated while Phe completely diverged from the rest of the AA.

Values of $\beta_{Trp\text{-}Src}$ were highly variable within and between groups, particularly for vascular plants (Table 1). Values were generally positive, except for $\beta_{Trp\text{-}Phe}$ in vascular plants, and $\beta_{Trp\text{-}Thr}$ in non-vascular autotrophs in most AA combinations. Also, mean $\beta_{Ile\text{-}Src}$ and $\beta_{Leu\text{-}Src}$ were negative in some cases, and mean $\beta_{Trp\text{-}Thr}$ in vascular plants were significantly higher than in non-vascular autotrophs (see ANOVA and Tukey's results in Table S6). For AA normalized to Met, the values were, in general, higher in vascular plants than in phytoplankton (except for $\beta_{Asx\text{-}Met}$ and $\beta_{Leu\text{-}Met}$). In contrast, $\beta_{Trp\text{-}Src}$ values were significantly higher in non-vascular groups compared to vascular plants when normalized by Lys (especially in phytoplankton) or Phe (in macroalgae and phytoplankton). No significant differences between groups were found for $\beta_{Trp\text{-}Ser}$ and $\beta_{Trp\text{-}Gly}$. Mean $\beta_{Glx\text{-}Phe}$ in non-vascular autotrophs were the same for both macroalgae and phytoplankton, but significantly different from that in vascular plants. Moreover, the SD of $\beta_{Glx\text{-}Phe}$ in vascular plants was almost twice the SD in non-vascular producers.

### 3.2. Source prediction by MixSIAR

Below, we briefly review the main findings of the 4 case studies. Details on source prediction and a full overview of results from MixSIAR is provided in Fig. 2 and Supplementary Appendix in Supplementary Material.

#### 3.2.1. Case study 1 (Molluscs–M)

In M1, MixSIAR with $TDF_A$ identified phytoplankton as the main source for blue mussels (median = 71%), and estimated a contribution of 24% macroalgae and 3% vascular plants. Almost equivalent values were found when using $TDF_B$. In M2, the model using $TDF_B$ identified phytoplankton as the main source for gastropods inhabiting brown algal colonies, with a median contribution of 56%, followed by macroalgae (37%) and vascular plants (5%). The application of $TDF_A$ also revealed phytoplankton as main source (68%), but detected a lower contribution of macroalgae. In turn, MixSIAR estimated a major contribution of vascular sources for the wood-boring mollusc (M3) using both TDFs (median values > 70%), although it was slightly higher for $TDF_B$ (76%).

#### 3.2.2. Case study 2 (green turtles–T)

Vascular sources were identified as the main component in the diet of T1 green turtles using either $TDF_A$ or $TDF_B$. In T2, the use of $TDF_B$ estimated median contributions of 50%, 33% and 15%, for macroalgae, phytoplankton, and vascular plants, respectively. However, a lower median contribution of macroalgae (16%) and higher contribution of phytoplankton (80%) were estimated when using $TDF_A$. Similarly, in T3 the model using $TDF_B$ identified a mixed diet of macroalgae (37%), phytoplankton (35%) and vascular plants (27%), corresponding to the expected diet, while the use of $TDF_A$ estimated a lower contribution of vascular plants (7%) and a higher contribution of phytoplankton (67%).

#### 3.2.3. Case study 3 (Zooplankton–Z)

For *Calanus pacificus* (Z1), the model using $TDF_B$ identified phytoplankton as the main source (90%), followed by macroalgae (7%) and

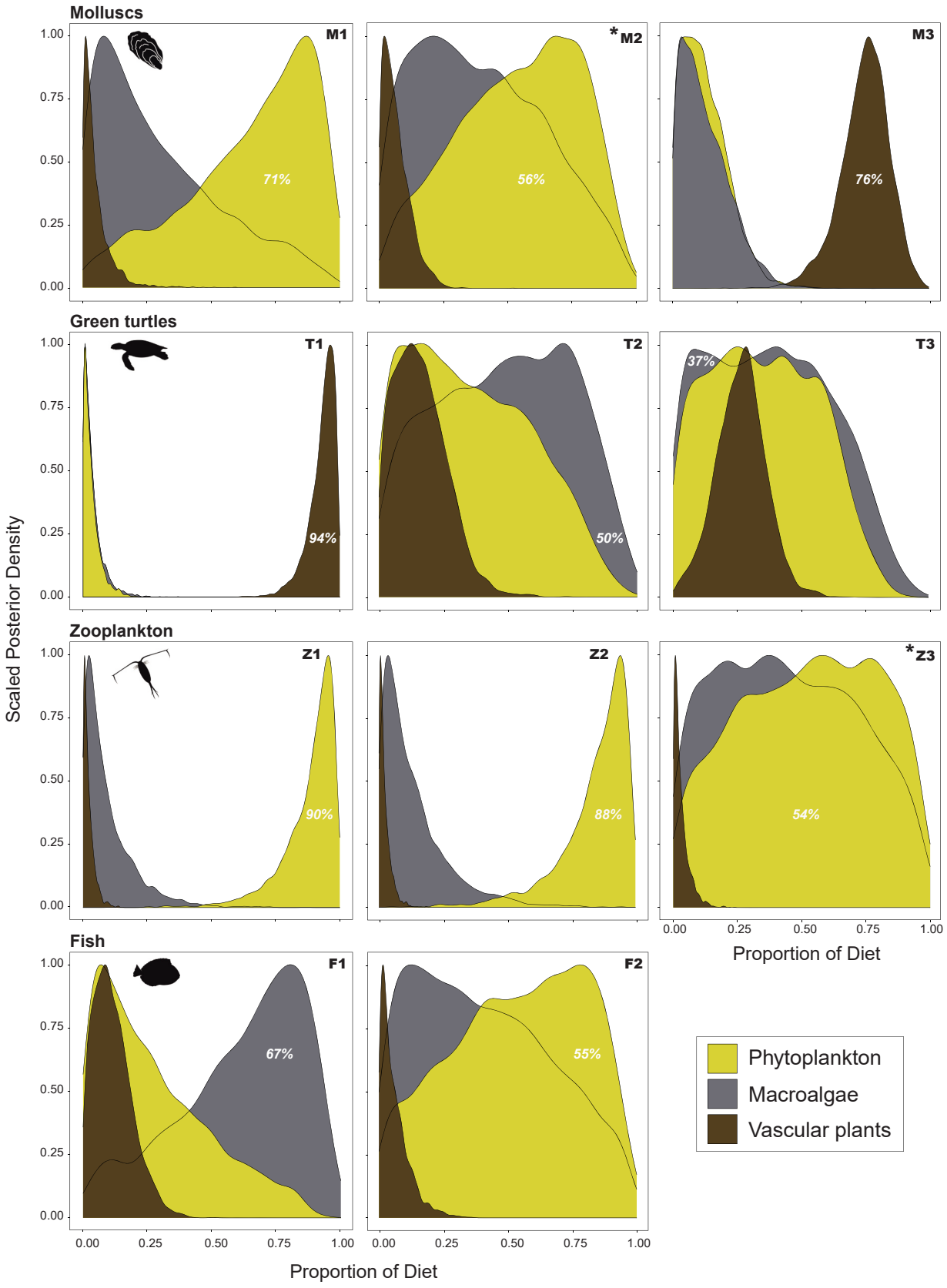


**Fig. 2.** Comparison of MixSIAR-estimated source contributions (as Bayesian credibility intervals and posterior densities) for different primary consumers based on outputs using consumer group-specific trophic discrimination factors (TDFs), except for M1, for which literature-based TDFs (McMahon and McCarthy 2016) were used. The percentage of the source with the highest contribution (median values in the models) is shown in each case. Asterisks indicate those case studies (i.e. M2 and Z3) in which MixSIAR yielded unreliable estimates for some of the sources based on the expected diet for these consumers.

vascular plants (2%). Dominance of phytoplankton contributions (88%) were also found for 40–2000 μm zooplankton (Z2), and for 100–300 μm zooplankton (Z3, 54%), with generally minor contributions of other sources. Similar diet proportions were also estimated using $TDF_A$ in Z1 and Z2. In the case of Z3, models estimated a higher proportion of macroalgae (58%), followed by phytoplankton (38%) and vascular plants (2%) when using $TDF_A$, but a higher proportion of phytoplankton (54%), followed by macroalgae (43%) and vascular plants (2%) when using $TDF_B$.

#### 3.2.4. Case study 4 (Fish–F)

Phytoplankton (91%), with minor contributions of macroalgae (7%) and vascular plants (1%) was the main component of the diet of F1 (*Odax pullus*) as determined using $TDF_A$, whereas the application of $TDF_B$ estimated a major contribution of macroalgae instead (67%), followed by phytoplankton (22%) and vascular plants (10%). In F2 (*Scarus* spp.), the models using either $TDF_A$ or $TDF_B$, estimated a major contribution of phytoplankton (73 and 55%, respectively), followed by macroalgae (22 and 38%), with a minor contribution of vascular items (3 and 4%).

### 3.3. Influence of trophic discrimination factor in source predictions

Application of either literature-based TDFs ($TDF_A$) or specific TDFs ($TDF_B$) in Bayesian multi-isotope mixing models provided reliable estimates of diet proportions for single-source consumers of phytoplankton (i.e., M1, Z1, and Z2) and vascular plants (M3 and T1). However, using specific TDFs generally produced slightly higher proportions of the main source expected (Fig. 3, Tables S2 and S3). In contrast, when macroalgae was assumed a major source item in the consumer's diet (M2 and T2), the use of literature-based TDFs consistently underestimated the proportion of macroalgae, while increased that of phytoplankton. When vascular plants were not the main potential source (usually <5%), the type of TDF did not affect the estimated contribution. However, for consumers with highly specialized diets (e.g. the wood-boring bivalves M3 or the green turtles T1), all models slightly underestimated the expected vascular contribution (Fig. 2).

In accordance with MixSIAR source predictions, the LDA plots (Fig. S1) classified molluscs M1 and M2, green turtles T2 and T3, zooplankton (Z1, Z2, and Z3) and fish (F1 and F2) samples within the mixing space for non-vascular autotrophs. In turn, molluscs M3 and green turtles T1 samples were included within the space for vascular plants.

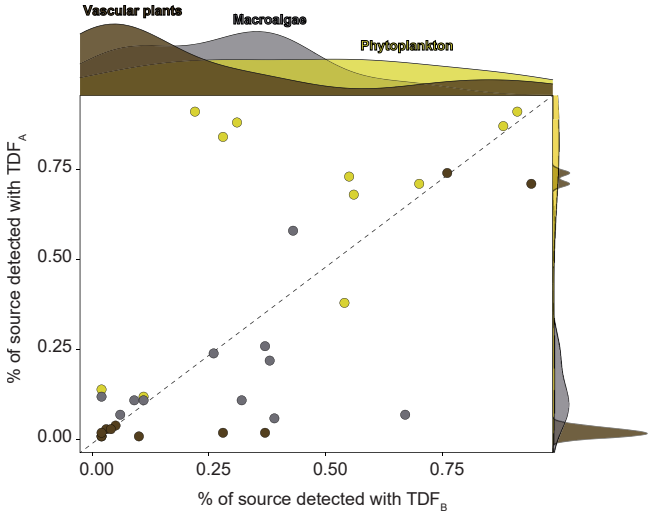


**Fig. 3.** Relationship between MixSIAR estimated source contributions for the primary consumers from the case studies (n = 33 median values) using literature-based trophic discrimination factors (TDFs) derived from McMahon and McCarthy (2016) (here $TDF_A$) and those estimated using specific TDFs for each consumer group (here $TDF_B$).

### 3.4. Influence of β and trophic discrimination factor in trophic position estimates

The TP estimates of the selected consumers were within the range of biologically realistic TPs in herbivores (i.e., $1.5 \geq TP \leq 2.5$), with SD usually ≤0.3 (Table 2). The $TP_{phyto}$ and $TP_{macro}$ estimates yielded similar values, but completely different from $TP_{vas}$, regardless the TDF used. $TP_{phyto}$ and $TP_{macro}$ were within realistic limits for non-vascular single-source primary consumers, but were clearly underestimated ($TP < 1.5$) for herbivores feeding exclusively on vascular plants (i.e., M3 and T1). In turn, both $TP_{vas}$ and $TP_{mix}$ yielded reliable estimates for M3 and T1, that were further improved by using $\beta_{vas}$ instead of $\beta_{mix}$. Overall, the $TP_{mix}$ approach, yielded realistic TPs regardless the TDF used. In particular, for green turtles in M3, as the unique example in our study with a mixed diet based on vascular and non-vascular items, the most realistic estimates were obtained using a $\beta_{mix}$.

## 4. Discussion

### 4.1. $\delta^{15}N_{AA\text{-}Phe}$ fingerprints and β estimates

The strikingly different $\delta^{15}N_{AA\text{-}Phe}$ fingerprints among major groups of primary producers resulted in a significant group separation for phytoplankton, macroalgae and vascular plants. This finding further demonstrates the utility of $\delta^{15}N_{AA\text{-}Phe}$ values to distinguish among vascular and non-vascular primary producers (Ramirez et al., 2021) but expands its applicability to the characterization and tracing of N basal sources in complex food webs by discriminating between phytoplankton and macroalgae. We found that any singular combination of two AA was unable to completely discriminate among the groups examined. However, the optimal separation between groups was achieved by using multiple both trophic and source AA, being Glx, Pro, Val and Gly the best diagnostic AA.

The characteristic variability in $\delta^{15}N_{AA\text{-}Phe}$ fingerprints across primary producers can be attributed to inter-group differences in biosynthetic pathways for these AA, since they can have large effects on $\delta^{15}N$ values (McCarthy et al., 2013; Ramirez et al., 2021). Theoretically, the isotopic fractionation of AA mainly depends on the kinetic isotope effect and the flow rate associated with transamination reactions, but also on the mechanisms of assimilation of inorganic N and on the biosynthetic pathways (Chikaraishi et al., 2010). Although the exact cause of the differences between groups is currently unknown, the studied AA must have been biosynthesized and/or metabolized through common pathways with almost the same kinetic isotope effect and flow rates, at least within groups with analogous $\delta^{15}N_{AA\text{-}Phe}$ fingerprints (Chikaraishi et al., 2007, 2009). This conclusion is supported by the grouping of Phe-normalized AA vectors in the nMDS according to their biosynthetic families, i.e. Ala, Leu and Val (derived from pyruvate), Glx and Pro (derived from α-ketoglutarate), and Gly (derived from 3-phosphoglycerate) (Morot-Gaudry et al., 2001; Yang et al., 2020). This observation is also consistent with reports of diagnostic $\delta^{13}C_{AA}$ fingerprints between evolutionary groups of primary producers (Larsen et al., 2013, 2020; Besser et al., 2022). Even though discussion at lower taxonomic levels than those considered here is beyond the scope of this study, we noted that $\delta^{15}N_{AA\text{-}Phe}$ patterns may contain further diagnostic variation. For instance, cyanobacteria and eukaryotic microalgae (as shown in McCarthy et al., 2013), but also macroalgae families, or C3 and C4 plants, could be discriminated. Although $\delta^{15}N_{AA\text{-}Phe}$ patterns are not significantly different between aquatic and terrestrial vascular plants (Ramirez et al., 2021), the clustering of the few available data on aquatic plants suggest that the aquatic and/or terrestrial environments may have induced certain differences in metabolic routing of those

**Table 2**
Variations in consumer trophic position (TP, mean ± SD) estimated for the case studies using different trophic discrimination factors (TDFs) and β values, assuming a) a single diet based on phytoplankton ($TP_{phyto}$), macroalgae ($TP_{macro}$) or vascular plants ($TP_{vas}$); and b) a mixed diet of the three sources ($TP_{mix}$). Biologically realistic TP estimates ($1.5 \geq TP \leq 2.5$) are shown in bold. The propagated errors (SD) in the estimates were computed taking into account inter-samples variation within each case study and the variability of β and TDF values.

| | | | *Single primary producer-sourced diet (a)* | | | | | | *Mixed primary producer-sourced diet (b)* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $TP_{phyto}$ | | $TP_{macro}$ | | $TP_{vas}$ | | $TP_{mix}$ | | | |
| | | | $TDF_A$ | $TDF_B$ | $TDF_A$ | $TDF_B$ | $TDF_A$ | $TDF_B$ | $TDF_A$ | $\beta_{mix\text{-}A}$ | $TDF_B$ | $\beta_{mix\text{-}B}$ |
| Case 1 | M1 | Molluscs | **2.1 ± 0.3** | **1.9 ± 0.3** | **2.1 ± 0.3** | **1.9 ± 0.2** | 3.6 ± 0.3 | 3.2 ± 0.2 | **2.2 ± 0.3** | 2.7 ± 1.3 | **2.0 ± 0.2** | 2.7 ± 0.6 |
| | M2 | Molluscs | **2.1 ± 0.3** | **1.9 ± 0.3** | **2.1 ± 0.3** | **1.9 ± 0.2** | 3.6 ± 0.3 | 3.2 ± 0.2 | **2.2 ± 0.3** | 2.7 ± 0.6 | **2.0 ± 0.2** | 2.5 ± 0.6 |
| | M3 | Molluscs | 0.4 ± 0.1 | 0.5 ± 0.1 | 0.4 ± 0.1 | 0.5 ± 0.1 | **2.0 ± 0.1** | **1.9 ± 0.1** | **1.6 ± 0.1** | −4.2 ± 0.4 | **1.6 ± 0.1** | −4.4 ± 0.4 |
| Case 2 | T1 | Green turtles | 0.3 ± 0.3 | 0.3 ± 0.3 | 0.3 ± 0.3 | 0.3 ± 0.3 | **1.8 ± 0.3** | **1.8 ± 0.3** | 1.4 ± 0.3 | −4.0 ± 0.5 | **1.7 ± 0.3** | −6.2 ± 0.5 |
| | T2 | Green turtles | **1.7 ± 0.6** | **1.6 ± 0.6** | **1.7 ± 0.6** | **1.6 ± 0.6** | 3.2 ± 0.6 | 3.1 ± 0.6 | **1.7 ± 0.6** | 2.8 ± 0.5 | **1.9 ± 0.6** | 1.6 ± 0.7 |
| | T3 | Green turtles | 1.4 ± 0.3 | 1.3 ± 0.3 | 1.4 ± 0.3 | 1.3 ± 0.3 | 2.9 ± 0.3 | 2.8 ± 0.3 | **1.5 ± 0.3** | 2.4 ± 0.6 | **1.7 ± 0.3** | 0.4 ± 0.6 |
| Case 3 | Z1 | Zooplankton | **2.2 ± 0.2** | **2.1 ± 0.2** | **2.2 ± 0.2** | **2.1 ± 0.2** | 3.7 ± 0.2 | 3.5 ± 0.2 | **1.8 ± 0.2** | 5.2 ± 0.3 | **2.1 ± 0.2** | 2.9 ± 0.3 |
| | Z2 | Zooplankton | **2.5 ± 0.3** | **2.4 ± 0.2** | **2.5 ± 0.2** | **2.4 ± 0.2** | 4.0 ± 0.3 | 3.8 ± 0.2 | **2.5 ± 0.2** | 2.9 ± 0.4 | **2.4 ± 0.2** | 2.9 ± 0.3 |
| | Z3 | Zooplankton | **2.4 ± 0.3** | 2.3 ± 0.3 | **2.4 ± 0.3** | 2.3 ± 0.3 | 3.9 ± 0.3 | 3.7 ± 0.3 | **2.4 ± 0.3** | 2.8 ± 0.7 | **2.3 ± 0.3** | 2.8 ± 0.7 |
| Case 4 | F1 | Fish | **2.2 ± 0.5** | **2.4 ± 0.5** | **2.2 ± 0.5** | **2.4 ± 0.5** | 3.8 ± 0.5 | 4.1 ± 0.5 | **2.2 ± 0.5** | 3.0 ± 1.1 | 2.6 ± 0.5 | 2.1 ± 0.6 |
| | F2 | Fish | **2.0 ± 0.2** | **2.1 ± 0.2** | **2.0 ± 0.2** | **2.1 ± 0.2** | 3.5 ± 0.2 | 3.8 ± 0.2 | **2.1 ± 0.2** | 2.7 ± 0.6 | **2.2 ± 0.2** | 2.6 ± 0.7 |

[a]

[a] The β value used in $TP_{phyto}$ and $TP_{macro}$ was 3.1‰, and in $TP_{vas}$ was −6.7‰. The $\beta_{mix}$ value used in $TP_{mix}$ was calculated with the source proportions estimated by MixSIAR in each case study using TDF and β values derived from the literature ($TDF_A$, $\beta_{mix\text{-}A}$), or species-specific values ($TDF_B$, $\beta_{mix\text{-}B}$). A common $TDF_A$ value was used for all case studies, ($TDF_A = 6.4$‰), while a specific TDF was used for each consumer group (i.e. $TDF_B = 7.7$‰ for M1 and M2, 6.9‰ for M3, 6.7‰ for T1, T2 and T3, 7.0‰ for Z1, Z2 and Z3, and 5.7‰ for F1 and F2). The propagated errors (±SD) in the estimates of $\beta_{mix}$ were computed taking into account the variability of $\beta_{phyto}$ (±1.9), $\beta_{macro}$ (±1.6) and $\beta_{vas}$ (±3.5), as well as the variability of estimated source proportions in each particular case.

diagnostic AA in plants. Since the mechanisms behind those differences are unclear, more research through controlled physiological studies is needed.

The increase of $\delta^{15}N_{Phe}$ towards vascular plants in the nMDS supports its use as indicator of lignin content, as suggested by previous studies (Kendall et al., 2019; Ramirez et al., 2021). Phenylalanine is biosynthesized in the Shikimate pathway and is the main precursor of various secondary metabolites and phenolic compounds in vascular plants via the phenylpropanoid pathway (Kendall et al., 2019; Yao et al., 2021). The Phe deamination to become cinnamic acid is the first step in the phenylpropanoid pathway involved in the production of lignin, which showed a positive correlation with $\delta^{15}N_{Phe}$ values (Kendall et al., 2019). In contrast, non-vascular autotrophs do not use this route (Ohkouchi and Takano 2014; Ohkouchi et al., 2017) and thus have low $\delta^{15}N_{Phe}$ values.

One practical issue highlighted by our analysis is the importance of $\delta^{15}N_{AA}$ normalization with Phe (i.e. the use of $\beta_{Trp\text{-}Phe}$ values) for inter-group comparison. A large number of the available $\delta^{15}N_{AA}$ data for phytoplankton and vascular plants are from organisms growing in controlled systems supplied with well-constrained N sources, which may influence the AA composition in some taxa especially dependent on N source and assimilation pathway (see references in Ramirez et al., 2021). Certain variability could also exist among samples collected from different habitats and different seasons, however the normalization of $\delta^{15}N_{AA}$ to Phe in our analysis reduced the effect of this variability, leading to an effective groups separation. Furthermore, $\beta_{Trp\text{-}Phe}$ values are independent of the isotopic composition of the inorganic N forms used by the primary producers, at least in algae (Chikaraishi et al., 2009; McCarthy et al., 2013), because all the absorbed forms are equally incorporated by trophic and source AA. Normalization using other source AA was also tested, but the best taxa separation was achieved using Phe.

Mean (±SD) $\beta_{Glx\text{-}Phe}$ values obtained for non-vascular (3.1 ± 1.8‰, n = 107 including combined data for phytoplankton and macroalgae, value not included in Table 1) and vascular primary producers (−6.7 ± 3.5‰, n = 309, Table 1) are in good agreement with the values described in Ramirez et al. (2021) for non-vascular (3.3 ± 1.8‰, n = 68) and vascular autotrophs (−6.6 ± 3.4‰, n = 152) respectively. New $\beta_{Glx\text{-}Phe}$ values reported in this study for non-vascular (2.8 ± 3.1‰, n = 43) and vascular primary producers (−6.3 ± 2.2‰, n = 4) are also within the range of variability previously found in the literature for this indicator (i.e. 3.2 ± 1.9‰, n = 68 for non-vascular and −6.5 ± 3.8‰, n = 308 for vascular autotrophs; data are from Table S1 excluding new reported values).

Because $\beta_{Glx\text{-}Phe}$ values for vascular plants are lower and display higher variability than those generally used for TP calculation, our results indicate that the classification of autotrophs in phytoplankton, macroalgae and vascular plants is more realistic than those based on aquatic vs. terrestrial (Chikaraishi et al., 2009, 2010), algal vs. vascular groups (Ohkouchi et al., 2017), or non-vascular vs. vascular groups (Ramirez et al., 2021). Indeed, the different $\beta_{AA\text{-}Phe}$ values for these groups of primary producers imply that TP determinations in consumers of food webs including sources from several groups would require a prior estimation of the mixed proportions of these sources in their diets to obtain a realistic $\beta_{mix}$ value.

### 4.2. Source prediction by MixSIAR

The use of wild organisms with potential, but not totally-known, dietary sources certainly prevents a quantitative rigorous assessment of the accuracy of the mixing model results. However, our results showed that Bayesian mixing models using $\delta^{15}N_{AA\text{-}Phe}$ values reproduced the expected contributions of different sources to primary consumers' diet, therefore revealing that their $\delta^{15}N_{AA\text{-}Phe}$ patterns contain information of the biosynthetic origin of N derived from specific groups of primary producers.

In the case of littoral blue mussels (M1), MixSIAR identified phytoplankton as the major primary source for their diet, which is consistent with the condition of primary consumers assumed from AA $\delta^{15}N$ values in Misarti et al. (2017), and is also in accordance with prior knowledge of a diet primarily based on phytoplankton, with minimal food web connection to macroalgae primary production and/or re-worked detritus (Larsen et al., 2013; Vokhshoori et al., 2014). However, models identified macroalgae as the second most important source for littoral gastropods (M2) after phytoplankton, even though there is evidence that these organisms specifically feed on brown macroalgae (namely *Sargassum filicinum* and *Undaria pinnatifida*) (Chikaraishi 2006). In turn, for wood-boring bivalves (M3), Yamanaka et al. (2015) demonstrated that these consumers are peculiarly adapted to feed on terrigenous woody materials (*Zelkova serrata* logs), which was well illustrated by MixSIAR results.

On the other hand, Caribbean green turtles (T1) are known to be

herbivorous with diets based predominantly on seagrasses, primarily *Thalassia testudinum* (Vander Zanden et al., 2013; Bjorndal, 2017). A major contribution of vascular items for their diet was accurately reported by MixSIAR. Yet, the diet of Hawaiian green turtles (T2) is less clear, although they seem to consume predominantly macroalgae and seagrasses (Arthur and Balazs 2008; Arthur et al., 2014), which is quite in agreement with the estimated contributions from MixSIAR. However, Eastern Pacific green turtles (T3) feed on a mixed diet of seagrasses and marine macroalgae/phytoplankton-derived nutrients, with a vascular contribution of ca. 25% (López-Mendilaharsu et al., 2005; Seminoff et al., 2021), also in accordance with MixSIAR estimated proportions, the vascular contribution in particular (i.e. 27%).

Zooplankton groups from Z1, Z2 and Z3 were selected from studies where the TP estimated from AA $\delta^{15}N$ values was ca. 2.0 ± 0.2 and herbivory was observed in all cases (Decima et al., 2013; Mompeán et al., 2016; Loick-Wilde et al., 2019). In Z1 and Z2, MixSIAR well estimated a major contribution of phytoplankton to zooplankton diet; however, in Z3, models failed to distinguish between phytoplankton and macroalgae.

Finally, in the case of coastal reef fish (F1 and F2), it is known that these species are strictly herbivores which graze on non-vascular items (Bradley et al., 2015; Sabadel et al., 2020). The first having a narrow diet niche based on macroalgae, e.g. *Durvillaea*, *Macrocystis*, etc. (Trip et al., 2014), and the second based on both phytoplankton and macroalgae (Bruce and Randall, 1984). According to this, MixSIAR identified reasonably well the expected contributions.

This knowledge advances our understanding of resource use in aquatic systems, complementing other tracer methods, as the $\delta^{13}C_{AA}$ fingerprinting (e.g. Larsen et al., 2020). The method is suitable to be used directly with primary consumers using appropriate TDFs, as its applicability to secondary consumers would require a prior knowledge of their TP to adjust consumer $\delta^{15}N_{AA}$ for trophic fractionation in MixSIAR. Besides, the transfer up the food web may alter the $\delta^{15}N_{AA\text{-}Phe}$ source information in high level consumers, so that additional studies are required to understand the traceability of these patterns across trophic steps. The main advantage of this procedure relies in the use of the same average β values provided in this study for the different primary producer groups, to estimate reliable $\beta_{mix}$ and thus improving TP estimations. This is not a trivial improvement, as most primary consumers in the sea are in fact omnivores (Chikaraishi et al., 2014). Even TP estimations in higher level consumers could be also improved because the $\beta_{mix}$ derived from primary consumers provides an integrated measure of the resource inputs at the base of the food web.

Overall, the results indicate that $\delta^{15}N_{AA\text{-}Phe}$ patterns in primary consumers cannot be fully interpreted without taking into account the variability of $\delta^{15}N_{AA\text{-}Phe}$ in primary producers supporting the underlying food web. Standard methods available to estimate relative contributions of basal sources to consumers' diets involve sampling, analysis and characterization of the underlying producers or the use of a combination of tracers such as $\delta^{13}C_{AA}$ and fatty acids (Hebert et al., 2016; Jarman et al., 2017; Larsen et al., 2020). However, all these methods are time-consuming and cost-intensive, and entail a number of additional limitations (Dalsgaard et al., 2003; Bowes and Thorp 2015). Thus, the use of pre-existent $\delta^{15}N_{AA\text{-}Phe}$ data on a suite of worldwide primary producers can be a complementary or even alternative solution to these approaches, offering a realistic representation of the widely variable N baseline sources (Chikaraishi et al., 2007, 2009; Ishikawa et al., 2018).

### 4.3. Influence of trophic discrimination factor in source predictions

The proposed method has, however, some methodological limitations. Even the most statistically advanced isotope mixing models, including MixSIAR, are highly sensitive to the TDFs used (Phillips et al., 2014; Stock et al., 2018). Therefore, the use of inaccurate TDFs clearly underscore the potential of $\delta^{15}N_{AA\text{-}Phe}$ fingerprinting to correctly estimate source contribution and, as a consequence, to calculate TPs. In our study, the source contribution estimated for the hypothetical single-source consumer groups using different TDFs best illustrated this aspect. Despite the general agreement of the results from the models using literature-based TDFs or consumer group-specific TDFs, the later typically estimated a higher contribution from the main potential source (either vascular or non-vascular), thus supporting the use of consumer group-specific rather than generic TDFs for estimating basal source proportions. However, specific TDFs derived from controlled-feeding experiments involving different taxa, TPs, food requirements, mode of N excretion, and conditions emulating natural environments are still rare (Chikaraishi et al., 2014; McMahon and McCarthy 2016). Alternatively, TDF values could be estimated from user-provided data using mixing models by implementing this functionality in the existing packages to improve model accuracy (Stock et al., 2018).

### 4.4. Influence of β and trophic discrimination factor in trophic position estimates

The $\beta_{Glx\text{-}Phe}$ value from the primary producers is strictly required to parameterize equation (1) for consumer TP calculation. However, the large $\beta_{Glx\text{-}Phe}$ variability among autotrophs, especially between vascular and non-vascular groups, is not accounted for within the conventional application of single species- or group-specific $\beta_{Glx\text{-}Phe}$ values. To determine TP of consumers in complex food webs supported by vascular plants and other primary producers, $\beta_{Glx\text{-}Phe}$ values must accurately reflect the mixture of producers through a $\beta_{mix}$. Thus, the use of CSIA-AA N-based fingerprinting combined with MixSIAR Bayesian mixing models allows for a preliminary estimation of $\beta_{mix}$ values and hence, for obtaining realistic estimates of TPs in complex food webs.

Uncertainty in $\beta_{Glx\text{-}Phe}$ values is expected to affect TP estimates of primary consumers more than those of higher-level consumers (Ramirez et al., 2021). Indeed, our study confirmed that, when the contribution from non-vascular sources is predominant, the use of a $\beta_{vas}$ substantially overestimated the expected TP = 2 for primary consumers (e.g. deeming TPs >3.5 for Case 3–Zooplankton and Case 4–Fish), regardless of the TDF used. While the use of either $\beta_{phyto}$ or $\beta_{macro}$ yielded TP estimates close to those reported in the original studies (i.e. TP = 2.0–2.2 for zooplankton and TP ~1.8 for fish). On the contrary, when there is a significant contribution from vascular sources, the use of $\beta_{phyto}$ or $\beta_{macro}$ substantially underestimated the expected TP, also independently of the TDF; while the use of $\beta_{vas}$ well approached to the TPs reported in the original studies (i.e. TP = 1.9–2.1 for molluscs M3 and TP = 1.7–2.1 for green turtles T1). In comparison, when consumers feed on a mixture of vascular and non-vascular sources, the TP calculated with a $\beta_{mix}$ better reflected the actual diet of the consumer (e.g. green turtles T3), yielding more realistic TP estimates for any TDF type used, compared to the TPs estimated in the original study (TP = 2.3 ± 0.2). Although these estimates appear to be more sensitive to β than TDF values, it should be noted that TDF also influence $\beta_{mix}$ estimates through MixSIAR, and therefore also have an indirect impact on the TP estimates. Thus, these results highlight the strong dependence on the source contributions, β and TDF values in TP studies, and support the use of appropriate $\beta_{mix}$ and specific TDFs.

Finally, it is important to acknowledge here that although there is evidence of different $\delta^{15}N_{AA\text{-}Phe}$ fingerprints between macroalgae and phytoplankton, MixSIAR was unable to properly distinguish both contributions in some cases (e.g. in molluscs M2 or zooplankton Z3). Among the possible causes of this failure are the use of inaccurate TDFs, the existence of unknown sources not included in the models, or the use of uninformative priors. In other cases (e.g. in molluscs M3 and green turtles T1), MixSIAR was able to distinguish vascular and non-vascular contributions, so that a $\beta_{mix}$ could be calculated using the relative contributions and mean $\beta_{Glx\text{-}Phe}$ of only two sources instead of three, since no significant differences between $\beta_{macro}$ and $\beta_{phyto}$, estimated from Glx and Phe, were found. Differentiation between macroalgae and phytoplankton contributions can be improved by using a multi-β value

calculated with several Trp and Src AA (Ohkouchi et al., 2017), as some $\beta_{Trp\text{-}Src}$ combinations (e.g., for Ala, Val or Gly) were significantly different between macroalgae and phytoplankton.

## 5. Conclusions

Our results showed distinctive $\delta^{15}N_{AA\text{-}Phe}$ fingerprints for primary producer groups that can be used to understand the origin and transfer of N sources to the food web. This source specificity of $\delta^{15}N_{AA\text{-}Phe}$ patterns is conserved at least in marine primary consumers, demonstrating the strong potential of CSIA-AA to preserve diagnostic information of the baseline resources in these organisms. Our results demonstrate that this predictive approach can be particularly valuable to distinguish vascular and non-vascular inputs to the diet of consumers inhabiting complex systems (e.g. estuaries, seagrass meadows, or freshwater systems receiving terrestrial vascular inputs). In these scenarios, the use of $\delta^{15}N_{AA\text{-}Phe}$ would allow for improved estimates of $\beta_{mix}$ values, and hence of TPs in consumers. Overall, this study is the first applying MixSIAR Bayesian mixing models based on multiple $\delta^{15}N_{AA\text{-}Phe}$ tracers to estimate the relative contribution of primary producer sources to consumers. Although we used MixSIAR to estimate N source contribution to primary consumers, this technique shows considerable promise to improve our knowledge of trophic dynamics also in higher-level consumers. Finally, to better characterize consumer source contributions within systems with complex primary producer assemblages using this method, we recommend that future research efforts be directed towards examining AA-specific TDFs in marine consumers throughout feeding-controlled experiments.

## Authors' contributions statement

RGS, IGV and AB conceived the ideas and designed the methodology; RGS collected and analysed the data; RGS led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

New isotopic data reported in this study can be accessed through the PANGAEA repository: https://doi.pangaea.de/10.1594/PANGAEA.942169 (García-Seoane et al. 2022).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2022.105792.

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