Mixing models and stable isotopes as tools for research on feeding aquatic organisms

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ABSTRACT: *Implementation of mixture models in isotopic analysis has been increasing due to the need to analyze sources and understand their variations in relation to consumers, which requires an accurate determination of their discriminant rate and fractioning. In this context, we presented the main concepts related to the application of stable isotopes and mixing models, with the aim to analyze the benefits and problems of these tools in surveys on nutrition of aquatic organisms. The predominant methods identified included application of the models IsoSource, MixSIR, SIBER and SIAR, with the use of C and N isotopes in research with aquatic organisms. Studies in both freshwater and marine environments were evaluated to determine the relative contribution of the main food items and understand trophic relationships between organisms. It can be concluded that the application of mixing models, with the stable isotope technique, is a promising tool for research on feeding aquatic organisms and understanding issues related to trophic ecology of these organisms.*
Key words: *food, nutrition, diets, fish.*

Modelos de mistura e isótopos estáveis como ferramentas para pesquisas sobre alimentação de organismos aquáticos

RESUMO: *Os modelos de mistura nas análises isotópicas vêm sendo cada vez mais utilizados devido à necessidade de analisar as fontes alimentares e compreender suas variações em relação aos consumidores, o que requer uma adequada determinação de suas taxas discriminantes e fracionamentos. Neste contexto, objetivamos apresentar os principais conceitos relacionados à aplicação de isótopos estáveis e modelos de mistura, visando analisar as vantagens e desvantagens destas ferramentas em pesquisas com nutrição de organsimos aquáticos. Foram identificados de forma predominante a aplicação dos modelos IsoSource, MixSIR, SIBER e SIAR, com a utilização dos isótopos de C e N em pesquisas com organismos aquáticos, tanto em ambientes dulcícolas como em ambientes marinhos para determinar a contribuição relativa dos principais itens alimentares e compreender relações tróficas entre organismos. Pode-se concluir que, a aplicação dos modelos de mistura com a técnica de isótopos estáveis compõe uma ferramenta promissora nas pesquisas sobre alimentação de organismos aquáticos e na compreensão de questões relacionadas à ecologia trófica destes organismos.*
Palavras-chave: *alimento, nutrição, dietas, peixe.*

INTRODUCTION

Analysis of the eating habits of individuals of a particular species makes possible to understand how food items are consumed, as well as how they are available and distributed in the environment. Furthermore, the quantity and quality of the assimilated food influence the allocation of nutrients to animal tissues and organs (SAMPAIO et al., 2001). To understand the contribution of food items in aquatic environments, data obtained from traditional and chemical analyses provides essential information to understand the trophic dynamics of communities and for the conservation of ecosystems (CORRÊA & SILVA, 2010; PESSOA et al., 2012; 2013a; 2013b; GAMBOA-DELGADO et al., 2013; FRANCO et al., 2014).

In recent decades, there has been increased research using techniques involving stable isotopes (DENIRO & EPSTEIN, 1977; 1978; 1981; FANTLE et al., 1999; FRY, 2006; 2008; BEZERRA et al., 2015; VIGHI et al., 2016; NACHEV et al., 2017). This analysis is widely used to investigate various

scientific issues pertaining to organisms and their ecosystems, especially concerning eating habits and availability of nutritional sources in life stages, whether in their natural environment or in captivity (PASQUAUD et al., 2010; GONDWE et al., 2011; 2012; KADYE & BOOTH, 2012; LETOURNEUR et al., 2013; RAO et al., 2015).

The tissue isotopic concentration of animals provides insight into their dietary history, and some authors propose that fish isotopic ratios are similar to the food they eat most (CAUT et al., 2009; BOECKLEN et al., 2011; FRANCO et al., 2014). Use of the stable isotope technique has some advantages over conventional approaches, such as analysis of gut contents and direct observations, especially because it is possible to determine more precisely the dietary components assimilated and used for animal growth (LI et al., 2013). Given the above, we aimed to present the main concepts related to application of stable isotopes and mixture models, aiming to analyze simultaneously the benefits and problems of these tools, especially in research on nutrition of aquatic organisms.

Concepts and application of stable isotopes

Isotopes are atoms that have the same numbers of protons, but have different numbers of neutrons (BISI et al., 2012; PHILIPPSEN & BENEDITO, 2013). Isotopic composition of ratios between elements of any substance is presented in the form of notation δ (delta), as the isotopic ratios of carbon and nitrogen ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) for example (BISI et al., 2012). These isotopic ratios are obtained using Equation 1, based on the natural abundance of elements used (LEAL et al., 2008; PHILIPPSEN & BENEDITO, 2013).

$$\delta (X) = [(R_{sample}/R_{standard})-1]^{*}1000 \qquad \text{Equation 1}$$

Where: X (‰) is the value of isotopic abundance of the chosen element, such as ^{13}C or ^{15}N, and R_{sample} is the relationship between the isotopic forms of the element, for example $^{13}C{:}^{12}C$ or $^{14}N{:}^{15}N$, and $R_{standard}$ is the standard used. For $\delta^{13}C$, the standard is the Peedee Belemnite (PDB) fossil of the mollusc *Belemnitella Americana* (DUCATTI et al., 2007). For $\delta^{15}N$, the accepted international standard is that of atmospheric air (NO_2), which is considered a mixture homogeneous isotopes on the earth's surface (DUCATTI et al., 2007).

Signatures of the natural abundance of isotopes can be used to find patterns and mechanisms at the level of a single organism, as well as to trace food webs, understand paleodietary studies and cycling of nutrients throughout the ecosystem, in both terrestrial and aquatic systems (FRY, 2008). However, the use of these techniques depends on fundamental issues, such as information on food sources and prey (FINLAY & KENDALL, 2007).

Isotopic techniques have provided better understanding on how organisms incorporate the elements they consume and have been use in several lines of research and with different taxonomic groups (PHILIPPSEN & BENEDITO, 2013). Among telements, the most used stable isotopes are carbon, nitrogen, sulfur, oxygen and hydrogen. They can be analyzed together or separately, depending on the purpose of the study (PEREIRA & BENEDITO, 2007).

Isotopic ratios of $\delta^{13}C$ and $\delta^{15}N$ are the most frequently used (MARTINELLI et al., 1988; MANETTA & BENEDITO-CECÍIO, 2003; PEREIRA & BENEDITO, 2007) especially in trophic ecology studies (PHILIPPSEN & BENEDITO, 2013). The isotopic ratios of C and N of the consumer, for example, present a trophic enrichment compared to the isotopic composition of the diet (BISI et al., 2012). Carbon isotopes function as markers, since they fractionate very little in the food chains, while nitrogen isotopes are used commonly in processes to estimate trophic positions (FRY et al., 1999; VANDER ZANDEN et al., 1999; TIUNOV, 2007; CAUT et al., 2009).

Stable isotopes undergo fractionations that occur during chemical, physical and biological processes, which result in different isotopic signatures for different biological materials (GANNES et al., 1998). This difference occurs when isotopes are fractionated in animal tissues. Due to their metabolic activity, species which feeds on different nutritional items, will present fractions and/or the discriminant rate relative to each type food consumed (DENIRO & EPSTEIN, 1978; FRY, 2006; MARTINELLI et al., 2009). Thus, contributions of food items are not transmitted directly and simply, but they take a specific time and have a certain complexity, requiring specific knowledge of fractionation or discriminant rate for each food item. This requires a more careful analysis for proper application of isotope data in mixing models.

Some authors refer to the discrimination factor as fractionation, fractionation factor or trophic enrichment (MARTINEZ DEL RIO et al., 2009; PHILIPPSEN & BENEDITO, 2013). Understanding concepts of fractionation, enrichment and discriminant rate, makes possible to use the mixing models to estimate and determine the relative contribution of food items consumed by aquatic organisms.

The term "fractionation" is in reality related to kinetic and equilibrium effects, which cause the differences and variations between reagents and products in chemical reactions (MARTINEZ DEL RIO et al., 2009; PHILLIPPSEN & BENEDITO, 2013). In this sense, these authors suggested the term "discrimination factor" (Equation 2) only be used when the work involves determining the difference in isotopic ratio between a particular consumer tissue and your food item. They also explain that the term "trophic fractionation" (Equation 3), should be used when analyzing the whole body of an individual in relation to their food sources (MARTINEZ DEL RIO et al., 2009; PHILLIPPSEN & BENEDITO, 2013). This distinction is essential to standardize each terminology and to provide proper application of discriminant rates and/or trophic fractionation in mixing models.

$\Delta = \delta$ animal tissue - δ food item   Equation 2

Δ trophic $= \delta$ whole body - δ food item   Equation 3

According to PHILIPPSEN & BENEDITO (2013), there is a noticeable increase in research on the variability of the discriminant factor, resulting from the popularization of the technique of stable isotopes and their relevance to ecology, combined with the development and improvement of technologies. These authors also pointed out that the recent perception, by part of researchers, that the use of a constant value was not ideal for all taxonomic groups, showing the need to understand the sources of variation for the discriminant factor, since these sources of variation compromise the performance of this technique as a research tool for animal feed.

Stable isotopes in research with aquatic organisms

In research using stable isotopes as indicators of the nutrition of a particular animal species, one aspect considered is how much a tissue type integrates and isotopically reflects the animal's diet. Thus, muscles are widely used in research due to ease in using and integrating the isotopic dietary signal (MARTINELLI et al., 2009). PHILIPPSEN & BENEDITO (2013) carried out a bibliographic analysis and verified that among the tissues analyzed with fish; the muscle was the most used (40.2%), followed by liver (14.6%) and the whole body (9.8%). Muscle is accepted as the most suitable tissue for stable isotope analysis, due to reduced amount of lipids, inorganic carbonates and less variation compared to other organs (liver, heart and fin) (PINNEGAR & POLUNIN 1999; SUZUKI et al., 2005; PHILIPPSEN & BENEDITO, 2013).

These other organs denote interest for use according to the type of research. The use of structures such as fins and scales become attractive because it is not necessary to sacrifice the animals used. Already the use of the whole body occurs when the species has a reduced body size that makes difficult to collect samples and specific tissues (SUZUKI et al., 2005; GERMAN & MILES, 2010; PHILIPPSEN & BENEDITO, 2013). However, PHILIPPSEN & BENEDITO (2013) showed that the use of the whole body is not the ideal sample, as it results in values with high isotopic variations (FOCKEN & BECKER, 1998). In this context, it is essential to have knowledge about species, environment and factors that influence the performance of this technique, because if the analytical problems and methodological approaches are ignored it may compromise the reliability of results.

FRANCO et al., (2014) explain that, despite his contribution in trophic studies, stable isotope analysis also has its limitations. These authors mention as potential problems isotopic fractionation ($\Delta\delta$) and the turnover rate, which is defined as the time required for the signature of a consumer to reflect the isotopic composition of prey and relies on biotic and abiotic factors (MANETTA & BENEDITO-CECÍIO, 2003; BOECKLEN et al., 2011).

It is known that the isotopic composition of food sources also varies seasonally, which may create the so-called problem of isotopic memory, related to turnover rates (MARTINELLI et al., 2009). Isotopic turnover it is a continuous renewal of chemical elements and their isotopes that make up the body tissue or the organism as a whole, which may occur through tissue renewal or by growth during tissue formation (DUCATTI, 2007). This change in the isotopic composition of the tissue depends on the assimilation of constituents ingested (CALDARA et al., 2010).

Another important factor considered by FRANCO et al. (2014) was the fractionation (Δ), which can also be denominated as discriminant factor or rate. Among the factors mentioned in the literature that cause variability in isotopic signatures referring to fractionation factor and/or discriminant rate, we can mention: type of diet (PHILIPPSEN & BENEDITO, 2013; HEALY et al., 2017); temperature, balance between diet and consumer, age of individuals (PHILIPPSEN & BENEDITO, 2013); metabolic activity (MARTINELLI et al., 2009); consumer tissue type (CAUT et al., 2009; HEALY et al., 2017); taxonomic group of the consumer and isotopic value of the diet (CAUT et al., 2009); nutritional stress, diet quality, body size, excretory mechanisms, feeding rate (HOBSON & WELCH, 1995; PONSARD & AVERBUCH, 1999; OVERMAN & PARRISH,

2001; PINNEGAR et al. 2001; VANDERKLIFT & PONSARD, 2003; MILL et al., 2007); the lipid content and the amino acid composition (WOLF et al., 2009); phylogeny, isotopic signature of sources and the error associated with measures within a species, with the importance of each source of element dependent variation (Nitrogen, Carbon) and taxonomic class (Aves, Mammalia) (HEALY et al., 2017). CAUT et al. (2009) still show in their review research that other factors (diet quality, type of excretion and trophic level) not considered in their research can influence the variations. Thus, sources of variation should be considered for determining discriminant rate and/or trophic fractionation more reliably, mainly in the application of these data in mixing models.

ZUANON et al. (2007) formulated diets based on grains from the C_3 and C_4 photosynthetic cycle's plants for analyzing the turnover of muscle carbon in Nile tilapia growth. Fish were fed the experimental diets ad libitum until the muscle $\delta^{13}C$ approached the carbon isotopic equilibrium, which occurred after 43 days. After the muscle $\delta^{13}C$ standardization period, fish had their diets changed (the ones fed on the C_3 diet, switched to the C_4 diet and vice-versa). Fish were fed ad libitum until the carbon isotopic equilibrium of muscle was reached again, after 57 days. Muscle $\delta^{13}C$ changes of Nile tilapia was verified and were consistent with the $\delta^{13}C$ of the supplied diets; nevertheless, the carbon isotopic equilibrium was reached only for the C_3 diet. These authors suggested that, the tissue metabolic activity can be evaluated by comparison of the carbon half-life values between different tissues of the same species or with the same tissue between different species.

As evidenced in research with juvenile pacu *Piaractus mesopotamicus*, FIOD et al. (2010) concluded that the isotopic composition of muscle tissue varied according to the food frequency, and fish fed two to three times a day presented a closer composition to the composition of the new food consumed. In the same way, other authors denote the importance of these studies for diverse research on the biology and ecology of different groups and species of aquatic organisms, which explore stable isotopes of various elements, with higher incidence of the $\delta^{13}C$ and $\delta^{15}N$ ratios, and low incidence of isotope ratios of $\delta^{34}S$, $\delta^{18}O$, $\delta^{66}Zn$ and $\delta^{202}Hg$ in these surveys (Table 1).

Some of this research was carried out with species of freshwater fish, both in their natural habitat and in captivity, especially with grass carp, silver carp (XIA et al., 2013), tambaqui, pacu (ABIMORAD & CASTELLANI, 2011) and Nile tilapia (GONDWE et al., 2011; 2012; RAO et al., 2015). These studies aimed to evaluate the contributions of natural and artificial foods, in order to try to reduce the supply of feed these fish are given and, consequently, make cultivation more sustainable (GONDWE et al., 2011; 2012; BRITO et al., 2014; ZORZAL-ALMEIDA & FERNANDES, 2014; MORAES, 2016). MORAES (2016), when using stable isotopes of C and N as indicators of the diet of the Nile tilapia (*O. niloticus*) cultivated in net-cage in a reservoir in the Semi-Arid region of Rio Grande do Norte, concluded that the natural food contributed predominantly in the growth of this species. This type of research fills in the gaps existing on nutritional aspects of cultivated species for assimilation of natural and artificial food, especially for production and cultivation in natural environments.

Other research has been developed with animals in their natural environment, in order to understand the relations and trophic interactions in their habitat (RAO et al., 2015; ENDJAMBI et al., 2016; NAMAN et al., 2016; NACHEV et al., 2017). These stable isotope surveys also allow more precise understanding the biology and ecology of different groups of aquatic organisms. Among these researchs, we can highlight the one developed by NAMAN et al. (2016) that used the technique of stable isotopes and mixing model to establish and understand the complexity of the trophic structure of pelagic fish and jellyfish in estuarine environments.

Mixing models for the analysis of stable isotopes

Mixture models have commonly been used in research involving the use of stable isotopes, both in ecology in the natural environment and in experimental research, with different species of aquatic organisms. Several programs have been developed for the use of mixing models in research, such as: IsoError (PHILLIPS, 2001), IsoConc (PHILLIPS & KOCH, 2002; KOCH & PHILLIPS, 2002), IsoSource (PHILLIPS & GREGG, 2003), IsotopeR (HOPKINS & FERGUSON, 2012), MixSIR (MOORE & SEMMENS, 2008), SIAR (PARNELL et al., 2010; 2013), SIBER (JACKSON et al., 2011), IsoWeb (KADOYA et al., 2012), MixSIAR (STOCK & SEMMENS, 2013), SISUS (ERHARDT, 2014), FRUITS (FERNANDES et al., 2014) and DEsiR (HEALY et al., 2017). In table 2, we present research that used mixing models as a statistical tool to determine the relative contribution of food items in the nutrition of aquatic organisms.

The IsoError and IsoConc programs make possible work with models targeted for two isotopes, with two or three food sources. In their work using the IsoError program, PHILLIPS & GREGG (2001)

Table 1 - Use of stable isotopes in research on feeding aquatic organisms.

Common name	Scientific name	Isotopes	Authors
Tilapia aurea	*Oreochromis aureus*	δ^{13}C	Schroeder (1983)
Nile Tilapia	*Oreochromis niloticus*	δ^{13}C; δ^{13}C and δ^{15}N.	Lochmann & Perschbacher (2000), Zuanon et al. (2007); Lochmann (2002), Kelly & Martínez del Rio (2010), Rao et al. (2015), Moraes (2016)
Tilapia	*O. karongae and O. shiranus*	δ^{13}C and δ^{15}N	Gondwe et al. (2012)
Tilapia	*Oreochromis niloticus*	δ^{13}C and δ^{15}N	Britton et al. (2009)
Catfish sharptooth african	*Clarias gariepinus*	δ^{13}C; δ^{13}C and δ^{15}N	Lochmann & Perschbacher (2000); Lochmann (2002), Kadye & Booth (2012)
Blue catfish	*Ictalurus furcatus*	δ^{13}C, δ^{15}N and δ^{34}S	MacAvoy et al. (2001)
Channel catfish	*Ictalurus punctatus*	δ^{13}C, δ^{15}N and δ^{34}S	MacAvoy et al. (2001)
Silver carp	*Hypophthalmichthys molitrix*	δ^{13}C and δ^{15}N	Li et al. (2013), Xia et al. (2013)
Bighead carp	*Aristichthys nobilis*	δ^{13}C and δ^{15}N	Li et al. (2013)
Pacu	*Piaractus mesopotamicus*	δ^{13}C and δ^{15}N	Fiod et al. (2010)
Japanese flounders	*Paralichthys olivaceus*	δ^{13}C and δ^{15}N	Tominaga et al. (2003)
Blue threadfin	*Eleutheronema tetradactylum*	δ^{18}O and δ^{13}C	Newman et al. (2011)
Whiting	*Merlangius merlangus*	δ^{13}C and δ^{15}N	Chouvelon et al. (2014)
European hake	*Merluccius merluccius*	δ^{13}C and δ^{15}N	Chouvelon et al. (2014)
Atlantic horse mackerel	*Trachurus trachurus*	δ^{13}C and δ^{15}N	Chouvelon et al. (2014)
Anglerfish	*Lophius piscatorius*	δ^{13}C and δ^{15}N	Chouvelon et al. (2014)
Artic Charr	*Salvelinus alpinus*	δ^{13}C and δ^{15}N	Murdoch et al. (2013)
Whitemouth croakers	*Micropogonias furnieri*	δ^{13}C and δ^{15}N	Mont'Alverne et al. (2016)
Barbels	*Barbus barbus*	δ^{13}C and δ^{15}N	Nachev et al. (2017)
Brown-marbled grouper	*Epinephelus fuscoguttatus*	δ^{13}C and δ^{15}N	Chen et al. (2012)
Sharks	Bull sharks (*Carcharhinus leucas*)and Tiger sharks (*Galeocerdo cuvier*)	δ^{13}C and δ^{15}N	Matich et al. (2010)
White Sharks	*Carcharodon carcharias*	δ^{13}C and δ^{15}N	Carlisle et al., (2012)
Sharks	Bonethead (*Sphyrna tiburo*), Atlantic sharpnose (*Rhizoprionodon terraenovae*), Bull Shark (*Carcharhinus leucas*), and White Shark (*Carcharodon carcharias*)	δ^{13}C and δ^{15}N	Olin et al. (2013)
Gastropods	*Lepetodrilus elevatus, Lepetodrilus pustulosus, Lepetodrilus ovalis, Eulepetopsis vitrea, Cyathermia naticoides, Peltospira delicata, Peltospira operculata* and *Rhynchopelta concêntrica*	δ^{13}C and δ^{15}N	Gaudron et al. (2015)
Oyster	*Crassostrea gigas*	δ^{13}C and δ^{15}N	Leal et al. (2008)
Bivalve mollusk	*Pinna nobilis Linnaeus*	δ^{13}C and δ^{15}N	Cabanellas-Reboredo et al. (2009)
Cladocerans	*Diaphanosoma spinolosum* and *Ceriodaphnia cornuta*	δ^{13}C and δ^{15}N	Caraballo et al. (2011)
Shrimp	*Litopenaeus vannamei*	δ^{13}C; δ^{15}N; δ^{13}C and δ^{15}N	Gamboa-Delgado & Le Vay (2009a); Gamboa-Delgado & Le Vay (2009b);Gamboa-Delgado et al. (2013; 2014)
Crayfish	*Orconectes propinquus* and *Orconectes rusticus*	δ^{13}C and δ^{15}N	Taylor & Soucek (2010)
Blue Crab	*Callinetes sapidus*	δ^{13}C and δ^{15}N	Fantle et al. (1999)
Common Terns	*Sterna hirundo*	δ^{13}C and δ^{15}N	Bond & Diamond (2011)
Balearic Shearwaters	*Puffinus mauretanicus*	δ^{13}C and δ^{15}N	Bond & Diamond (2011)
Little penguin	*Eudyptula minor*	δ^{13}C and δ^{15}N	Chiaradia et al. (2014)
Green sea turtle	*Chelonia mydas*	δ^{13}C and δ^{15}N	Seminoff et al. (2006); Bezerra et al. (2015)
California sea otter	*Enhydra lutris nereis*	δ^{13}C and δ^{15}N	Newsome et al. (2012)
Arctic marine mammals	Walruses (*Odobenus rosmarus*), Bearded seals (*Erignathus barbatus*),Ringed seals (*Pusa hispida*), and Polar bears (*Ursus maritimus*),	δ^{13}C, δ^{15}N and δ^{66}Zn	Jaouen et al. (2016)
Fin whale	*Balaenoptera physalus*	δ^{13}C and δ^{15}N	Vighi et al. (2016)
Biota	Plant (*Potamogeton pectinatus*),Mosquito larvae (*Chironomidae*), Amphipod (*Gammaridea*),Backswimmers (*Notonectidae*), Snail (*Radix sp.*), Juvenile and adult fish (*Gymnocypris namensis* and *Gymnocypris waddelli*),Ruddy shelduck eggs (*Tadorna ferruginea*), and Clams (*Sphaerium sp.*)	δ^{13}C, δ^{15}N and δ^{202}Hg	Xu et al. (2016)

Source: Prepared by the authors based on the mentioned literature.

Table 2 - Use of mixing models in research with aquatic organisms.

Common name	Scientific name	Mixture Models	Authors
Pelagic fish and jellyfish	Juvenile Chinook salmon (*Oncorhynchus tshawytscha*), Three-spined stickleback (*Gasterosteus aculeatus*), juveniles Chum salmon (*O. keta*), Pacific herring (*Clupea pallasii*), Surf smelt (*Hypomesus pretiosus*); Sea gooseberry (*Pleurobrachia bachei*)Water jelly (*Aequorea* spp.) Cross jelly (*Mitrocoma* spp.), and Lion's mane (*Cyanea capillata*)	SIBER	Naman et al. (2016)
Fin whale	*Balaenoptera physalus*	SIBER	Vighi et al. (2016)
Sharks	Bonnethead (*Sphyrna tiburo*), Atlantic sharpnose (*Rhizoprionodon terraenovae*), Bull Shark (*Carcharhinus leucas*), and White Shark (*Carcharodon carcharias*)	SIBER	Olin et al. (2013)
Oyster	*Crassostrea gigas*	IsoSource	Leal et al. (2008)
Crayfish	*Orconectes propinquus* and *Orconectes rusticus*	IsoSource	Taylor & Soucek (2010)
White shark	*Carcharodon carcharias*	MixSIR	Carlisle et al. (2012)
California sea otter	*Enhydra lutris nereis*	MixSIR	Newsome et al. (2012)
Common Terns	*Sterna hirundo*	SIAR	Bond & Diamond (2011)
Balearic Shearwaters	*Puffinus mauretanicus*	SIAR	Bond & Diamond (2011)
Gastropods	*Lepetodrilus elevatus, Lepetodrilus pustulosus, Lepetodrilus ovalis, Eulepetopsis vitrea, Cyathermia naticoides, Peltospira delicata, Peltospira operculata* and *Rhynchopelta concêntrica*	SIAR	Gaudron et al. (2015)
Little penguin	*Eudyptula minor*	SIAR	Chiaradia et al. (2014)
Green sea turtle	*Chelonia mydas*	SIAR	Bezerra et al. (2015)
Nile Tilapia	*Oreochromis niloticus*	MixSIAR	Moraes (2016)

explain that to use stable isotopes to determine the relative importance of various sources, which is equally important to understand the proportions of the sources that are less divergent in the isotopic composition. They explain that sensitivity analyses indicated that the uncertainty of estimates of the proportions are affected by the difference in signature isotopes between sources, by variability of the mixture signatures and by the size of the sample.

PHILLIPS & KOCH (2002) demonstrated that IsoConc is based on a geometric interpretation of distance between vertices that form a mixing triangle and a triangle of food sources. In theory, there should be a visual relation on the graph between the mixing triangle and the triangle of sources, which explains whether there is a relationship or not between the consumer and each food item. Using the program, the authors realized that the IsoConc model corresponded successfully to the known proportions of food sources in relation to the species used in the research. ROBBINS et al. (2002) justify that the use of this type of model is a multi-step process, with increased numbers of assumptions and contributions factors to provide. However, KOCH & PHILLIPS (2002) argue that a simple model is just as likely to produce

errors as a more complex model. In addition, mixing models that are statistically more complex are highly sensitive to possible variations on the discrimination factors used (BOND & DIAMOND, 2011).

PHILLIPS et al. (2014) explain, based on the original IsoConc model, that the concentration dependence of the isotopic elements was incorporate in SIAR, in IsotopeR, MixSIAR and FRUITS. For this reason, these authors showed that, when stable isotope analyses are performed, elemental concentrations are also determined (so researchers should make sure to request these data from the laboratory) to consider the dependence of concentration and make it possible to use the models.

HOPKINS & FERGUSON (2012) performed tests of comparative application between IsotopeR and the Bayesian models IsoError, IsoConc, IsoSource, SIAR and MixSIR. They concluded that the interface of IsotopeR (in R) provides researchers with a tool for analysis using mixture of models with stable isotopes (SIMMs) and that the model is also applicable for use in paleontology, archeology and forensic studies, as well as for estimating pollutants. KADOYA et al. (2012) concluded that the IsoWeb model, in general, it provides a promising method

for the reconstruction of quantitative food webs from stable isotope information. However, these authors showed that the current version of IsoWeb does not estimate a network of energy flow, and that it is necessary to develop a structure that can determine a power flow network integrating stable isotopes and other information, such as biomass and metabolic rate of each species.

According to KARNOVSKY et al. (2012), the SISUS mixing model (Stable Isotope Sourcing using Sampling) is a model with frequent-based option. This model considers the rate of discrimination (fractionation), concentration and assimilation efficiency (ERHARDT, 2014).

JACKSON et al. (2011) in turn, described the SIBER (Stable Isotope Bayesian Ellipses in R) as a metric model that analyzes the isotope dataset based on Bayesian ellipses. FRUITS (*Food Reconstruction Using Isotopic Transferred Signals*) is presented as a model capable of manipulating dietary routing that makes it possible to provide a platform that simplifies the incorporation *a priori* information, including information from ecological, archaeological, biochemical or physiological sources (FERNANDES et al., 2014).

To make estimates on the relative contribution of food items more concretely using mixture models, HEALY et al. (2017) developed the DEsiR (*Discrimination Estimation in R*) which is still defined as SIDER (HEALY, 2017). The DEsiR estimates the discriminant rates and includes the functionality to consider the associated error in the construction of phylogenetic branching using the mulTree package (GUILLERME, 2016). These authors tested this model using values of the C and N isotopic ratios for different species of birds and mammals, which makes it possible for other researchers to make estimates for groups and species not yet studied.

In their research, NEWSOME et al. (2012) argued that this mixing models used a linear framework to determine a unique mathematical solution for the relative contributions of "n + 1" prey sources using "n" isotope systems (e.g., $\delta^{13}C$ or $\delta^{15}N$, or both). When evaluating the applicability of one of these models, BENSTEAD et al. (2006) performed tests about contributions from food sources in a mangrove region using IsoSource. They described a statistical model that calculates the contributions in intervals, being useful to demonstrate when a source is not important for a food web. NEWSOME et al. (2012) clarified that Bayesian models, such as MixSIR and SIAR, were developed with the constructive capacity

of IsoSource and allowed users to include information on isotope variation in potential sources and trophic factors of discrimination,and allowed the input of prior information of resources or habitat use. However, HOPKINS & FERGUSON (2012) explained that; although, MixSIR can calculate dietary estimates, it may present very narrow intervals, because the model did not explain the variation between individuals and other sources of error.

Latest models such as SIAR and MixSIR implement a Bayesian approach to mixing models with stable isotopes, and allowed users to incorporate variability in discrimination factors (Δ) or even in the values of the isotopic ratios (δ) between prey and consumers (BOND & DIAMOND, 2011). But the recent MixSIAR model allowed incorporation of hierarchical population structure and other continuous variables in the estimation of assimilated items by consumers (PHILLIPS et al., 2014), in addition to allowing the use of discriminant rates for different food items. This model is an enhanced version resulting from the contribution of researchers who had already developed other models such as MixSIR and SIAR, and, consequently, incorporates the criteria and advances of previous models.

These current Bayesian blend models employ one resampling to determine the probability of contributions from potential sources (NEWSOME et al., 2010; 2012). In general, for each source a proportional contribution vector random is proposed, from a complete set of sources (WARD et al., 2011). This type of statistical approach occurs in the MixSIAR model, for example, which uses the Monte Carlo method based on *a posteriori* simulations by Markov chains (MCMC). Markov chains are defined as stochastic processes that have the purpose of modeling systems and describing the probabilistic movement between a series of data through simulations (ALBERT, 2009).

When the chains are initiated at different locations in the state-space vector, each chain will converge to its equilibrium distribution or stationary distribution, which can be observed through convergence tests (GAMERMAN & LOPES, 2006). According to STOCK & SEMMENS (2013), MixSIAR showed three of these tests: GEWEKE (1992), GELMAN-RUBIN (1992) and HEIDELBERGER-WELCH (1983). The convergence evaluated by the method of GEWEKE (1992) performed a division of variables generated by simulation, with respect to the two means in the quantiles (0.1 and 0.5). Thus, this test used the comparison of the initial and final values of the Markov chain to verify convergence faults;

therefore, with the hypothesis of nullity tested, values of probability below 0.05 indicated evidence contrary to the convergence of chains (BORBA et al., 2011).

For the GELMAN-RUBIN test, (GELMAN-RUBIN, 1992; GELMAN et al., 2014) scale reduction factor "R" can be used as an indicator of convergence. GELMAN & RUBIN (1992) and GELMAN et al. (2014) determine if there has been convergence for values using the following rule: $1 \leq R \leq 1.1$. In addition, if the values of R are greater than 1.1, it is necessary to execute the process of tracking by chains again in order to improve the convergence of the data set, and to perform a better evaluation of the data used in the research.

The HEIDELBERGER-WELCH (1983) diagnostic test, evaluates the null hypothesis of stationarity of chains using two simultaneous tests. If the null hypothesis is rejected, for a given value, the test is repeated after discarding the 10% of the initial values of the sequence. If the hypothesis is rejected again, plus 10% of the initial values are discarded and thus successively until they discarded the 50% of the initial values (DELFINO et al., 2011). However, DELFINO et al., (2011) explained that if the hypothesis is rejected again, it indicated that a larger number of iterations is required. Otherwise, the initial number of iterations discarded indicated as the "burn-in" size. According to these authors, if one considers that a sequence of values sampled has reached the stationarity, the Half-Width test should be applied to verify the estimated average being calculated with a pre-specified accuracy and, thereby, convergence will be guaranteed when the null hypothesis is accepted in both tests.

CONCLUSION

The ^{13}C and ^{15}N isotopes and mixing models have been used successfully in several studies that aim to determine the type of diet consumed and the trophic level of aquatic organisms. However, it is essential that all data inserted in these models (discriminant factor, information on the aquatic ecosystem, species and its food sources) are obtained and properly determined in accordance with the previously defined objectives. In this context, the use of these tools enables: (I) a better understanding of the real contribution of food items (natural, artificial, autochthonous and/or allochthonous) in the nutrition of aquatic organisms, (II) adequate understanding of biology and ecology of the species studied and (III) establishment of interactions and trophic relationships between groups and species in their habitat. We concluded that application of the technique of stable

isotopes with the application of mixing models composes an efficient tool to understand how aquatic organisms use food resources in their natural environment or in the cultivation environment, and how they contribute to its growth and development at different stages of life.

In animal nutrition, it becomes increasingly necessary to develop new research that includes isotopic analyses in conjunction with other types of chemical markers, especially environmental impact analyses, such as how to quantify concentrations of heavy metals based on the food ecology of a species. There will be a progressive increase in the application of mixing models as authors have become more selective and increasingly consider the inherent problems of the method. Among these problems, there is the problem of isotopic memory, related to the turnover rates and the different sources of variation that influence discriminant rates, and in the fractionation factor. Thus, consider the inherent problems of the method, ensures the reliability in the data analysis and in the results obtained by these researches.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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