Temporal stability in fish assemblage isotopic niches: insights from a conservation reference river in southeastern Brazil

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Abstract

Globally, aquatic ecosystems are facing the highest levels of anthropogenic pressures ever. Thus, the characterization of environments with little or no impact becomes critically important for the development of sound conservation efforts. The Santo Antônio River, a tributary of the Doce River, has already been proposed as a priority area for conservation as it is an important free-flowing river with high biodiversity in the Rio Doce basin. This study aimed to characterize the trophic structure of the fish assemblage in the Santo Antônio River using stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes. We evaluated the temporal stability of isotopic metrics using data from sampling conducted in 2020 and 2022. Over the two years, a total of 24 fish species were collected. The fish community exhibited stability in isotopic parameters, despite temporal variation in species composition. The overall isotopic niche of the community varied minimally, showing substantial overlap (44%) between the two years values. The most important resources sustaining the community remained the same for most feeding guilds. Omnivores and invertivores primarily consumed aquatic invertebrates in both years, while piscivores relied on fish. Detritivores and herbivores fed mainly on periphyton in 2020 and on suspended material in 2022. Trophic diversity metrics remained stable. In general, despite some variations in collected species and assimilated items, the isotopic structure remained stable over time. Thus, these parameters can serve as a reference tool for comparison with other impacted sites in the basin and in so much-needed recovery efforts within the Doce River basin.

Keywords: Doce River; Ichthyofauna; Stable Isotopes; Layman Metrics; Carbon; Nitrogen

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Introduction

Water stands as a natural resource of utmost significance for human civilization. Initially employed in agriculture and livestock management, its utilization progressively extended to industrial sectors with the surge in urbanization (Kummu et al. 2011; Feio et al. 2021). Consequently, more than half of aquatic ecosystems are currently under a substantial threat due to anthropogenic activities (Dudgeon et al. 2006; Vörösmarty et al. 2010; ICMBio 2018). Several categories of impact have undergone extensive investigation, encompassing the invasion of exotic species, habitat degradation, aquatic pollution, alteration of flow regimes, and overexploitation of communities (Malmqvist and Rundle 2002; Dudgeon et al. 2006). Recent contributions to this discourse have introduced additional perspectives, such as the cumulative effect arising from the interaction among diverse pressures, and considerations about emerging pollutant sources (Craig et al. 2017; Pelicice et al. 2017; Reid et al. 2019; Torremorell et al. 2021).

Due to the escalating and persistent impact of polluting activities and anthropogenic impacts, it is crucial to comprehend the functioning of environments that still exhibit little or no anthropogenic alteration (Bailey et al. 2004). These environments, defined as reference sites, provide a baseline from which changes can be assessed, aid in discerning the effects of human activities on ecosystems, and serve as models to guide restoration and conservation efforts (Dodds and Oakes 2004; Stoddard et al. 2006; Alagona et al. 2012). However, pristine environments are becoming increasingly rare, leading to the utilization of less disturbed sites as surrogate reference areas (Stoddard et al. 2006; Kosnicki et al. 2014). Therefore, the characterization of these areas, taking into account the specific attributes of each studied environment, encompassing physical, chemical, and biological aspects (Bailey et al. 2004), is of paramount importance.

The Doce River basin, located in the southeastern region of Brazil, has a pronounced history of exploitation and alteration of its environments, attributed to various anthropogenic activities (Vieira 2009). Intensive land use, deforestation, untreated sewage pollution, eutrophication of watercourses, and river siltation (PIRH - Doce 2010; ANA 2016), along with the historical mining exploration (Hatje et al. 2017), have significantly transformed this drainage. In the year of 2015, the Fundão dam in Mariana, MG, experienced a huge rupture resulting in the contamination of the Doce River bed with 66 million m^3 of iron mining tailings (Governo do Estado de Minas Gerais 2016), further aggravating the various pre-existing impacts affecting this basin. As a result of this rupture, which was deemed the most significant environmental disaster in Brazil (Hatje et al. 2017), considerable attention has been directed towards the Doce River basin, prompting the beginning of a series of environmental recovery actions. However, the conditions prior to the historical impacts to which the river has been subjected remain unknown, emphasizing the urgent need for a better understanding of potential reference ecosystems, including more pristine tributaries of the basin.

One of the tributaries of the Doce River, the Santo Antônio River holds significant ecological importance in the region and has been proposed as a priority area for conservation (Drummond et al. 2005), and an important area of free-flowing river (IEF 2021). Recognized for its high biodiversity and as a crucial lotic remnant (Vieira

2006; dos Santos and de Britto 2021), it serves as a habitat for half of the native fish fauna in the Doce River basin. Specifically, 57 species, constituting 50% of the total native fauna in the Doce basin, are found in this river (Vieira 2006; Bueno et al. 2021). Notably, one species, *Henochilus wheatlandii*, has its current occurrence area restricted to the Santo Antônio river basin (Vieira et al. 2000), while others are endangered species, including *Hypomasticus thayeri*, *Steindachneridium doceanum*, *Brycon dulcis*, and *Phochilodus vimboides* (Vieira 2006; Sales et al. 2018; Biodiversitas 2021).

Among other characteristics of a reference site, it is crucial to understand the functioning of trophic dynamics (Zanden et al. 2016), delineating the primary sources of energy that sustain local communities and its trophic structure. The flow of energy within a system can be examined through the analysis of stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes of resources and consumers of a community (Peterson and Fry 1987; Syväranta et al. 2011). Through the isotopic composition of carbon, it becomes possible to trace the carbon sources incorporated by consumers, as they will reflect the carbon signature of the consumed food resource (Post 2002a). The nitrogen isotopic composition enables the identification of the trophic level of organisms, as there is an enrichment in the nitrogen signature of the consumer compared to its resource (Post 2002b). The nitrogen composition can also be employed to trace pollution impacts, as changes in nitrogen concentrations in its various forms (nitrate, nitrite, ammonia) in the water may be reflected in the nitrogen signatures of aquatic organisms (Wang et al. 2016; de Carvalho et al. 2021).

Among aquatic organisms, fish constitute a widely distributed group, with species occupying various trophic levels (Karr 1981). The isotopic composition of fish communities provides a valuable tool for evaluating various ecological aspects. These include seasonal variations in trophic niches and niche overlap in floodplain fish in the

Amazon (Azevedo et al. 2022), the impact of artificial ecosystems and non-native species on the trophic niches of Mediterranean fish (Toutain et al. 2024), the influence of different land-use types on the trophic structure of stream fish (Carvalho et al. 2017), and how environmental gradients and human disturbance affect fish isotopic niches (Wang et al. 2021). Regarding disturbances, studies demonstrate that variations in food resource signatures can have a significant influence on the trophic parameters of the community, particularly in disturbed environments (de Carvalho et al. 2021, 2022).

Studies conducted in a watershed under significant anthropogenic influence show that fish communities in reference condition environments exhibit greater stability in their trophic niches (de Carvalho et al. 2021). Furthermore, the trophic structure of fish communities in these reference condition rivers is more similar to each other (Alonso et al. 2020; de Carvalho et al. 2023) and streams (Carvalho et al. 2017). The authors also highlight the importance of improving knowledge about aquatic environments in reference conditions, as this can inform the development of recovery strategies for disturbed aquatic ecosystems (Alonso et al. 2020).

In this study, the objective was to assess the trophic structure of the fish assemblage in the Santo Antônio River using stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes. Through samplings in two distinct periods (2020 and 2022), we evaluated whether different isotopic metrics are temporally stable, allowing for their potential use as a baseline for the Doce River basin. Thus, our specific objectives were a) to characterize the trophic structure of the fish assemblage and the trophic position of each species for each sampling year; b) to analyze the niche occupied by the fish assemblage; c) to identify which resources are most important for the fish assemblage in each year; d) to compare trophic diversity indices between the two years. We tested the hypothesis that, although trophic levels and assimilated items by each species may change between years, parameters of the trophic structure such as trophic diversity metrics of a fish assemblage in a minimally impacted environment should remain stable. With this information, we aim to isotopically characterize the trophic structure of the fish assemblage in the most significant reference site of the Doce River basin and identify possible metrics that can be used for comparison with regions degraded by the dam collapse.

Material and Methods

Study area

The Doce River basin is located in two Brazilian states: Minas Gerais (86%) and Espírito Santo (14%), covering an area of 83,431 km² (ANA 2016). The confluence of the Carmo and Piranga rivers forms the main course of the Doce River, which flows for 888 km before reaching its mouth in the Atlantic Ocean (ANA 2016). The dry season extends from April to September, while the rainy season lasts from October to March. The Atlantic Forest biome dominates the basin, covering 98% of its area, with the remaining portion consisting of Cerrado vegetation (ANA 2016). The main tributaries of the Doce River include the Piranga, Piracicaba, Suaçuí Grande, Suaçuí Pequeno, Caratinga, Manhuaçu, Guandu, Santa Maria do Rio Doce, São José and Santo Antônio rivers (PIRH - Doce 2010). In 2015, the collapse of the Fundão dam in Mariana, Minas Gerais, Brazil, profoundly affected the main channel of the river. The release of mining tailings flowed downstream, ultimately reaching the Atlantic Ocean. This disaster caused the destruction of 1,469 hectares of riparian vegetation, sediment deposition in floodplain areas (Fernandes et al., 2016), and the death of 11 tons of fish (Governo do Estado de Minas Gerais, 2016). It had severe impacts on both freshwater and marine fauna. Additionally, the event resulted in the loss of 19 human lives and significant socio-economic repercussions for the region (Fernandes et al., 2016)

The Santo Antônio River watershed is entirely located in the state of Minas Gerais, covering an area of 10,429.46 km² (PIRH - Doce 2010). The main left bank tributaries are the Peixe and Guanhães rivers, while on the right bank, are the Preto, Itambé and Tanque rivers (PIRH - Doce 2010). The Santo Antônio River originates in the Serra do Espinhaço, in the municipality of Congonhas do Norte, and flows for approximately 280 km until it joins the Doce River in the municipality of Naque (PIRH - Doce 2010). The majority of the basin is situated within the Atlantic Forest biome, a biodiversity hotspot and one of the most fragmented biomes globally (Myers et al. 2000; de Lima et al. 2020). The Santo Antônio River was sampled at a location near the municipality of Santo Antônio do Rio Abaixo, Minas Gerais, in two different years (Fig. 1). Sampling took place in August/September of 2020 and 2022.

Data sampling

Fish sampling:

Fish sampling was conducted using gill nets, trawl nets and sieves. Two sets of gill nets (mesh sizes of 3-16 cm and 10 m in length) were deployed continuously for a period of 12 hours. Trawl nets (5 m in length with a 1mm mesh) were employed for sampling in shallow and littoral areas. Sieves (80 cm in diameter with a 1 mm mesh) were used in macrophyte banks near the shoreline. After sampling, all individuals were measured, weighed, and identified. In the field, a portion of the muscle tissue was excised and immediately frozen until laboratory processing to prevent decomposition. The aim was to sample tissue from at least five individuals of each species for isotopic analysis. The sampled species were categorized into trophic guilds (detritivore, herbivore, omnivore, invertivore, and carnivore/piscivore), according to the literature (Online resource 1).

All sampling procedures were authorized by SISBIO – *Sistema de Autorização e informação em Biodiversidade* - License No. 10327-3, and by the Ethics Committee on Animal Use of the Universidade Federal de Lavras (CEUA – UFLA), protocol No. 034/21.

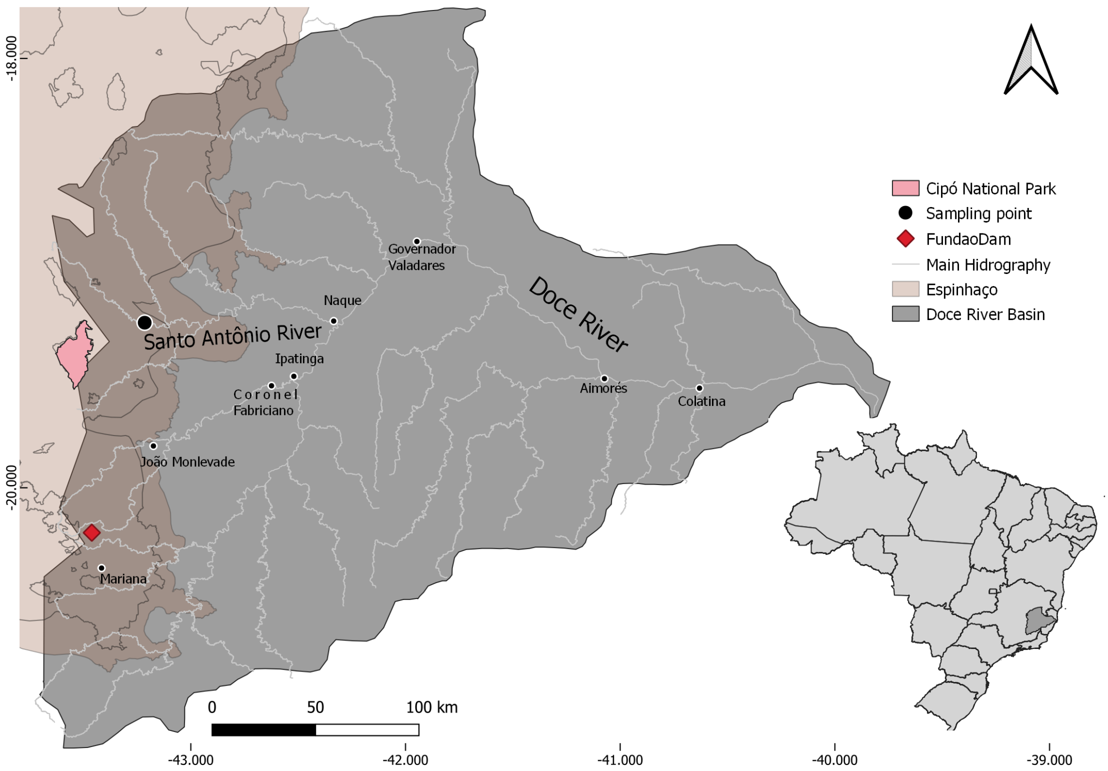

Fig. 1 Location of the fish and food resources sampling point in the Santo Antônio River, Minas Gerais, Brazil. The dark-gray area represents the Doce river basin area

Food Resources Sampling:

Samples of potential fish food items were also sampled for isotopic analysis. Resources sampled in both years included filamentous algae, coarse particulate organic matter (CPOM), terrestrial and aquatic invertebrates, suspended material, and periphyton. To address potential gaps in food resources that were not sampled in 2020, new resources were collected in the second sampling in 2022, including grasses, macrophytes, riparian vegetation, manure, and fine particulate organic matter (FPOM). The collection of five samples for each resource was standardized. Samples of macrophytes, CPOM, terrestrial invertebrates, filamentous algae, grasses, riparian vegetation, and manure were arbitrarily, i.e. systematically, sampled at the sampling site. Each resource sample was collected at least 5 meters apart from the other to minimize pseudoreplication. Aquatic macroinvertebrates were sampled using Surber nets and sieves (80 cm in diameter with a 1mm mesh) and were collected to encompass different taxa in each sample. FPOM samples were collected by resuspending sediment and passing this material through a phytoplankton net (45 µm mesh). Periphyton was sampled by scraping rocks with the aid of a brush, and the collected material was stored in plastic containers with distilled water. Suspended material was sampled using a phytoplankton net (45 µm mesh) placed in the water column for a period of three minutes. The samples were frozen until laboratory processing.

Isotopic Analysis:

In the laboratory, fish tissue samples were lyophilized for 24 hours, ground into a uniform powder, and stored in Eppendorf tubes. Liquid samples (periphyton, FPOM, and suspended material) were filtered using a filtration apparatus connected to a vacuum pump with pre-burned quartz filters (Whatman® QMA). Subsequently, the filters with the samples were dried in an oven at 40°C for a period of 24 hours. The remaining samples were lyophilized for 24 hours, then ground and stored in Eppendorf tubes for subsequent isotopic analysis. After the preparation of the materials, they were sent for isotopic analysis. In 2020, the samples were analyzed at *Centro de Energia Nucelar na Agricultura* (CENA) at the *Universidade de São Paulo* (USP). In 2022, the analyses were conducted at the *Central de Análises e Prospecção Química* (CAPQ) of the *Universidade Federal de Lavras* (UFLA). For isotopic analysis, 1 mg of fish and resource material was used. For liquid sample resources, 35 mg of material was used.

To determine the isotopic ratio in 2020, we used a continuous-flow isotope ratio mass spectrometer (CF-IRMS); with a Carlo Elba analyzer coupled to a Delta Plus mass spectrometer (Thermo Scientific). In 2022, we used a Delta V Plus Isotope Ratio Mass Spectrometer (Thermo Fisher) coupled with a Flash IRMS elemental analyzer interfaced with ConFlo IV. The results were expressed as relative differences in international reference systems, using the delta (δ) notation, in parts per thousand (‰), and calculated using the following formula (Eq. (1)):

$$\text{Eq. (1) } \delta X = \left[\left(\frac{R sample}{R standard}\right) - 1\right] x \; 10^3$$

where X is ^{13}C or ^{15}N, and R is the isotopic ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (Barrie and Prosser 1996). The International Atomic Energy Agency standards IAEA-N1 and IAEA-N2 were used for nitrogen in both years, NBS-19 and NBS-22 for carbon in 2020 and Certified Reference Material CRM 0002 of sucrose of sugarcane for carbon in 2022.

Statistical Analyses

The trophic structure of the fish assemblage in the Santo Antônio River was characterized for each year using bi-plot graphs (x-axis: $\delta^{13}C$ and y-axis: $\delta^{15}N$) with the isotopic signatures of potential food resources and sampled fish. The bi-plot graph was represented in two ways: A) Mean values of the isotopic signature for each species and the mean and standard deviation of food resources; and B) Mean values and standard deviation of the isotopic signature of the species. In the first representation, it is possible to observe which basal resources are more assimilated by the fish assemblage, while in the second representation; we can more clearly assess intraespecific variations in isotopic compositions. To test for differences between the basal resources isotopic signature over the two study years we performed T-tests or Mann-Whitney tests depending on the normality of the data.

The trophic position of the species was calculated for each year using the Bayesian package "tRophicPosition" in R (Quezada-Romegialli et al. 2018). The model employed was the "complete two-source baseline model," with benthic macroinvertebrates and periphyton being considered as baselines. The model ran with 20000 iterations and 20000 adaptative samples. The isotopic fractionation for muscle tissue was $\Delta^{15}N$: 2.9 ± 0.32‰ and $\Delta^{13}C$: 1.3 ± 0.3‰ (McCutchan et al. 2003).

To assess potential differences in the isotopic niche of trophic guilds during the two sampling periods, we employed the Standard Ellipses Area (SEA). SEA was calculated for the whole fish assemblage in each year, and for the trophic guilds of each year. The SEA represents the isotopic niche and illustrates the richness and uniformity of the resources consumed by the population (Bearhop et al. 2004). Graphical representation of the ellipse areas and the degree of niche overlap was conducted using the SIBER package (Jackson et al. 2011) in the R program.

The contribution of each food resource to the species' diet was calculated for each year using Bayesian stable isotope mixing models with the MixSIAR package (Stock and Semmens 2016) in the R program (R Core Team 2024). Food resources selected for the partitioning analysis were based on the feeding habits of each species, as described in the literature. The species were grouped into the following feeding groups: detritivores, herbivores, invertivores, omnivores and carnivores/piscivores. For the carnivore/piscivore guild, the selected resources included fish, terrestrial and aquatic invertebrates. For the omnivore and invertivore guilds, the considered resources were periphyton, terrestrial and aquatic invertebrates, and filamentous algae. Lastly, for the detritivore and herbivore trophic guilds, the considered resources were filamentous algae, coarse particulate organic matter (CPOM), suspended material, and periphyton.

To assess trophic diversity between the two sampling periods, we calculated, for each year, the metrics proposed by Layman et al. (2007): Carbon range (CR): the carbon amplitude used by the species; Nitrogen range (NR): the nitrogen amplitude used by the species; Total area (TA): the area occupied by species in isotopic space; Centroid distance (CD): a measure of trophic diversity; Mean nearest neighbor distance (MNND): a measure of trophic redundancy; Standard deviation of the nearest neighbor distance (SDNND): represents the distribution of trophic niches in fish communities. The first four metrics mentioned above are related to the spacing of species in the biplot space and measures trophic diversity. The last two metrics give information about species relative positions to each other and are used to estimate trophic redundancy (Layman et al. 2007).

A recent study conducted in degraded areas of the Doce River has demonstrated average variations of 60% in Layman metrics between years (Fráguas et al. in press). In the same study, niche overlap reached a maximum of 41% in those areas. Such values were considered as “references” for assessing both stability and the degree of overlap.

Results

A total of 129 fish samples, representing 24 species, were analyzed in the Santo Antônio River (Table 1). In 2020, samples from 76 individuals belonging to 16 species were analyzed, and in 2022, a total of 53 samples representing 18 species were analyzed. Among the 24 collected species, ten were present in both sampling years, six species were collected only in 2020, and eight species were collected only in 2022. Among the 24 species collected over the two sampling years, five were classified as detritivores, two as herbivores, four as invertivores, nine as omnivores, and four as carnivores/piscivores, based on the literature.

In 2020, *Hoplias intermedius* and *Oligosarcus solitarius*, piscivorous species, occupied the top trophic position, sharing their resources (Fig. 2a). However, in 2022, the invertivore *Trichomycterus tantalus* took the top position, presenting a carbon signature not explored by any other species at the top of the assemblage (Fig. 2b). The herbivorous species *Henochilus wheatlandii* showed the largest intraspecific variation in both years, with individuals exhibiting markedly different carbon signatures. Throughout both years, *Astyanax lacustris* occupied a similar basal isotopic area on the graphs (Fig. 2c and d). The assemblages sampled in both years had $\delta^{13}C$ values ranging from -25 to -15‰ and $\delta^{15}N$ values ranging from 8 to 12‰ (Fig. 2). Along with *T. tantalus,* species such as *Hisonotus thayeri* and *Phalloceros uai*, collected only in 2022, occupied peripheral positions compared to the rest of the fish assembly in the Santo Antônio River (Fig. 2d).

Table 1 Means and standard deviations of carbon and nitrogen isotopic signatures of fishes and resources sampled in the Santo Antônio River in 2020 and 2022. Means of resources marked with * were statistically different between years by the T-test. p<0.05.

Year of sampling	2020							2022						
Samples		Size (cm)		$\delta^{13}C$		$\delta^{15}N$			Size (cm)		$\delta^{13}C$		$\delta^{15}N$	
	N	Mean	SD	Mean	SD	Mean	SD	N	Mean	SD	Mean	SD	Mean	SD
Detritivores	**13**	**12.26**	**7.95**	**-17.38**	**0.57**	**11.76**	**0.58**	**12**	**11.10**	**5.30**	**-18.78**	**1.82**	**11.45**	**0.96**
Delturus carinotus (La Monte, 1933)	3	20.43	0.98	-17.14	0.38	12.66	0.06	2	12.25	3.88	-20.08	1.33	11.97	0.51
Hypostomus luetkeni (Steindachner, 1877)	5	16.7	2.43	-17.4	0.75	11.71	0.2	4	17.12	1.31	-17.35	0.63	10.41	0.20
Parotocinclus sp.	5	2.94	0.19	-17.5	0.53	11.29	0.32							
Euryochus thysanos Pereira & Reis, 2017								5	7.58	0.92	-18.59	1.20	12.28	0.23
Hisonotus thayeri Martins & Langeani, 2016								1	2.40	-	-22.79	-	10.32	-
Herbivores	**5**	**15.08**	**6.95**	**-18.81**	**3.41**	**10.22**	**0.68**	**7**	**14.20**	**5.01**	**-18.53**	**2.18**	**11.05**	**1.25**
Henochilus wheatlandii Garman, 1890	5	15.08	6.95	-18.81	3.41	10.22	0.68	2	12.8	8.06	-18.11	4.87	9.91	2.31
Hypomasticus mormyrops (Steindachner, 1875)								5	14.76	1.56	-18.70	1.02	11.51	0.33
Invertivores	**14**	**12.37**	**9.39**	**-18.94**	**1.84**	**11.90**	**0.72**	**7**	**15.48**	**7.07**	**-17.86**	**2.20**	**11.80**	**1.11**
Pachyurus adspersus Steindachner, 1879	8	19.62	4.84	-19.77	1.8	12.14	0.95	5	19.24	2.04	-17.29	2.11	12.08	0.25
Trichomycterus aff. *alternatus* (Eigenmann 1917)	6	2.7	0.63	-17.32	1.14	11.51	0.33							
Phalloceros uai Lucinda, 2008								1	1.80	-	-17.60	-	9.41	-
Trichomycterus tantalus Reis, Vieira & de Pinna 2022								1	10.40	-	-20.95	-	12.84	-
Omnivores	**35**	**6.21**	**2.82**	**-19.58**	**2.02**	**11.17**	**0.99**	**20**	**5.88**	**2.22**	**-18.24**	**2.28**	**10.81**	**0.85**
Astyanax lacustris (Lütken, 1875)	4	9.15	1.96	-22.3	1.26	9.75	0.91	2	9.70	0.28	-22.73	1.21	8.76	0.47
Brycon opalinus (Cuvier, 1819)	6	3.65	0.25	-17.56	2.36	10.17	0.4							
Characidium timbuiense Travassos, 1946	3	4.56	0.51	-20.01	0.31	12.03	0.04							
Hypomasticus copelandii (Steindachner, 1875)	1	3.10	-	-20.85	-	11.78	-	2	3.30	-	-18.06	2.07	10.34	0.29
Knodus moenkhausii (Eigenmann & Kennedy, 1903)	5	3.62	0.35	-20.51	0.63	11.52	0.4	5	3.18	0.10	-16.27	0.87	10.78	0.14
Psalidodon sp.	5	4.94	0.46	-20.29	0.92	11.82	0.27							
Characidium cf. *krenak* (Oliveira-Silva, 2022)								1	5.6	-	-18.42	-	11.02	-
Geophagus brasiliensis (Quoy & Gaimard, 1824)	11	9.04	1.86	-18.84	1.43	11.73	0.76	5	6.84	0.85	-16.97	1.56	10.96	0.11
Psalidodon fasciatus (Cuvier, 1819)								5	6.62	0.55	-19.71	0.68	11.64	0.45
Piscivores	**9**	**17.92**	**14.44**	**-18.86**	**0.61**	**13.16**	**0.98**	**7**	**14.30**	**8.52**	**-19.09**	**1.67**	**12.08**	**1.15**
Hoplias intermedius (Günther, 1864)	2	42.50	3.53	-18.89	0.16	13.71	0.09	1	30	-	-17.91	-	12.00	-
Oligosarcus argenteus Günther, 1864	4	9.25	4.55	-18.67	0.82	12.62	1.34	5	11.32	4.71	-19.60	1.75	12.22	1.37
Oligosarcus acutirostris Menezes, 1987	3	13.10	2.81	-19.09	0.57	13.51	0.26							
Crenicichla lacustris (Castelnau, 1855)								1	10.50	-	-17.73	-	11.47	-

Year of sampling	2020							2022						
Samples		Size (cm)		$\delta^{13}C$		$\delta^{15}N$			Size (cm)		$\delta^{13}C$		$\delta^{15}N$	
	N	Mean	SD	Mean	SD	Mean	SD	N	Mean	SD	Mean	SD	Mean	SD
Feeding resources	**30**			**-25.46**	**4.17**	**5.70**	**2.42**	**55**			**-24.04**	**5.72**	**4.56**	**2.30**
Filamentous Algae	5			-29.27	4.9	5.94	2.97	5			-19.65	10.22	6.17	0.65
CPOM	5			-30.27	0.95	1.96	0.73	5			-30.71	0.79	2.17	1.38
Terrestrial invertebrates	5			-26.28	3.91	6.15	3.78	5			-24.27	5.82	6.65	2.15
Aquatic invertebrates	5			-24.42*	1.33	7.88	0.67	5			-20.26*	3.27	7.74	0.58
Suspended matter	5			-23.41	3.16	6.32*	0.63	5			-23.64	2.08	4.00*	2.01
Periphyton	5			-20.58	2.95	6.34*	0.62	5			-20.48	1.13	4.14*	0.27
FPOM	5			-23.99	0.74	5.29*	0.38	5			-22.85	1.35	4.13*	0.44
Grass								5			-26.04	6.59	4.58	3.33
Macrophyte								5			-28.02	1.20	4.39	1.73
Riparian vegetation								5			-30.51	0.90	2.32	2.56
Manure								5			-18.07	1.29	3.83	1.76

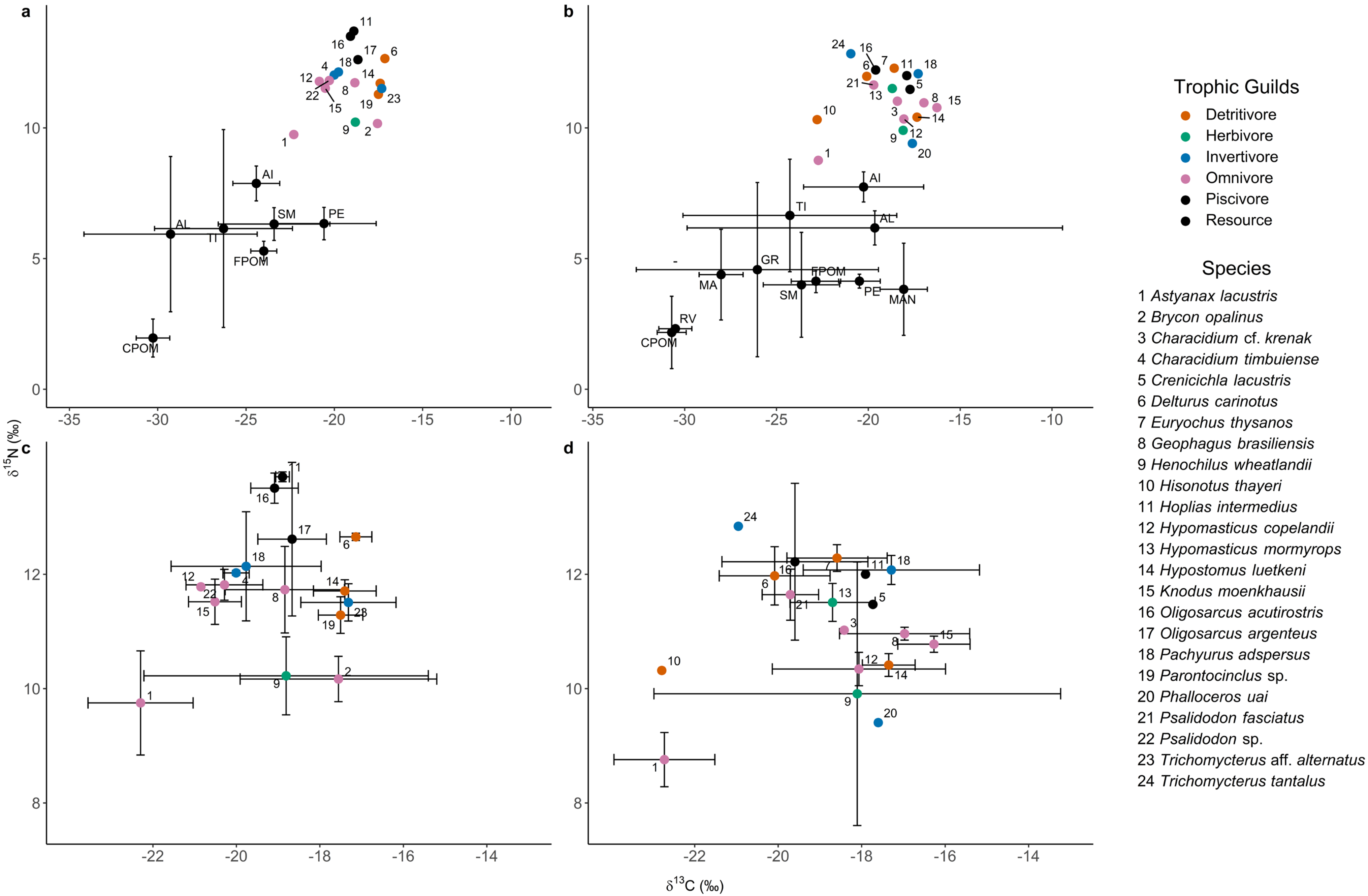
a
b
c
d
δ15N (‰)
δ13C (‰)
Trophic Guilds
Detritivore
Herbivore
Invertivore
Omnivore
Piscivore
Resource
Species
1 *Astyanax lacustris*
2 *Brycon opalinus*
3 *Characidium* cf. *krenak*
4 *Characidium timbuiense*
5 *Crenicichla lacustris*
6 *Delturus carinotus*
7 *Euryochus thysanos*
8 *Geophagus brasiliensis*
9 *Henochilus wheatlandii*
10 *Hisonotus thayeri*
11 *Hoplias intermedius*
12 *Hypomasticus copelandii*
13 *Hypomasticus mormyrops*
14 *Hypostomus luetkeni*
15 *Knodus moenkhausii*
16 *Oligosarcus acutirostris*
17 *Oligosarcus argenteus*
18 *Pachyurus adspersus*
19 *Parontocinclus* sp.
20 *Phalloceros uai*
21 *Psalidodon fasciatus*
22 *Psalidodon* sp.
23 *Trichomycterus* aff. *alternatus*
24 *Trichomycterus tantalus*
AI
AL
SM
PE
TI
FPOM
CPOM
GR
MA
MAN
RV

Fig. 2 Trophic structure of the fish assemblage in the Santo Antônio River in 2020 (a and c) and 2022 (b and d). X-axis: carbon isotopic signature, Y-axis: nitrogen isotopic signature. a) and b) Bi-plot of means and standard deviation of fishes and food resources. c) and d) Bi-plot with means and standard deviation of fishes. Resources: Filamentous algae (AL), periphyton (PE), coarse particulate organic matter (CPOM), aquatic invertebrates (AI), terrestrial invertebrates (TI), and suspended material (SM), manure (MAN), grass (GR), macrophyte (MA), and riparian vegetation (RV)

The species *A. lacustris*, *H. wheatlandii*, and *Hypomasticus copelandii* maintained their basal trophic positions during both years of sampling. The piscivorous species – *Oligosarcus argenteus, H. intermedius* and *O. solitarius* - showed a higher trophic position in both years, accompanied by a detritivorous species, *Delturus carinotus*, in 2020 and *H. thayeri* in 2022 (Fig. 2; Online resource 2). The guilds of invertivores, detritivores, and omnivores, representing the majority of the community species, occupied intermediate positions, except for one species in each trophic guild (*P. uai*, *H. thayeri*, and *A. lacustris*, respectively), which held lower positions in the trophic chain (Online resource 2). In 2020, the trophic positions ranged between 3.14 and 4.96. The majority of species occupied intermediate trophic positions. In 2022, trophic positions ranged between 3.06 and 5.01, trophic positions were more evenly distributed in this year (Fig. 3).

Comparing the trophic niches of the assemblages in the years 2020 and 2022, it there was a niche overlap of 44% between the two years (Fig. 4), with similar amplitude and distribution of $\delta^{13}C$ and $\delta^{15}N$ values in both sampling periods. However, the trophic niche occupied by each guild in 2020 exhibits a more well-defined structure (i.e., more delimited and less overlapping niches) (Fig. 5). In 2020, the herbivores' niche appeared broader and more basal. In the intermediate position are the niches of invertivores, omnivores, and detritivores, with some overlap among them. At the top, there is the more restricted niche of piscivores (Fig. 5a). In contrast, in the year 2022, the distinction between guilds was not as clear-cut, with niche overlap among all studied guilds (Fig. 5b).

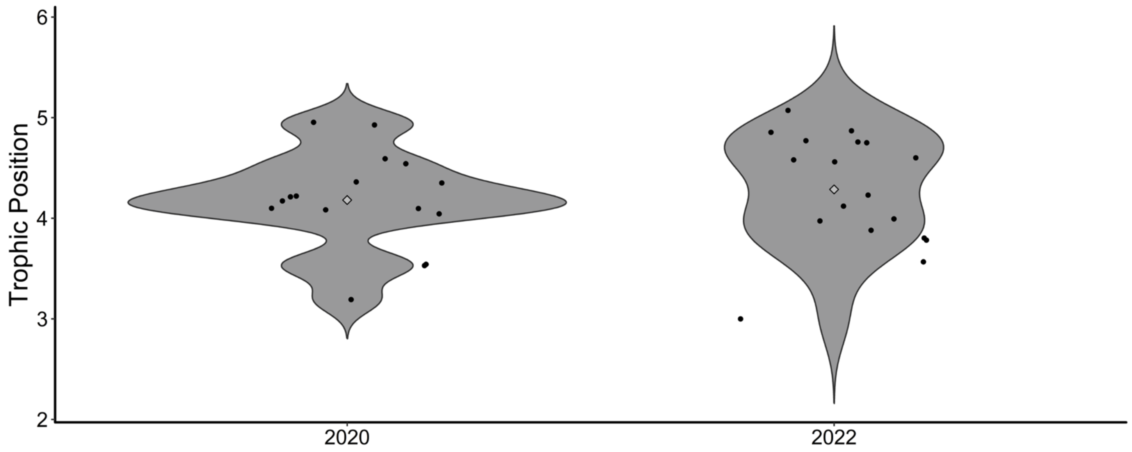

Fig. 3 Violin plot illustrating the distribution of trophic positions for the fish communities in 2020 and 2022. The gray area represents the values distribution; clear circle indicates the mean, black dots indicate species trophic positions in both years.

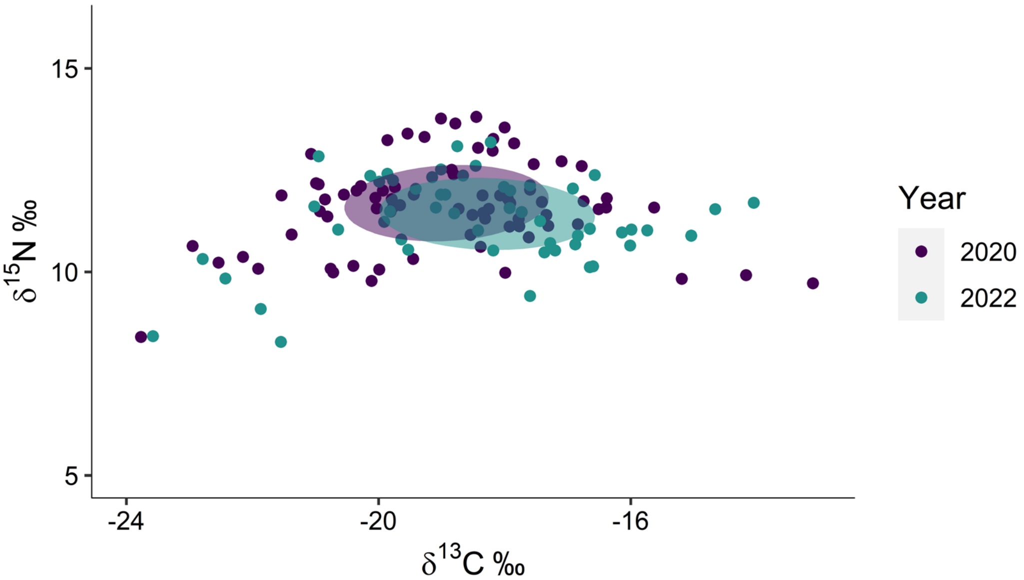

Fig. 4 Ellipses (Standard Ellipse Area - SEA - calculated using a 40% confidence interval) representing the trophic niche of the fish assemblage sampled in 2020 and 2022

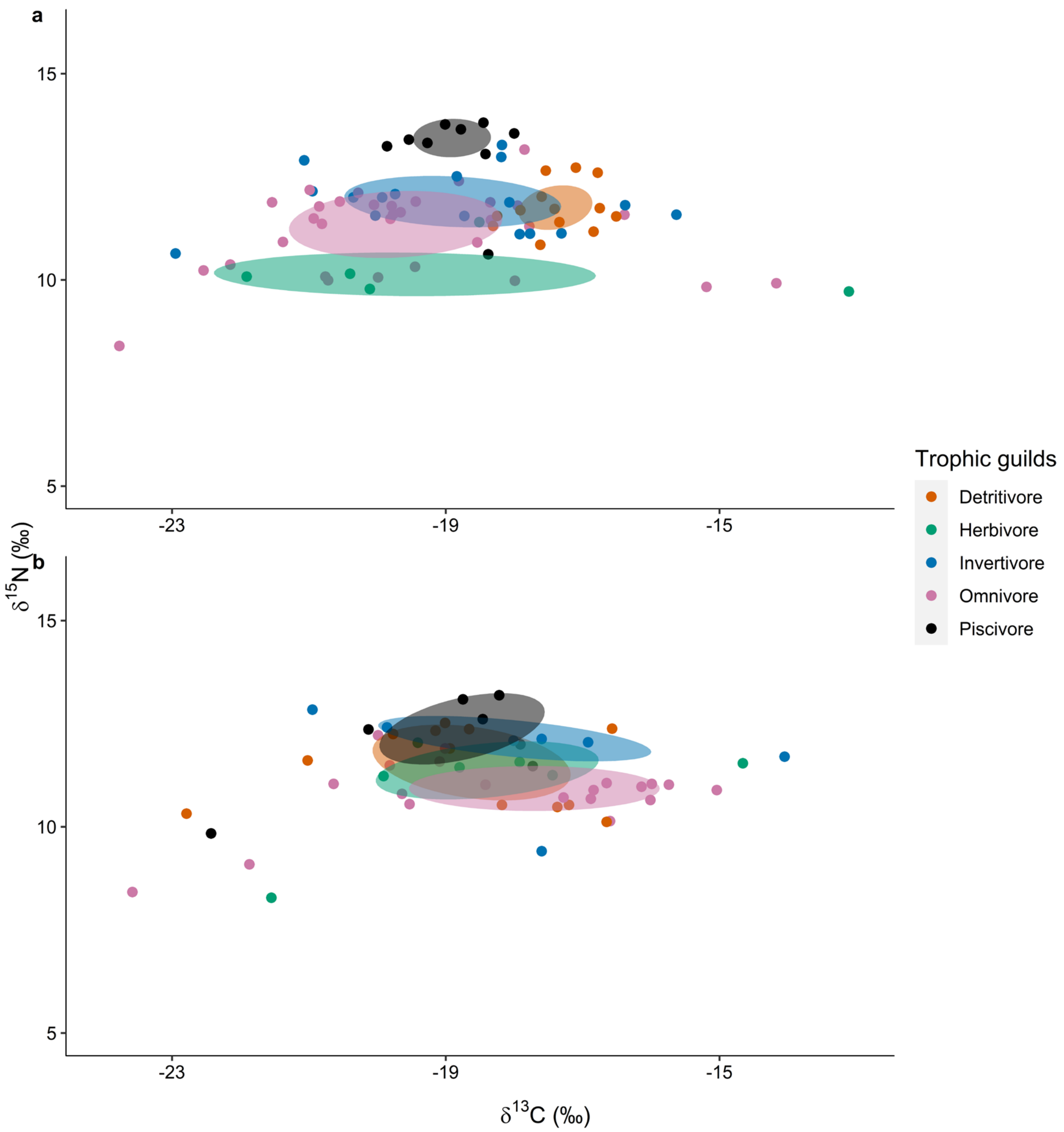

Fig. 5 Ellipses (Standard Ellipse Area - SEA - calculated using a 40% confidence interval) representing the trophic niche of fish guilds sampled in the Santo Antônio River: a) 2020 and b) 2022

The assimilated food resources by the fish assemblage did not change between the sampling years for three of the five evaluated feeding guilds. Aquatic invertebrates were the most assimilated item by omnivores and invertivores both in 2020 and 2022, and fish were the primary resource for the piscivorous guild in both sampling years. In 2022, there was an increase in the assimilation of invertebrates in the diet of piscivores. For detritivores and herbivores, periphyton was the most assimilated resource in 2020, while suspended material had greater importance in 2022. A notable difference between the two sampling years is that in 2020, the most consumed items overall represented half or more of the assimilated energy by the species. In contrast, in 2022, despite observing the highest importance of one food item over the others, the proportion of resource assimilation showed a more even distribution among the analyzed resources (Fig. 6, Online resource 3). Despite the variation in proportions of assimilation by most trophic guilds, different Layman metrics showed similar values in both sampled years. Regarding spacing of the species, there was little variation for carbon and nitrogen ranges and trophic diversity between years (< 15%) and greater variation for total area (41%) (Table 2).

Table 2 Layman's metrics for the years 2020 and 2022. CR = Carbon range, NR = Nitrogen range, TA = Total area, CD = Centroid distance mean, MNND = Mean nearest neighbor distance, and SDNND = Standard deviation of nearest neighbor distance.

Year	CR	NR	TA	CD	MNND	SDNND
2020	5.155	3.962	11.685	1.615	0.621	0.594
2022	6.520	4.085	17.968	1.787	0.729	0.351

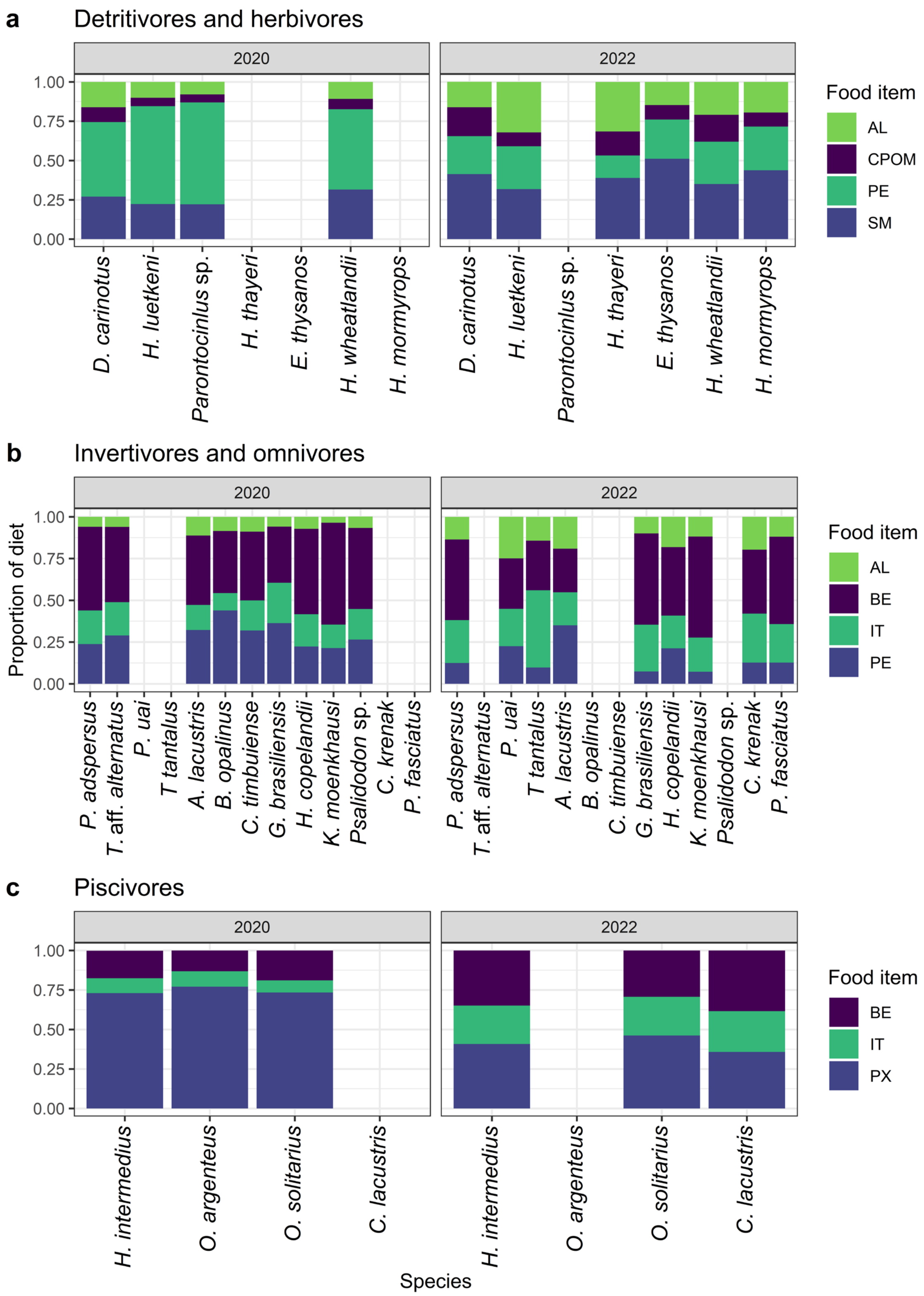

Fig. 6 MixSIAR partition analysis presenting the mean proportions of assimilated food resources for each fish species sampled in the Santo Antônio River. Resources: Filamentous algae (AL), coarse particulate organic matter (CPOM), aquatic invertebrates (BE), terrestrial invertebrates (IT), periphyton (PE), fish (PX), and suspended material (SM)

Discussion

Despite some variation in species composition sampled each year, the fish assemblage of the Santo Antônio River stability across various metrics of isotopic space over the two years of sampling, corroborating the central hypothesis of our study. There was substantial niche overlap between the fish assemblage in both years, despite slight variations in trophic levels and assimilated items for each species. The resources sustaining the assembly remained stable for omnivores and invertivores, which consumed aquatic invertebrates, and for piscivores, which mainly consumed fish. Herbivores and detritivores assimilated mainly periphyton in 2020 and suspended matter in 2022.

Fish sampling represents an assessment of a subset of local species, especially in large rivers (Argent and Kimmel 2005; Lapointe et al. 2006). Therefore, it was expected that the sampled community would not be the same between the two years of sampling. Thus, by analyzing both years of sampling together, we obtain a better representation of the fish community in the region, encompassing 45% of all native species in the Santo Antônio river basin (Vieira 2006). Even with such sampling differences, we observed significant overlap between the isotopic niches of the fish assemblage in both years, as has also been observed in other reference river conditions (de Carvalho et al. 2021). The overlap of niches indicates the stability of trophic conditions in the local fauna, which continues to exploit similar resources over time (Brush et al. 2016).

The community, in general, showed a higher number of species with trophic positions at intermediate levels, with a smaller number of organisms at the base and top of the food chain. This trend was also observed in rivers with good environmental quality in the das Velhas River basin (de Carvalho et al. 2023). This pattern is not observed in disturbed areas; instead, in environments affected by sedimentation, the trophic position of fish tends to rise proportionally with the intensity of sedimentation impact (Burdon et al, 2020). Several factors influence the length of the food web in an environment, such as resource availability, ecosystem size, and disturbance (Post 2002a; Sabo et al. 2009; McHugh et al. 2010). These factors tend to vary according to the ecosystem and the interaction of intrinsic and extrinsic factors within the community (Ward and McCann 2017). Disturbance can affect the length of the food chain in different ways. The food chain length can extend in regions with intermediate levels of disturbance (Power et al. 1996), while shorter chains occur in areas with either no disturbance or excessive disturbance. Constant and extreme disturbance events reduce food chain length by selecting only species tolerant to continuous changes. In contrast, regions without constant disturbances, as is the case of the Santo Antônio River, tend to have shorter food chains, as they allow the establishment of species with more predation protective mechanisms (Post 2002a). Describing the trophic positions of species in areas with low disturbance levels can be useful for future comparisons with more impacted sites throughout the Doce River basin.

For fish communities, it is expected the piscivore guild is at the top, followed by omnivores and invertivores in intermediate positions, and herbivores and detritivores in more basal positions (Jepsen and Winemiller 2002). The same pattern was observed in the Santo Antônio River for the piscivore, invertivore, omnivore, and herbivore guilds. However, detritivores presented more intermediate signatures, along with omnivores and invertivores. The consumption of detritus colonized by microbiota (bacteria, fungi, and protozoa) can increase the trophic position of detritivores (Steffan et al. 2017), thus explaining the observed variation. It is worth noting that species were categorized into trophic guilds based on literature information, which may occasionally deviate from the current biology of local populations. These studies considered stomach content analyses, providing information on the organism's short-term diet and not taking into account what is absorbed by the organism through long-term diet (Rybczynski et al. 2008). For instance, the omnivorous species *Astyanax lacustris* occupied a more basal position than other species in its guild, resembling the signature of herbivorous species. This hypothesis is supported by works indicating the herbivorous tendency of this species (Cassemiro et al. 2002; Pereira et al. 2016).

The isotopic composition of a community also provides information about the niche breadth of species (Newsome et al. 2007). Piscivores and detritivores exhibited a smaller trophic niche area, indicating a specialization on a narrower range of resource. On the other hand, the guilds of herbivores, omnivores, and invertivores, which are more horizontally elongated, suggest the consumption of resources with more varied carbon signatures. Omnivores and invertivores assimilated different feeding resources on even proportions in 2022 and the herbivores showed differences in food resources between the sampling years, suggesting greater than expected dietary plasticity (Abelha et al. 2001). Despite morphological constraints within these guilds for exploring certain food resources, a change in the most consumed resources is still possible in response to environmental variations (Ibañez et al. 2007). The herbivorous species *Henochilus wheatlandii*, endemic to the Santo Antônio River, exhibited trophic plasticity during the two years of sampling, primarily consuming periphyton and suspended material. Despite this species consuming items with a wide variation in carbon signature, it occupied a unique and similar space in the bi-plot graph in both sampling years. The pronounced trophic ontogeny that occurs in this species (Vieira 2006), can explain the great variation in carbon sources since we collected individuals with different body sizes.

Two food resources were most important in sustaining the detritivore and herbivore guild: periphyton and suspended material. The significance of the former source deserves special attention. The Santo Antônio River is located in the Rio Doce basin, where one of the largest mining waste dam failures occurred (Hatje et al. 2017). In areas affected by mining waste, periphyton growth was impacted, negatively influenced by the presence of mining tailings in the environment (Zorzal-Almeida and Fernandes 2021). Moreover, periphyton has been used as a bioindicator in studies indicating changes generated by increased water turbidity (Laird et al. 2021) and the presence of metals (Vidal et al. 2021). Given that periphyton played a significant role in sustaining the fish community in the Santo Antônio River, at least in one of the sampling years, further studies may investigate whether the impact caused by mining tailings on periphyton in the rest of the basin alters the feeding resources available for the fish community. In 2022, higher water turbidity values in the Santo Antônio River (IGAM 2022) may be related to increased suspended material, as turbidity can affect periphyton growth (Ren et al. 2021; Laird et al. 2021). The increase in turbidity observed in the Santo Antônio River corresponds with the higher precipitation levels recorded for the Doce River basin in 2022 compared to 2020 (IGAM 2022).

Invertivorous and omnivorous fish primarily consumed aquatic macroinvertebrates in both sampling years. Within these feeding guilds analyzed in 2022, only a few species did not present higher consumption of macroinvertebrates, consuming all the items in similar proportions. Only one species, *Trichomycterus tantalus*, collected exclusively in 2022, showed greater assimilation of terrestrial invertebrates. The strict preference of the species for this feeding resource can explain the unique space that the species occupies in the biplot. The consumption of terrestrial invertebrates by fish underscores the importance of preserving riparian vegetation around water bodies (Pusey and Arthington 2003). Piscivores showed fish as the most important resource. In 2022, the fish assemblage consumed resources in more homogeneous proportions. Therefore, some resources had a greater contribution to the energy assimilated by fish. Omnivores and invertivores additionally consumed terrestrial insects, algae, and periphyton. Meanwhile, piscivores also consumed terrestrial and aquatic invertebrates. Natural shifts in environmental conditions, such as increased precipitation and water levels, affect the abundance and availability of food resources (Abujanra et al. 2009; Silva et al. 2014). The proportional consumption of resources is associated with the observed overlap in the isotopic niches of feeding guilds in 2022, as certain resources such as algae, periphyton, and both aquatic and terrestrial invertebrates were consumed by more than one trophic guild.

Despite some alterations in the trophic positions, resources exploited by the assemblage and the species sampled each year, the overall isotopic niche of the community remained consistent over the two years. An additional aspect worth exploring in future studies is the comparison between two rainy seasons to assess whether the trophic structure in these locations remains stable despite the influence of seasonality. Carbon and nitrogen ranges and trophic diversity had little variations over the years. The change observed in total area was influenced by species with signatures at extreme values for $\delta^{13}C$ and $\delta^{15}N$. In the 2022 sampling, *Hisonotus thayeri* exhibited lower carbon signatures, with values close to -23‰; *Phalloceros uai*, showing nitrogen-depleted signatures, with values close to 9.50‰; and *Trichomycterus tantalus*, with a carbon signature close to -20‰, showing new isotopic spaces relative to the 2020 condition. In addition to the increase in total area, the slight increase in trophic diversity (CD) reflects greater spacing of species in the second year of sampling. Carbon and nitrogen ranges showed slight increases between the years. The extent of trophic redundancy in the fish assemblage is represented by the metrics of MNND and SDNND (Layman et al. 2007). We observed a slight decrease in trophic redundancy in 2022 (higher MNND values), as species explore different resources like those mentioned above for *H. thayeri*, *P. uai*, and *T. tantalus*. The decrease in the value of SDNND indicates that niches are more evenly distributed, as we observed higher niche overlap between feeding guilds in 2022. The Cipó river, a neighboring reference condition river in Rio das Velhas basin, has similar characteristics and is also located in the Espinhaço mountain range. The stability of the isotopic niche was also observed between rainy and dry seasons (de Carvalho et al. 2021). In this study, we observed minor variations in trophic diversity metrics between the two years. In regions impacted by mining tailings, sites closer to the dam collapse exhibited significant temporal instability, showing pronounced fluctuations across all analyzed trophic diversity metrics (Fráguas et al. in press). Layman metrics proved to be effective indicators of ecological conditions in streams spanning a gradient of anthropogenic habitat degradation (Horka et al. 2023). Carbon Range (CR) and Nitrogen Range (NR), which represent the diversity of resources utilized by the community and the variation in trophic levels, respectively, were associated with habitat variability both within the stream and in adjacent areas. Total Area (TA), reflecting the overall niche space, was influenced by nutrient concentrations in the water, which can modify the variability and quality of food resources. Additionally, biological and chemical oxygen demand, driven by nutrient enrichment, were found to influence Trophic Diversity (CD) and Trophic Redundancy (MNND). Thus, all six metrics proposed by Layman may serve as important indicators of ecological alterations in impacted systems.

Conclusion

This work presents the isotopic characterization of the fish assemblage in Santo Antônio River, a biodiversity-rich river in reference conditions. The use of stable isotope data allowed for the generation of robust information regarding the trophic structure of the fish assemblage. Our data underline the overall stability of fish community metrics and general parameters of the isotopic structure may characterize relatively undisturbed conditions, despite isotopic variations in some species. These parameters can be used not only as a baseline in the face of various impacts (PIRH - Doce 2010) affecting the basin but also to monitor the effects of basin recovery efforts elsewhere in this basin.

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