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1 **Space-time dynamics in monitoring neotropical fish communities using eDNA**  
2 **metabarcoding**

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23 **ABSTRACT**

24 The biodiverse Neotropical ecoregion remains insufficiently assessed, poorly managed, and  
25 threatened by unregulated human activities. Novel, rapid and cost-effective DNA-based  
26 approaches are valuable to improve understanding of the biological communities and for  
27 biomonitoring in remote areas. Here, we evaluate the potential of environmental DNA  
28 (eDNA) metabarcoding for assessing the structure and distribution of fish communities by  
29 analysing water and sediment from 11 locations along the Jequitinhonha River catchment  
30 (Brazil). Each site was sampled twice, before and after a major rain event in a five-week  
31 period and fish diversity was estimated using high-throughput sequencing of 12S rRNA  
32 amplicons. In total, 252 Molecular Operational Taxonomic Units (MOTUs) and 34 fish  
33 species were recovered, including endemic, introduced, and previously unrecorded species for  
34 this basin. Spatio-temporal variation of eDNA from fish assemblages was observed and  
35 species richness was nearly twice as high before the major rain event compared to afterwards.  
36 Yet, peaks of diversity were primarily associated with only four of the locations. No  
37 correlation between  $\beta$ -diversity and longitudinal distance or presence of dams was detected,  
38 but low species richness observed at sites located near dams might that these anthropogenic  
39 barriers may have an impact on local fish diversity. Unexpectedly high  $\alpha$ -diversity levels  
40 recorded at the river mouth suggest that these sections should be further evaluated as putative  
41 “eDNA reservoirs” for rapid monitoring. By uncovering spatio-temporal changes, unrecorded  
42 biodiversity components, and putative anthropogenic impacts on fish assemblages, we further  
43 strengthen the potential of eDNA metabarcoding as a biomonitoring tool, especially in regions  
44 often neglected or difficult to access.

45 **Keywords:** eDNA, biodiversity assessment, fish, freshwater, Brazil, river

## 46 **1 INTRODUCTION**

47           Despite covering less than 1% of the Earth's surface, freshwater habitats harbour over  
48 40% of global fish diversity (Nelson, 2006; Dudgeon et al., 2006). Fish from rivers, lakes, and  
49 wetlands provide essential protein subsistence for a large proportion of human populations  
50 worldwide (FAO, 2012; McIntyre et., 2016), and are increasingly affected by anthropogenic  
51 impacts (e.g. habitat modification, fragmentation, climate change; Vörösmarty et al., 2010;  
52 Grill et al., 2019). Because of the global impact to freshwater ecosystems, their associated  
53 vertebrate populations are declining at alarming rates (83% decline since 1970; WWF, 2018),  
54 and their conservation and management are a priority for global biodiversity (IPBES, 2019).  
55 Nevertheless, despite broad agreement on the requirements to understand and monitor  
56 biodiversity and ecological networks in freshwater habitats (Socolar et al., 2015), our  
57 comprehension of biodiversity conservation in this realm lags behind terrestrial and marine  
58 environments (Jucker et al., 2018).

59           The Neotropical region harbours one of the greatest freshwater fish diversities in the  
60 world (approximately 30% of all described freshwater fish species), and is currently facing  
61 unprecedented levels of anthropogenic pressure. In this region, conservation and management  
62 actions in freshwater habitats are challenging due to a lack of infrastructure leading to  
63 sampling constraints, as well as a shortage of taxonomic expertise to fully characterise this  
64 megadiverse ichthyofauna (Reis et al., 2016). In Neotropical countries such as Brazil, fish  
65 biodiversity assessment relies on sampling using traditional survey methods (e.g. gill nets and  
66 traps) followed by morphological identification, which might be selective, harmful, and have  
67 low detection rates for rare and elusive species and small life-stages (Becker et al., 2015;  
68 Sales et al., 2018).

69           Use of specific fishing practices coupled with the remoteness and large geographic  
70 extension of most catchments, has meant that Neotropical rivers have not been sufficiently  
71 surveyed for baseline estimates of fish diversity. Underestimation of fish diversity resulting  
72 from low sampling efficiency may provide biased metrics and hamper management and  
73 conservation plans (Trimble & van Aarde, 2012), including recovery plans for damaged  
74 ecosystems (Sales et al., 2018). In addition, with a significantly reduced investment in  
75 scientific research and conservation (Thomé and Haddad, 2019), there is an urge to move  
76 towards more cost-effective methods to estimate biodiversity at a broad scale (i.e. detecting  
77 and monitoring multiple species simultaneously in vast areas).

78           Molecular approaches offer a universal key to identify, assess and quantify  
79 biodiversity, especially in biodiversity-rich and understudied ecosystems and regions  
80 (Schwartz et al., 2006). One of the most effective approaches to circumvent the limitations of  
81 traditional surveys in mega-diverse systems is the use of DNA barcoding and metabarcoding  
82 (Gomes et al., 2015; Cilleros et al., 2019). Sequencing DNA traces present in the water  
83 (environmental DNA or eDNA) can now be reliably used to detect species presence (Deiner et  
84 al., 2017) and, to some extent, abundance (Doi et al. 2017; Ushio et al. 2018; Shelton et al.,  
85 2019). Recently, Cilleros et al. (2019) demonstrated the efficiency of eDNA metabarcoding in  
86 providing spatially extensive data on freshwater fish biodiversity in French Guiana, and a  
87 better discrimination of assemblage compositions when compared to traditional sampling. We  
88 recently showed the influence of sampling medium, as well as sampling preservation and  
89 time, on the reconstruction of ichthyofaunal assemblages in a Brazilian catchment, inferred  
90 through eDNA (Sales et al., 2019). Nevertheless, the vast majority of eDNA metabarcoding  
91 biomonitoring studies remain concentrated in temperate regions, in established and fairly  
92 well-accessible environments (Handley et al., 2019; McDevitt et al., 2019).

93 In this study, we use eDNA metabarcoding to unravel patterns of fish diversity in a  
94 poorly studied Brazilian catchment, the Jequitinhonha River Basin (JRB). This catchment  
95 belongs to the east Atlantic basin complex, characterised by a high number of species  
96 endemism (Reis et al., 2016). Until 2010, the known ichthyofauna of this catchment included  
97 63 described fish species (including 10 introduced species and five endangered species, Rosa  
98 & Lima 2008; Andrade-Neto, 2010), making this river a relatively low biodiversity ecosystem  
99 when compared to its neighbouring basins. This reduced species richness had been linked to  
100 historical geological and geographical features (Andrade-Neto, 2010). However, the  
101 geological history of the JRB is very similar to that of adjacent basins (e.g. Doce and Mucuri  
102 river), which led to the consideration that more contemporary factors may explain the low  
103 biodiversity in the catchment, including the lack of adequate surveys and impact from  
104 anthropogenic activities. The Jequitinhonha region is known to be affected by severe  
105 droughts, the impact of dams in the main river course and tributaries, and the occurrence of  
106 introduced species (Sales et al., 2018). Thus, an inadequate baseline survey of the basin might  
107 still account for a great number of native and cryptic species yet to be described for this  
108 catchment (Jerep et al., 2016; Dutra et al., 2016; Nielsen, Pessali & Dutra, 2017).

109 Furthermore, as other semi-arid and arid regions, the Jequitinhonha faces great  
110 variation in water availability (i.e. long dry periods and sudden heavy rain periods; Leite et  
111 al., 2010). However, the influence of precipitation in fish assemblages dynamics have not  
112 been evaluated in this context.

113 Here, we aimed to test whether this DNA-based method can estimate community  
114 structure along the course of this anthropogenically-impacted river and thus be proposed for  
115 use in future biomonitoring purposes. Specifically, we hypothesise that: i) fish community  
116 composition varies across sampling medium (sediment and water samples), ii) biodiversity

117 estimates (alpha and beta-diversities) can be obtained in the absence of taxonomic  
118 assignments, iii) the detection of hidden diversity and alien species can be greatly improved  
119 by expanding regional DNA reference libraries; iv) spatio temporal fluctuation of fish  
120 assemblages can be explained by anthropogenic impacts and rapid seasonal changes. To  
121 address these questions, here we assessed fish diversity, spatially (along the river stem and in  
122 two tributaries) and temporally (before and after heavy precipitation) using eDNA  
123 metabarcoding.  
124

## 125 2 MATERIALS AND METHODS

126

### 127 *2.1 Study Area*

128 The Jequitinhonha River basin (JRB, Figure 1), Southeast Brazil (17°S, 43°W), flows  
129 between two biodiversity hotspots ('Cerrado' and the Atlantic Forest) and is characterised by  
130 a tropical climate and environmental heterogeneity. The main river flows over 1,082 km, from  
131 its source in Serro, at an elevation of 1200 m, to its outlet in the Atlantic Ocean at the locality  
132 of Belmonte. The main river stem is interrupted by two large dams built for hydroelectric  
133 power generation: the Irapé, the tallest dam in Brazil, built in 2006, and the Itapebi,  
134 established in 2002.

135

### 136 *2.2 Historical data and local reference database construction*

137 A compiled species list was built by retrieving all papers available using a Google  
138 Scholar search with the terms "fish" and "Jequitinhonha", combined with a search in  
139 Portuguese language journals (applying the terms "peixe", "Jequitinhonha", "ictiofauna").  
140 The final list included data from research papers as well as compiling information on species  
141 occurrence from unpublished environmental reports (Table S1, Supplemental information).

142 To enhance the available reference sequence database in order to obtain a better  
143 taxonomic assignment, we retrieved all 12S rRNA mitochondrial gene fish sequences  
144 available from GenBank and sequenced 55 additional neotropical species (Table S2).  
145 Information regarding sample preparation and sequencing is provided in the Supplemental  
146 information.

147

### 148 *2.3 eDNA sampling and processing*



149 Two sampling campaigns were conducted at 11 sites during a five-week interval (first  
150 sampling period: 22/01 to 01/02/2017; second sampling: 19/02 to 01/03/2017). Between the  
151 two sampling campaigns, a major precipitation event (from 2.1-50 mm in the first sampling  
152 event to 100-250 mm in the second sampling event - CPTEC/INPE, 2018) occurred. Sites  
153 included locations on the main river (nine) and one on each of two of the major tributaries  
154 (the Itacambiruçu river and the Araçuaí river; Fig. 1). At each site, six water samples of one  
155 liter each and two sediment samples (~25 mL each) each were collected. Sediments samples  
156 were preserved in ethanol and kept cold during the sampling. At the time of sampling proper  
157 storage conditions of samples in tropical field conditions had been untested. Therefore, we  
158 split half of the water samples (N=3) and stored them on ice in a cooling box while for the  
159 other samples (N=3) the cationic surfactant benzalkonium chloride (BAC) was added at a  
160 final concentration of 0.01% as a preservation buffer to suppress the degradation of DNA by  
161 microorganisms (Yamanaka et al. 2017). The effect of storage treatment (ice vs BAC) on  
162 MOTU diversity recovery was significant only for samples obtained during the first  
163 campaign. Still, despite significant ( $p = 0.016$ ) only 2% of the variance was explained,  
164 whereas no significant difference was found for samples obtained during the second campaign  
165 (Sales et al. 2019), all replicates were used for downstream analyses in this study. In total, 132  
166 water samples and 44 sediment samples were analysed.

167 Laboratory work was conducted following Sales et al. (2019) and all information is  
168 detailed in the Appendix included in the Supplemental information. In brief, DNA was  
169 extracted from filtered water and sediment samples, amplification of the 12S rRNA fragment  
170 was obtained using the MiFish-U primer set (Miya et al., 2015), and sequencing was  
171 conducted including two separate multiplexed libraries (Library 1/LIB1 – first sampling  
172 event; Library 2/LIB2 – second sampling event) in one Illumina MiSeq platform run. Detailed

173 procedures to control for contamination are also described in Supplemental information.

174

#### 175 ***2.4 Bioinformatic analyses and taxonomic assignment***

176 The metabarcoding bioinformatics pipeline used for data analysis was based on the  
177 OBITools software suite (Boyer et al., 2016), following the protocol described in Sales et al.  
178 (2019). Clustering was conducted using a step-by-step aggregation method (SWARM, Mahé  
179 et al., 2014) applying a clustering value of  $d=1$  (detailed information on evaluation of  
180 different clustering values can be found on Supplemental information). Molecular operational  
181 taxonomic units (MOTUs) and the inferred species (based on at least 97% of similarity  
182 with reference sequences; Sales et al., 2020) richness were compared among the three  
183 obtained datasets.

184 For the diversity analyses (species richness and  $\beta$ -diversity), we applied a conservative  
185 approach and treated our results as presence/absence-based as suggested by Li et al (2018).  
186 Often MOTUs are used as a proxy for species, however, the correlation between these two  
187 classifications of diversity are not straightforward. Richness in MOTUs is highly influenced  
188 by the occurrence of cryptic species and by the thresholds applied during the bioinformatic  
189 analyses (Pawlowski et al., 2018), which may cause an overestimation of true richness (e.g.  
190 inflation of different MOTUs belonging to the same species due to natural intraspecific  
191 variability, PCR amplification and/or sequencing errors). On the other hand, richness based on  
192 MOTUs being assigned to a species may be underestimated due to the lack of a complete  
193 reference database or due to a low taxonomic resolution of the target gene fragment analysed.

194 To verify whether the inferred community diversity patterns significantly varied  
195 because of the species assignment process, two datasets were used for estimating community  
196 metrics of  $\alpha$ - and  $\beta$ -diversities. Specifically, the filtered dataset included only MOTUs that

197 could be identified to the rank of species, whereas the non-filtered dataset included all  
198 MOTUs retrieved after quality filtering steps. The filtered dataset is a subset of the total  
199 MOTU diversity recovered, and thus it provides a more conservative overview for known fish  
200 diversity (Li et al., 2018).

201 A species name assigned to each MOTU might not correspond exactly to the species  
202 occurring in the JRB (based on the compiled species list; Table S1) because when the correct  
203 species is not present in the reference database, the taxonomic assignment is based on the  
204 closest congeneric species. In this case, species not previously reported for this basin are  
205 marked with an asterisk in order to highlight that the species herein included might be an  
206 indicative of occurrence of the genus and not the exact species present in this river basin.

207 Statistical analyses were performed in R v3.5.1 (R Core Team 2019). Replicates were  
208 pooled (water= 6 samples per site, sediment= 2 samples) before the following statistical  
209 analyses. Species richness ( $\alpha$ -diversity) was estimated as the total number of MOTUs  
210 (unfiltered dataset), or number of MOTUs assigned to species level (filtered dataset), at each  
211 sample site.  $\beta$ -diversity was obtained by generating a distance matrix based on the Jaccard  
212 coefficient, using the *vegdist* function implemented in *vegan* 2.5-2 (Oksanen et al. 2013). The  
213 Jaccard distance is based on presence or absence of species (value of 0 means both samples  
214 share the same species whereas 1 means samples have no species in common). Principal  
215 Coordinates Analysis (PCoA) was used to determine the relationship between distance and  
216 sites in the  $\beta$ -diversity matrix (*cmdscale* function) and the correlation between  $\beta$ -diversity and  
217 longitudinal distance and the  $\beta$ -diversity and presence of physical barriers (dams) was tested  
218 using a Mantel test (Li et al., 2018). The geographic distance matrix between sites was  
219 estimated using the road route because the road follows the river course and thus, this distance  
220 would provide a better estimate when compared to linear distance between two sample

221 locations. The matrix used for testing the influence of physical barriers was constructed by  
222 weighting distance values between sites according to the existence of barriers (e.g. 0 – no  
223 physical barrier between sites, 1- one barrier between sites and 2 – two barriers). To examine  
224 the potential effect of seasonality on community composition, a Permutational Multivariate  
225 Analysis of Variance (PERMANOVA) applying the Jaccard dissimilarity index was  
226 performed through the function ‘adonis’ (vegan 2.5-2 R package).

227         Even after our extensive effort to supplement the reference database for taxonomic  
228 assignment improvement, most of the MOTUs recovered were not identified to species level  
229 (see above) and, thus, a great portion of biodiversity information that could be used for  
230 diversity assessments is not included in the filtered dataset. To verify the total diversity  
231 recovered and to visualize the community data, we used a hierarchical structure of taxonomic  
232 classifications, in the R package Metacoder (Foster et al., 2017). This package, designed for  
233 metabarcoding data, provides “heat tree” plots using statistics associated with taxa (e.g. read  
234 abundances) and allows for a visual comparison between samples that takes into account their  
235 taxonomic/phylogenetic diversity. Venn diagrams were obtained by comparing the orders and  
236 families included in the compiled species list, and orders and families detected in each of the  
237 eDNA datasets (filtered and non-filtered) using BioVenn (Hulsen, Vlieg, & Alkema, 2008).

238

### 239 3 RESULTS

240 Our extensive review of both published and non-published literature sources resulted  
241 in 111 species records for the JRB (Table S1). A total of 55 additional Neotropical species  
242 were sequenced (Table S2) and included in the reference database alongside with all 12S  
243 rRNA mitochondrial gene fish sequences available on GenBank.

244 We obtained 16.1 million raw reads (LIB1 - 6,399,823; LIB2 - 9,704,699) in one  
245 Illumina MiSeq run (See Supplemental information for details). After quality control,  
246 clustering and all initial filtering steps, 2056 (LIB1) and 967 (LIB2) MOTUs were kept, with  
247 154 and 59 MOTUs being assigned to species with at least 97% similarity respectively. The  
248 number of retained MOTUs varied considerably between filtered and unfiltered datasets and  
249 for several species, more than one MOTU was also recovered (Figure 2, Table S4 and Table  
250 S5).

251

#### 252 ***3.1 Taxonomic assignment***

253 Based on the combined data (including all filtered datasets – species  $\geq 97\%$  similarity  
254 with reference sequence) detected fish diversity included six orders, 20 families, 28 genera  
255 and at least 34 fish species (Table S5). Characiformes (n=12) and Siluriformes (n=12) were  
256 the two orders represented by the largest number of species identified and all the remaining  
257 orders were comprised by less than five species.

258 A comparison between species identified by eDNA detection and closely related  
259 species reported for the JRB suggests that several congeneric species (e.g. *Leporinus*,  
260 *Prochilodus*, *Trichomycterus*) are not discernible using our generally applied bioinformatic  
261 threshold of  $\geq 97\%$  similarity due to a lack of taxonomically informative variation in the ~170  
262 bp fragment of the 12 rRNA gene, for these groups (Table S6).

263 Comparing the data obtained for both sampling times (Figure 3, Table S7), four  
264 species were detected only during the first sampling (*Australoheros facetus*, *Cyprinus*  
265 *carpio*\*, *Hypostomus* sp., *Trichomycterus* sp.), whilst *Coptodon zilli*\* and *Hoplias*  
266 *intermedius* were detected only in the second sampling.

267 Sediment samples failed to detect five species (*Australoheros facetus*, *Cyprinus*  
268 *carpio*\*, *Hypostomus gymnorhyncus*\*, *Poecilia reticulata*, *Trichomycterus* sp.), whilst water  
269 samples detected all species present in the sediments. Analyses of water and sediment samples  
270 demonstrated the occurrence of both widely distributed as well as less abundant species.  
271 Several taxa (e.g. *Leporinus* sp., *Prochilodus* sp., *Rhamdia quelen*) were detected in both  
272 water and sediment samples in most of sampling sites, in at least one sampling campaign, and  
273 therefore seem to have a broad geographic distribution in the JRB.

274 A remarkable result obtained by eDNA included the detection in all analysed sites of  
275 species rarely reported in traditional sampling studies (e.g. *Crenicichla* sp., Figure 3). Also we  
276 may highlight, the occurrence of putative new records for this basin including invasive  
277 species such as the dourado - *Salminus brasiliensis*\* and pacamã - *Lophiosilurus alexandri*\*.  
278 Furthermore, some species, including native and non-indigenous species, were restricted to a  
279 few locations (e.g. native: roncador *Wertheimeria maculata* (sample sites 1, 3, 8 and 10); non-  
280 indigenous: oscar *Astronotus ocellatus* (sample site 7); chameleon cichlid *Australoheros*  
281 *facetus* (sample site 11); tilapias *Coptodon* sp.\* (sample sites 1 and 2); or were detected in  
282 only one campaign (e.g. *Australoheros facetus*, *Coptodon* sp.\*, carp *Cyprinus carpio*\*, wolf  
283 fish *Hoplias intermedius*, pleco *Hypostomus gymnorhyncus*\*, pencil catfish *Trichomycterus*  
284 sp.).

285 The filtered dataset provides a potentially more conservative estimate of fish diversity  
286 at the rank of species because many MOTUs could not be assigned a name using the 97%

287 similarity threshold. Fish diversity depicted by the heat trees based on all detected MOTUs  
288 (i.e. the unfiltered dataset) shows that diversity remains especially high for the Order  
289 Characiformes, as many families appear to be comprised of several MOTUs (e.g.  
290 Anostomidae, Prochilodontidae; Figure 4). Comparisons between the filtered and unfiltered  
291 datasets demonstrated that a conservative approach (i.e. using filtered data) might lead to a  
292 biodiversity information loss since it greatly reduces the diversity in MOTUs recovered and  
293 fails in detecting orders and families known to occur in this catchment but that were not  
294 identified up to the species level (Figure 5).

295

### 296 ***3.2 Species richness and $\beta$ -diversity***

297 During the first campaign, highest MOTU richness was found in water samples from  
298 the most upstream (site 1) and downstream (site 11) sampling sites, followed by sampling  
299 sites 4 and 8 (Figure 6A). The lowest number of MOTUs was recovered for sample site 7.  $\beta$ -  
300 diversity patterns showed similarities between sample sites 4 and 11, and sample sites 1 and 8,  
301 whereas sample site 7 showed the most distinct fish assemblage when compared to all  
302 locations. Environmental DNA recovered from water samples collected three weeks later,  
303 demonstrated that species richness among sites fluctuate in time in this catchment (Figure  
304 6B), with generally greater homogeneity in the species richness amongst all sample sites in  
305 the late sampling event. Still, the most upstream and downstream locations (1, 2, 10, 11),  
306 alongside sample site 8, still harboured the highest number of species.

307 Data recovered from sediment samples provided a different overview of species  
308 richness and  $\beta$ -diversity. Overall, the number of species recorded for sediment samples was  
309 lower compared to water samples in the first campaign (Figure 6C). Sample site 1 had a much  
310 lower species richness compared to water samples along with sampling sites 2, 4, 8, 9, 10. An

311 increase in the species richness was detected for sampling sites 3, 5 and 7, while sample sites  
312 11 and 8 were confirmed as highly species-rich locations. In the second campaign (Figure  
313 6D), when compared to data recovered from water samples, six sample sites (1, 2, 6, 8, 9, 10)  
314 had a lower species richness, while higher values were obtained for sample sites 3, 4, 7.

315 Over time, the pattern of harbouring the highest species richness appeared relatively  
316 constant in sites 1 and 11 for both sampling media, except in the first campaign where fewer  
317 species were detected in location 1 for sediment. Yet, the most downstream location kept an  
318 almost stable species richness in both sampling media for both sampling campaigns.

319 Longitudinal distance had a negligible effect on  $\beta$ -diversity amongst sample sites ( $p$ -  
320 value  $> 0.05$ , Table 1) and the presence of physical barriers (e.g. dams) also did not show a  
321 significant influence on  $\beta$ -diversity of different sample types (water and sediment, Table 1). A  
322 positive significant correlation was found between filtered and unfiltered datasets, for both  
323 water and sediment (Table 1). The community structure varied significantly between the two  
324 sampling campaigns (before vs after the intense rain event) as indicated by results of  
325 PERMANOVA for both sampling media (water:  $R^2 = 0.64$ ,  $p=0.004$  and sediment:  $R^2$   
326  $= 0.25$ ,  $p=0.0009$ ).

327 For both sampling media, despite the variation in taxa richness showed by both  
328 datasets, the pattern of  $\alpha$ -diversity variation among sample sites obtained for filtered (species)  
329 and unfiltered (MOTUs) datasets were still quite congruent (Figure 7). However, for sediment  
330 samples collected in the first campaign, sites 3 and 11 had a greater MOTU diversity when  
331 compared to all nine remaining locations (Figure 7C). Despite also being the most species  
332 rich sites, the great amount of MOTUs obtained and not assigned indicates that a great  
333 diversity remains hidden in this sampling medium. Also, as demonstrated by the PCoA  
334 (Figure 7C), in the first campaign these sites had a more distinct fish assemblage when



335 compared to the others. Furthermore, a higher resolution was obtained for the unfiltered  
336 dataset as a more segregated sample clustering is evident in the PCoA ordination. Sediment  
337 samples from the first campaign exhibited a peculiar clustering, with highly diverse samples  
338 in 3 and 11 strongly separated from all other sites.  
339

340

341 **4 DISCUSSION**

342 The understanding of species distribution and the processes shaping spatial variation  
343 and community composition are crucial for applying sustainable management schemes and  
344 ensure timely conservation of biodiversity, especially for endemic and threatened species.  
345 Such actions also require methods that allow for rapid and robust detection of biodiversity at  
346 different spatial scales (Kelly et al., 2014). Here, we used eDNA metabarcoding of water and  
347 sediment samples to investigate fish community variation over time along the course of a  
348 Neotropical river.

349 We found that eDNA metabarcoding applied to understanding fish distributions in a  
350 neotropical setting greatly enhanced our ability to not only measure richness along the course  
351 of a large river, but also to reveal hidden diversity and putative unrecorded species invasions.  
352 The compiled list of species (N=111) reported for the JRB herein was higher than previously  
353 recorded (N=63) in 2010 (Andrade-Neto, 2010), and our thorough evaluation of all possible  
354 taxonomic information available at the time of our study estimates the occurrence of more  
355 than 80 species in this catchment (Andrade-Neto, 2010; Godinho et al., 1999).

356 Previous studies have demonstrated the importance of expanding reference databases,  
357 specially for understudied taxonomic groups and areas (Schenekar et al., 2020; Weigand et al.,  
358 2019). In comparison with the previous eDNA study conducted in the JRB, the extension of  
359 our reference database through the inclusion of sequences from 55 additional fish species led  
360 to a much improved taxonomic assignment. The extended database allowed the detection of  
361 several species previously missing from the available genetic reference databases (e.g.  
362 endemic species, *Wertheimeria maculata*), similar to results found by Schenekar et al. (2020)  
363 in a re-evaluation of a eDNA metabarcoding study in Volga headwaters. Still, our molecular

364 assessment based on eDNA metabarcoding demonstrates that, as of yet, there may be even  
365 more species yet to be recorded and putting the richness of this basin on par with other closely  
366 adjacent basins thought to harbour higher diversity. These results demonstrate our current  
367 lack of understanding of tropical diversity in many systems and corroborates that new DNA  
368 based methods are ideal in generating new baselines for biodiversity monitoring.

369

#### 370 **4.1 Introduced and native species**

371 Environmental DNA metabarcoding allows the detection of multiple species  
372 simultaneously, including species not expected to occur in an area (Deiner et al., 2017),  
373 helping to track biological invasions and providing an early warning of species introduction.  
374 Here, almost 30% of the taxa detected by eDNA were non-indigenous species, including  
375 species not reported yet for this catchment. To our knowledge, previous records of *Salminus*  
376 *brasiliensis* and *Lophiosilurus alexandri* occurrence in the JRB are absent from the literature.  
377 These are commercially important species, already introduced for fishery purposes in several  
378 Brazilian basins (Vitule et al., 2014). Hence, their occurrence in the JRB is not necessarily a  
379 surprise. However, it raises concerns about the ecological consequences of such unmanaged  
380 introductions. Biodiversity loss is not only restricted by species disappearance, but also by a  
381 reduction in ecosystem services due to an increase of biological similarity between areas (i.e.  
382 species loss or increase through biological introductions leading to biotic homogenization;  
383 Rahel, 2000).

384 It has been widely documented that analysis of eDNA surpasses traditional methods  
385 for assessment of biodiversity and detection of invasive species (Schmelzle & Kinziger, 2016;  
386 McDevitt et al., 2019). The only cyprinid previously documented in this basin was  
387 *Hypophthalmichthys molitrix*. Herein, we registered the presence of *Cyprinus carpio*, another

388 species that has been widely introduced to Brazilian waters (Alves et al., 2007).  
389 Environmental DNA metabarcoding also detected various species of tilapia (*Oreochromis* sp.  
390 and *Coptodon zilli*). The impacts of tilapia invasion are well known worldwide, and all  
391 species show high invasive potential, including in Neotropical countries (Cassemiro et al.,  
392 2017).

393 Our study also detected remarkable cases, such as the native species *Crenicichla* sp.  
394 The genus *Crenicichla* is one of the most species rich among the South American Cichlids,  
395 where it is known to widely occur. However, the genus is still lacking an improved  
396 taxonomic resolution and conservation status evaluation (Kullander & de Lucena, 2006). In  
397 2006, an expedition applied extensive sampling efforts to collect *Crenicichla* sp. in the  
398 Jequitinhonha, without any success, and this species was only documented in 2009 by an  
399 environmental report based on traditional sampling and morphological identification  
400 (Kullander & Lucena, 2006; Intertechne, 2009). An issue reported worldwide, is that even  
401 when monitoring programmes are conducted, most of the data obtained are often not  
402 published or made available and thus remain inaccessible to further scientific studies  
403 (Lindenmayer & Likens, 2009; Revenga et al., 2005). Here, eDNA metabarcoding data  
404 revealed that this species might be present at several locations in the JRB, indicating a  
405 possible large geographical distribution.

406 Taxonomic issues are often present in monitoring programs and the risk of  
407 misidentification exists, regardless of the method applied (i.e. traditional sampling,  
408 morphological identification, eDNA; Radinger et al., 2019; Jerde, 2019). Erroneous  
409 identifications might also be present in the reference databases, especially in highly biodiverse  
410 regions such as the Neotropics, where the amount of unknown and undescribed taxa and the  
411 occurrence of cryptic species represent substantial issues. As demonstrated in previous

412 studies, identification of some species might be problematic when using eDNA  
413 metabarcoding based on the 12S fragment employed here, due to its lack of taxonomic  
414 resolution and the incompleteness of the reference databases (Yu et al., 2012; Eiler et al.,  
415 2013). Because a gene tree is not necessarily related to a species tree, the phylogenetic  
416 resolution it provides can be obscured for groups of taxa. The imperfect taxonomic resolution  
417 might allow the multiple assignment of congeneric species (i.e. one species being  
418 concomitantly assigned to its multiple congeners) when several reference sequences are  
419 available (please see example of *Prochilodus* sp. below). In contrast, when the reference  
420 database is not complete for all species occurring in the area, several MOTUs belonging to  
421 distinct species might be assigned to and erroneously identified as the single closely related  
422 species available in the database (Sales et al., 2020). For instance, most MOTUs belonging to  
423 *Prochilodus* sp. could not be assigned to species level due to a high similarity among  
424 orthologous sequences from congeneric species. This poses a conservation issue, since  
425 *Prochilodus argenteus* is an invasive species in the JRB, and is believed to have recently  
426 diverged from the endemic species *P. hartii* (Melo et al., 2018). Henceforth, due to the  
427 conservative criteria applied to analyse the data, the number of species detected is surely  
428 underestimated.

429         Six anostomids are described for the JRB, and here we identified one of these species  
430 (*Megaleporinus garmanii*), but also identified two species not previously reported (*Leporinus*  
431 *copelandii* and *Hypomasticus mormyrops*). The only previous record of *Leporinus copelandii*  
432 was deemed as an historical error (Andrade-Neto, 2010). Cilleros et al. (2019), despite using a  
433 different 12S fragment, also reported the limitations in the taxonomic assignment of species  
434 belonging to the genus *Leporinus*, therefore our data set is unable to clarify the nuances  
435 within this group.

436

437 **4.2 Anthropogenic impacts and species richness**

438 Ecological communities vary in time and space, and the monitoring of these dynamics  
439 is essential for conservation purposes (Bálint et al., 2018). In the JRB, spatial and temporal  
440 fluctuations in fish assemblages inferred from eDNA were detected.

441 The sites comprising the highest fish diversity in this basin were represented by  
442 locations characterized by different anthropogenic influences. The most upstream site  
443 (Mendanha) is located in a less populated and more pristine region (Table S8, Supplementary  
444 Material), near two areas of natural preservation (State Parks Biribiri and Rio Preto). The  
445 other two sampling sites (Almenara, 8, and Belmonte, 11) are located near more densely  
446 populated cities and impacted areas (i.e. due to the deforestation and mining activities,  
447 siltation increases towards the river mouth and represents one of the greatest impacts in the  
448 Jequitinhonha river - IBGE, 1997). Almenara, is a particularly impacted area, and during the  
449 sampling had a low water level and accumulation of sediments, which might have contributed  
450 to increasing the eDNA concentration and accumulation, and therefore increasing the species  
451 diversity recovery, despite the low environmental quality.

452 Among the sites showing the lowest species richness included the reservoirs (3 – José  
453 Gonçalves, 9 – Salto da Divisa) and the first sites located downstream of the dams (5 –  
454 Coronel Murta and 10 – Itapebi; Figs. 6 and 7). The longitudinal distance and presence of  
455 barriers did not explain community variation ( $p>0.05$ ); however, the presence of dams is a  
456 well known fish diversity reduction factor since these barriers greatly impact the environment  
457 (i.e. modification of physical and ecological characteristics of the habitats, such as  
458 modifications in water flow, nutrient dynamics, water quality and temperature; Pelicice &  
459 Agostinho, 2007; Pompeu et al., 2012). Still, changes in fish distribution and communities

460 composition may also arise from plenty of distinct alterations and complex interactions in the  
461 impounded environment (Agostinho, Pelicice & Gomes, 2008).

462 Environmental DNA metabarcoding offers a promising tool for evaluating the  
463 impoudment's impact on fish distribution and thus, in this context, futher investigation  
464 (including increasing spatial and temporal replicates) are recommended since anthropogenic  
465 impacts might still have an influence on fish diversity distribution in this river basin.

466

### 467 **3.3 Seasonal changes in fish assemblages**

468 Seasonal changes driven by natural factors (e.g. water flow, rainfall) could also  
469 contribute to explain assemblage variation even over a short time frame (i.e. weeks) as mobile  
470 species, such as fish, can rapidly disperse and vary their distribution in response to changing  
471 abiotic conditions (Arrington & Winemiller, 2006; Fitzgerald et al., 2017). Furthermore, fish  
472 ecology and behaviour may also influence the variation in eDNA recovery, as seasonal  
473 changes can lead to increased DNA shedding rates due to factors such as spawning events,  
474 growth of juveniles or even temporal changes in fish metabolism (Maruyama et al., 2014;  
475 Buxton et al., 2017).

476 Water availability shows a great temporal variability in semi-arid and arid regions,  
477 with short, but intense, rainfall episodes followed by long dry periods (Leite et al., 2010). The  
478 JRB is inserted in a semi-arid region and in the first sampling campaign it was facing a severe  
479 drought. Before the second sampling campaign, an increase in the average accumulated  
480 rainfall (from 2.1-50mm in the first sampling event to 100-250 mm in the second sampling  
481 event; CPTEC/INPE, 2018) might have contributed to a higher evenness in MOTU  
482 richness/fish diversity amongst sample sites (regarding the contemporary species richness  
483 inferred through water samples; Figs. 6 and 7). The climatic and hydrological changes

484 followed by the onset of the rainy season usually triggers the start of fish migration in the  
485 semi-arid regions (Chellappa et al., 2003; Chellappa et al., 2009). An increased water volume  
486 and subsequently higher connectivity of aquatic habitats might stimulate the dispersal and  
487 result in reduced densities of organisms (Fitzgerald et al., 2017). Previous studies have  
488 demonstrated that compositional changes in accordance with seasonal variations can be  
489 inferred through eDNA for fish communities (Sigsgaard et al., 2017, Hayami et al., 2020).  
490 Here, the comparison between the two sampling campaigns showed a significant influence of  
491 seasonality on community composition for both water ( $p=0.004$ ) and sediment ( $p=0.0009$ )  
492 datasets. These results might suggest that freshwater fish assemblages in tropical habitats may  
493 vary significantly between dry and wet seasons, corroborating with previous published eDNA  
494 studies. Besides the apparent homogenization found after the rainfall event, an important  
495 factor to take into consideration is the reduction of diversity recovered in the second  
496 campaign when compared to the first. The ecology of DNA might play an important role  
497 regarding this matter, as eDNA molecules could be more diluted in the water column  
498 decreasing the detectability of some species (e.g. rare or less abundant species).

499 Higher inhibition levels due to seasonality are also considered as important factors  
500 when investigating eDNA recovery. Plant-derived substances, often present in water and  
501 sediment samples, are recognised as natural PCR inhibitors. After heavy raining events, an  
502 increased accumulation and degradation of leaf litter might have increased the availability of  
503 these substances through the river, and thus contaminating environmental samples and  
504 decreasing eDNA detection rates. However, as in this study we strived to minimise PCR  
505 inhibition in eDNA samples, it is reasonable to expect that this process would only have a  
506 minor impact on the seasonal pattern observed.

507



#### 508 4.4 eDNA transport and species richness

509 Another factor we need to take into account is eDNA transport from locations  
510 upstream from our sample sites. This transport could lead to an overestimation of species  
511 richness recovered for each sample site, and, the species identification per site therefore does  
512 not mean that the species themselves are present there at the time of collection (Barnes &  
513 Turner, 2016; Deiner et al., 2014). Still, eDNA transport distances may vary between river  
514 systems due to abiotic and biotic factors (e.g. temperature, pH, bacterial load, or seasonal  
515 changes such as drought or intense rainfall periods; Deiner et al., 2016). Most of the studies  
516 evaluating the effect of eDNA downstream transportation reported travel distances of few  
517 kilometers, whereas, a travel distance higher than 100km was demonstrated by Pont et al.  
518 (2018) for a high discharge ( $\text{m}^3/\text{s}$ ) river system. Still, despite the eDNA downstream  
519 transportation, the latter study demonstrated the capability of eDNA in providing an accurate  
520 snapshot of fish assemblage composition in a large river and finally, suggested that a distance  
521 of around 70 km would be enough to limit the potential noise of eDNA transport. Therefore,  
522 despite having a high discharge rate (average of  $409 \text{ m}^3/\text{s}$ ), the approximate distance between  
523 sites was 100 km and thus, the influence of eDNA transport on species detected at each site  
524 might not be considered as a great concern here. However, as no study has been conducted in  
525 Brazilian lotic environments focusing on understanding eDNA transport and diffusion, it is  
526 difficult to draw sound conclusions regarding this matter and so, additional studies focusing  
527 on the information recovered from eDNA in large neotropical rivers might contribute to  
528 expand the knowledge of its complex spatiotemporal dynamics.

529 The high  $\alpha$ -diversity values found for the site located at the river mouth (site 11,  
530 Belmonte) deserves some consideration since this region has marine influence (including the  
531 detection of one marine family, Engraulidae, by sediment samples in this sample site, Figure

532 4) and its abiotic characteristics (e.g. increased salinity) would be expected to restrict the  
533 occurrence of some freshwater species. A hypothesis that could explain the detection of  
534 species not expected to occur in this area includes eDNA transport and accumulation. Species  
535 shed DNA constantly, which can be available in the water column or bound to superficial  
536 sediment. A higher concentration and longer persistence of fish eDNA in the sediments might  
537 contribute to eDNA molecule resuspension which might affect inferences from aqueous DNA  
538 in both spatial and temporal scales (Turner et al., 2015; Graf & Rosenberg, 1997; Bloesch,  
539 1995;).

540 Due to the fragmentation of the Jequitinhonha River, this site (site 11, Belmonte) is  
541 located in a region characterized by a high level of sediment trapping  
542 ([freeflowingriver.org/maptool/](http://freeflowingriver.org/maptool/)) and possibly, this segment can act as an “eDNA reservoir”  
543 due to the accumulation of molecules transported throughout the river. In addition to that, an  
544 increase in water flow and tidal movements can also cause eDNA particle resuspension  
545 (increasing the probability of retrieving old eDNA from the sediment beds – Jamieson et al.,  
546 2005), which, associated with the resistance applied by the incursion of the marine waters into  
547 the river, can contribute to retain and resuspend the eDNA accumulated in this area, making it  
548 available in the water column. Considering this, river mouths should then be further  
549 investigated as putative eDNA reservoirs since it could contribute in future sampling  
550 strategies focusing on obtaining a snapshot of the entire fish community at a large scale.

551 Bioinformatics and technical aspects also play an important role in diversity recovery  
552 from eDNA samples, and the existing trade-off between uncertainty and stringency may be  
553 carefully considered when interpreting eDNA results as it might lead to false negative or false  
554 positive detections (Evans et al., 2017; Grey et al., 2018). Regarding the analysed datasets, the  
555 filtered data is considered as a subset of the total diversity recovered and showed a lower

556 diversity at the order and family levels. However, the significant positive correlation between  
557 datasets demonstrated that  $\beta$ -diversity is not influenced by the filtering criteria applied as  
558 much as the effect of sampling medium or sampling time. As suggested by Li et al. (2018),  
559 the filtered dataset provided a more conservative overview of fish diversity, compared to the  
560 unfiltered dataset and thus did not detect several families and orders known to be present in  
561 this catchment.

562 Fish diversity depicted by the heat trees based on the unfiltered data shows that a  
563 hidden diversity might be present, especially for the Order Characiformes, as many families  
564 appear to comprise several MOTUs (e.g. Anostomidae, Prochilodontidae). This likely reflects  
565 the presence of multiple genera/species such as in the Anostomidae, known to harbour at least  
566 seven species in this basin, which are absent from the reference sequence databases.  
567 Therefore, to avoid underestimating the biodiversity, and reduce ambiguity in eDNA-based  
568 species detection, we stress the importance of coordinating morphological surveys alongside  
569 DNA assessments. Most importantly, there is also a need of increasing efforts towards  
570 building more complete genetic reference databases, ideally composed of whole  
571 mitochondrial genomes, as the lack of reference sequences has been considered as a great  
572 hindrance to fullfill the potential of eDNA metabarcoding in assessing biodiversity rich  
573 ecosystems (Cilleros et al., 2019; Sales et al., 2020).

574

575

**576 5 CONCLUSIONS**

577           Given the unprecedented rates of population and species decline and the increasing  
578 anthropogenic impacts on freshwater communities, the importance of a rapid, robust and  
579 efficient monitoring program has never been more in need for this ecosystem. Here we  
580 illustrated eDNA ecology when analysing an entire river basin from the headwater to the river  
581 mouth, and highlighted some of the challenges of applying eDNA metabarcoding in spatio-  
582 temporal ecological studies, including recommendations for future work. Understanding  
583 eDNA metabarcoding dynamics is an important step to make it a complementary monitoring  
584 tool to traditional methods. This enhancement can improve the applicability of eDNA  
585 metabarcoding for biomonitoring purposes in Brazilian freshwaters and therefore, allow the  
586 detection of elusive, rare or patchily distributed species and provide data for neglected and  
587 difficult to access localities.

588

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598

599

**600 DECLARATION OF COMPETING INTEREST**

601 The authors declare that they have no known personal relationships or competing financial  
602 interests that could have influenced the work conducted in this study.

603

**604 AUTHOR CONTRIBUTIONS**

605 NGS, OSW and SM designed the study. NGS carried out the fieldwork. NGS and OSW  
606 performed the laboratory work and the bioinformatics. NGS analysed the data primarily, with  
607 contributions from ADM, IC, KD and KP. All authors discussed the results and implications.  
608 NGS drafted the manuscript, all authors provided manuscript input and contributed in  
609 discussion that developed the study.

610

**611 DATA ACCESSIBILITY**

612 Raw data are available in the Dryad Digital Repository  
613 (<https://doi.org/10.5061/dryad.4mw6m9073>). The reference sequences are available on NCBI  
614 under the following accession numbers MT901385 - MT901477.

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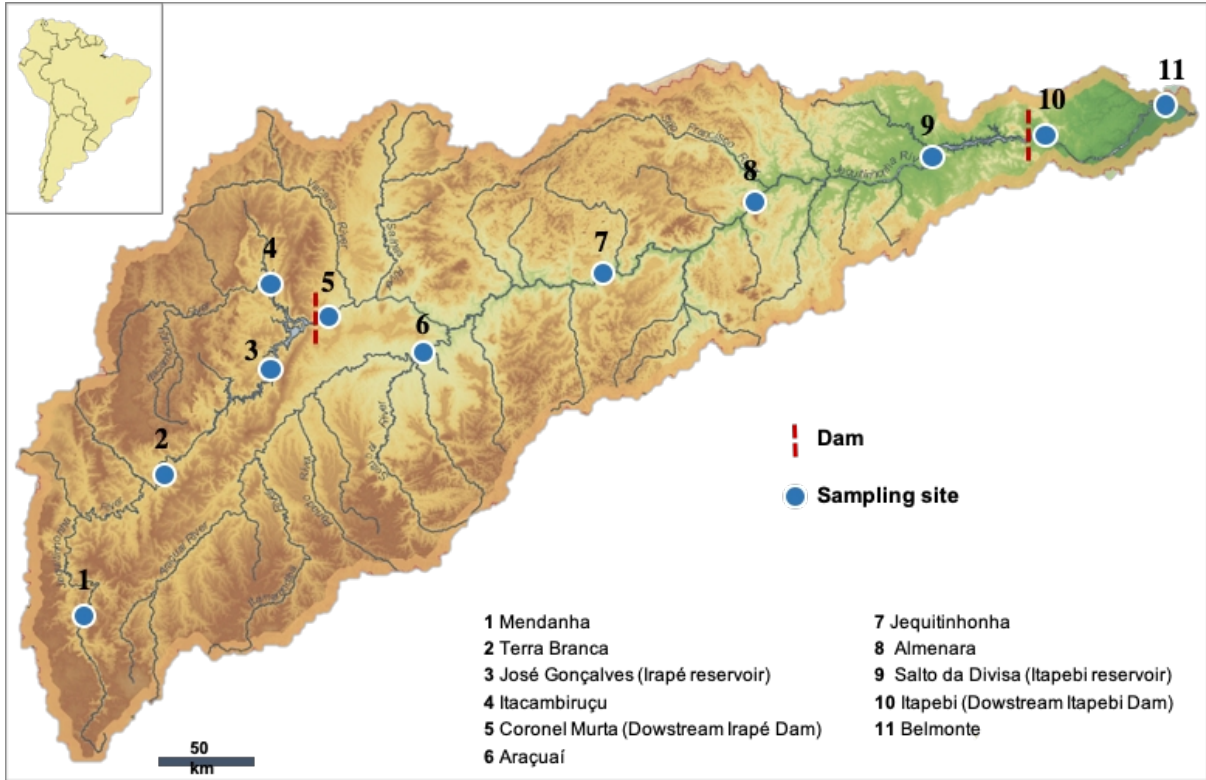


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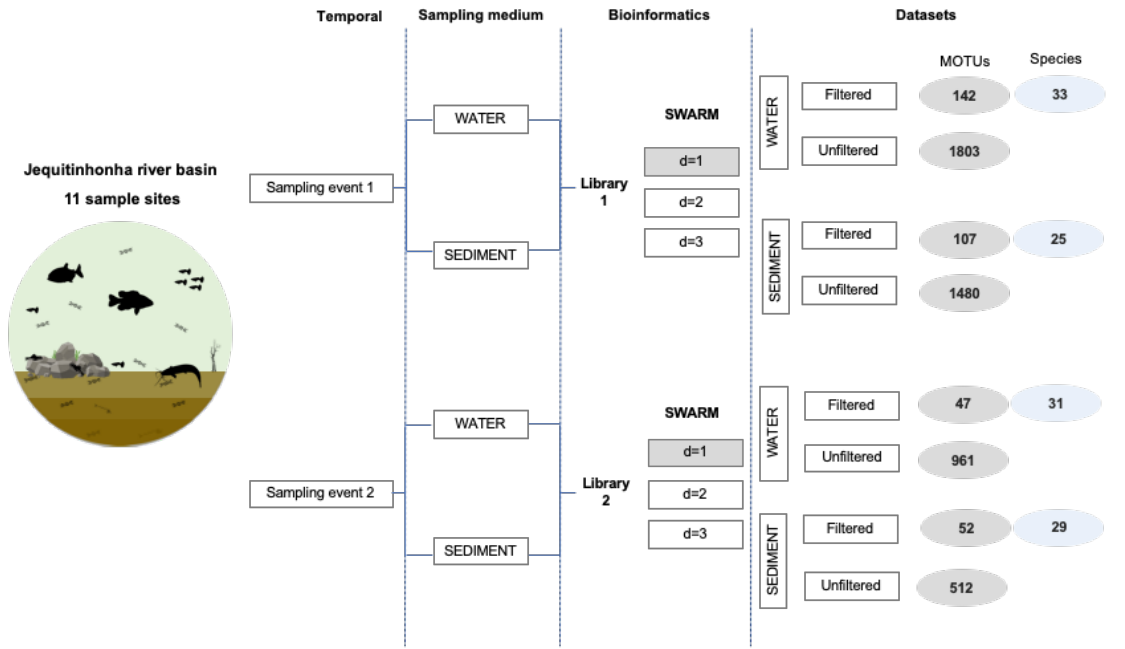
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889 FIGURE 1 | The Jequitinhonha river basin, including sampling sites used in the study, dams  
 890 and respective hydrological regions.

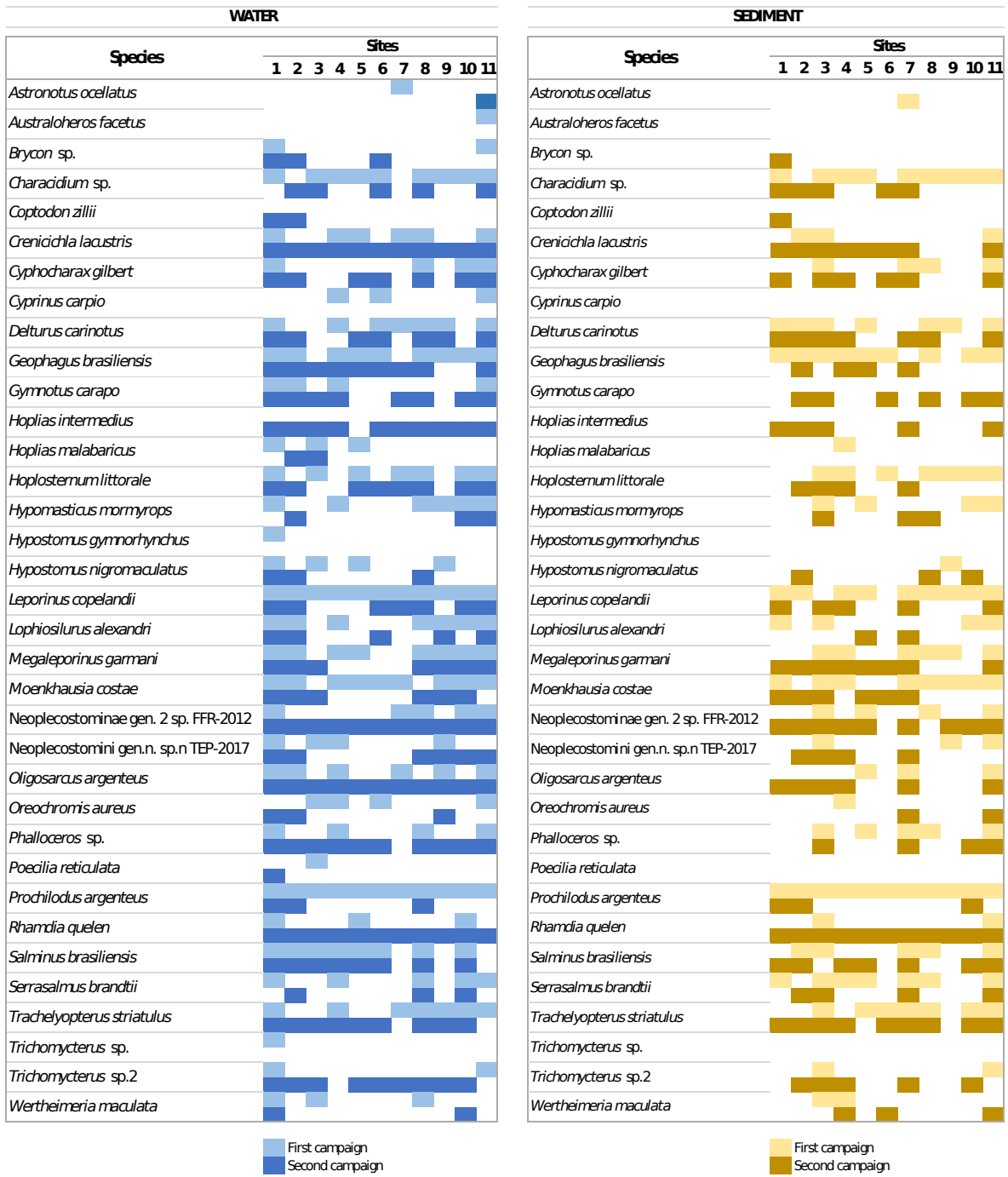
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894 FIGURE 2 | Workflow illustrating the methods used in this paper and respective number of  
 895 MOTUs retrieved in each dataset analysed, and the final number of species assigned with  
 896 >0.97 identity



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899 FIGURE 3 | Species distribuon in the Jequitinhonha River Basin, according to sampling  
900 media and campaign.

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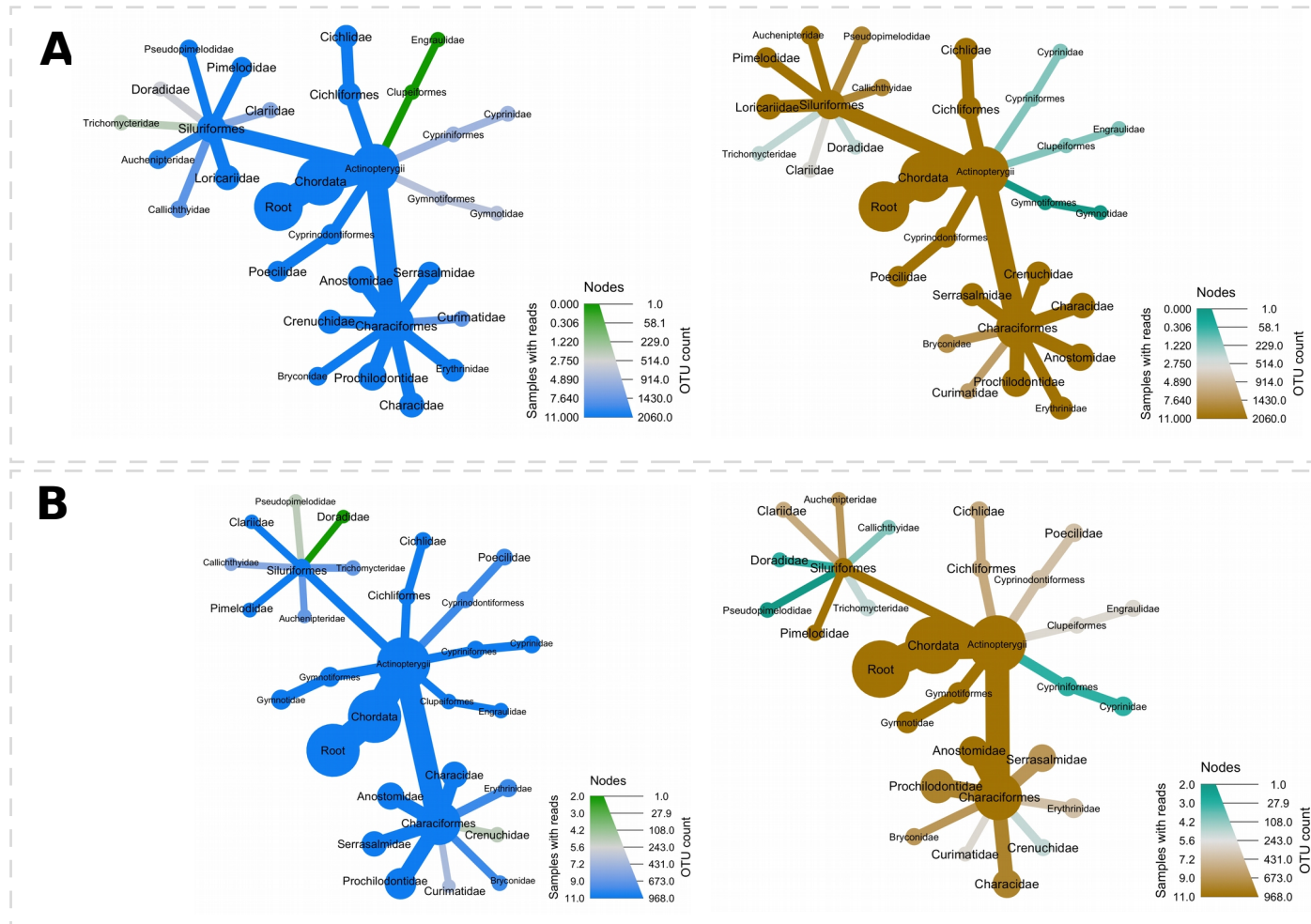
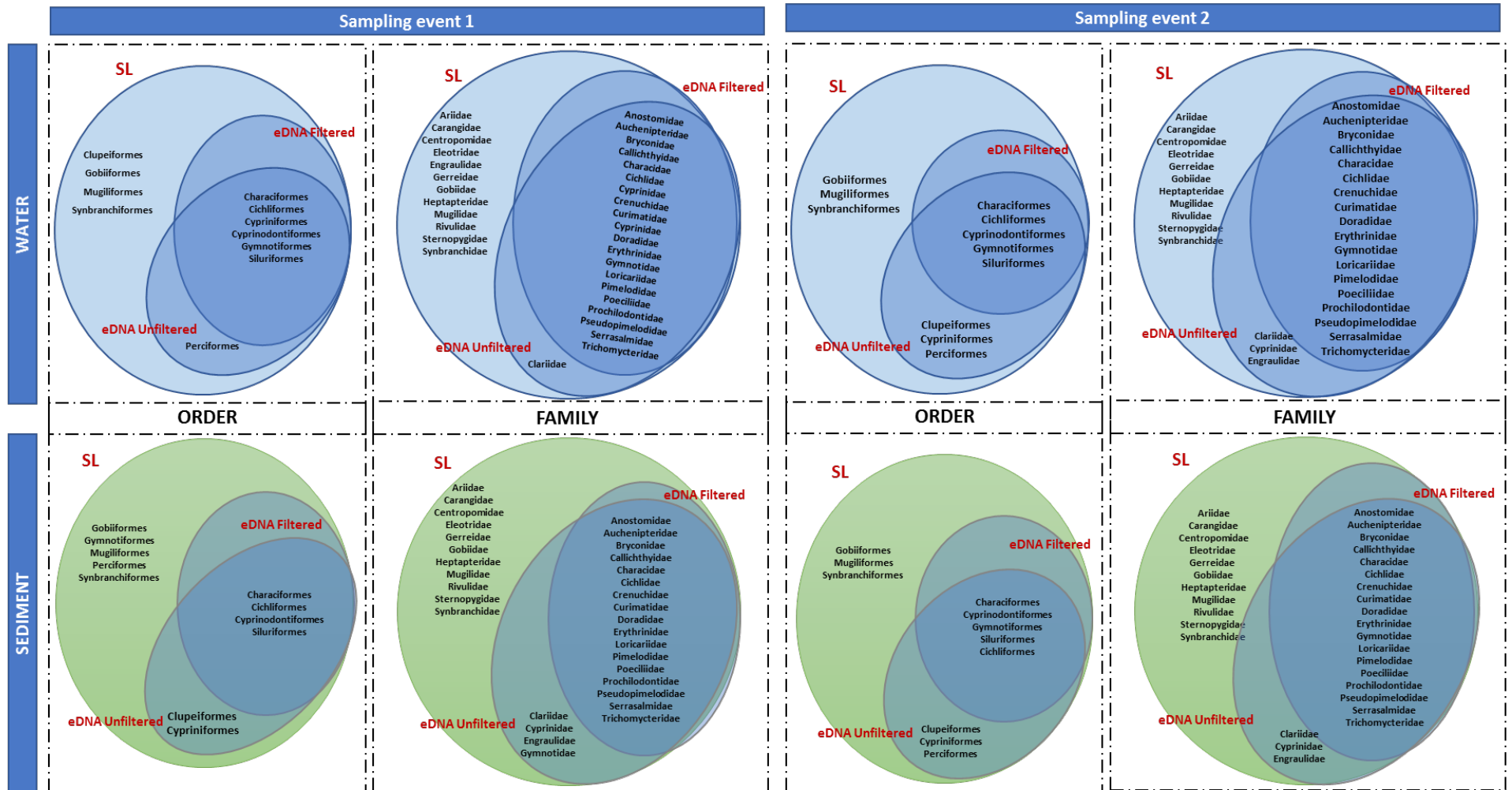


FIGURE 4 | Heat trees displaying the fish diversity recovered for Jequitinhonha River Basin using eDNA metabarcoding unfiltered datasets, during the first (A) and second (B) campaigns. Blue = Water samples; Brown = Sediment samples. Size and colour of nodes and edges are correlated with the read abundance of taxa in each analysed dataset, and edges include the number of sites the taxon was detected ('sample with reads') and respective number of OTUs recovered.





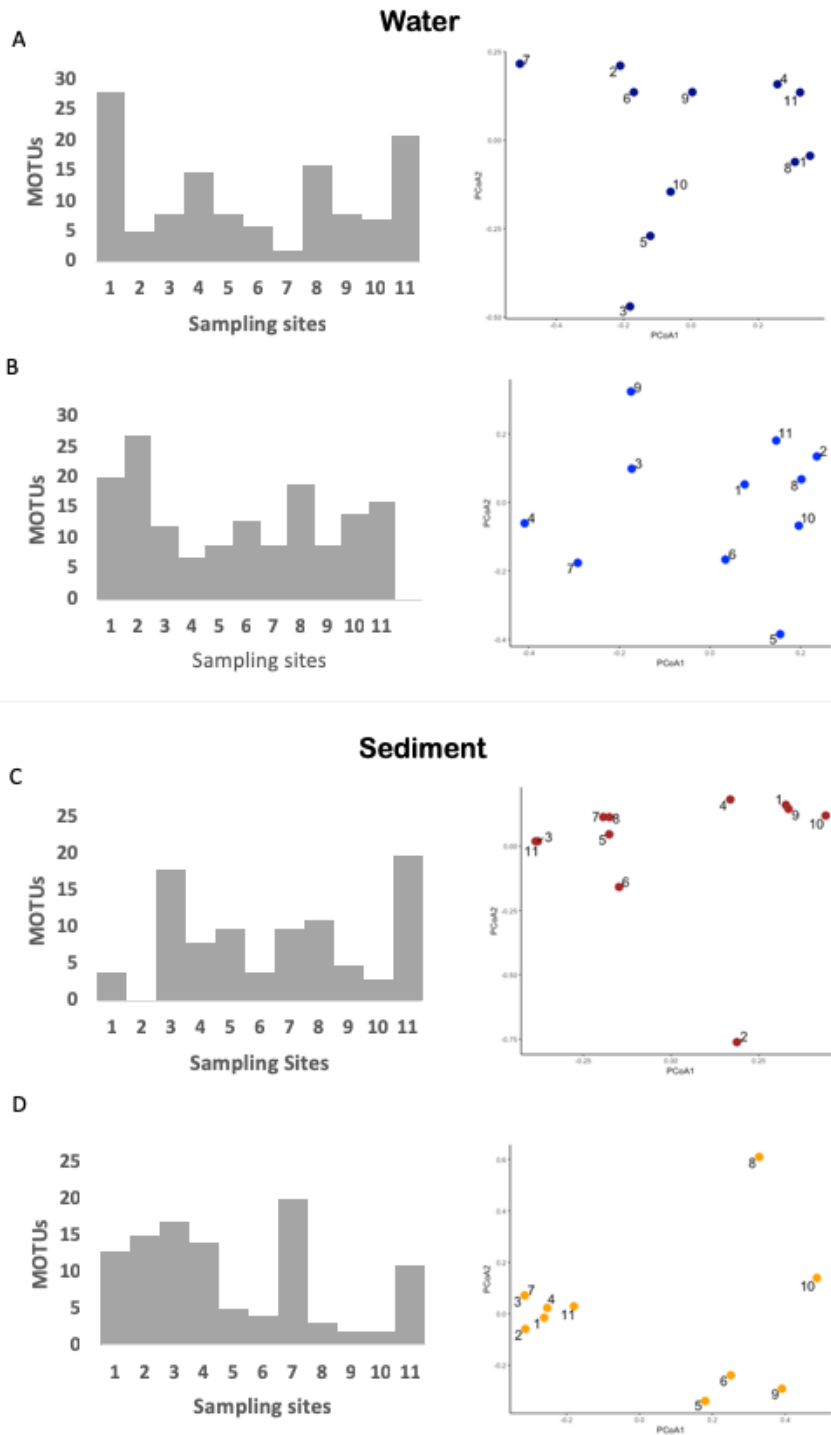
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FIGURE 5 | Venn diagram of fish orders and families comparing the data included in the species list based on traditional sampling (SL) to eDNA detected in distinct sampling media (water vs sediment); sampling campaign; and datasets analysed (unfiltered vs filtered).

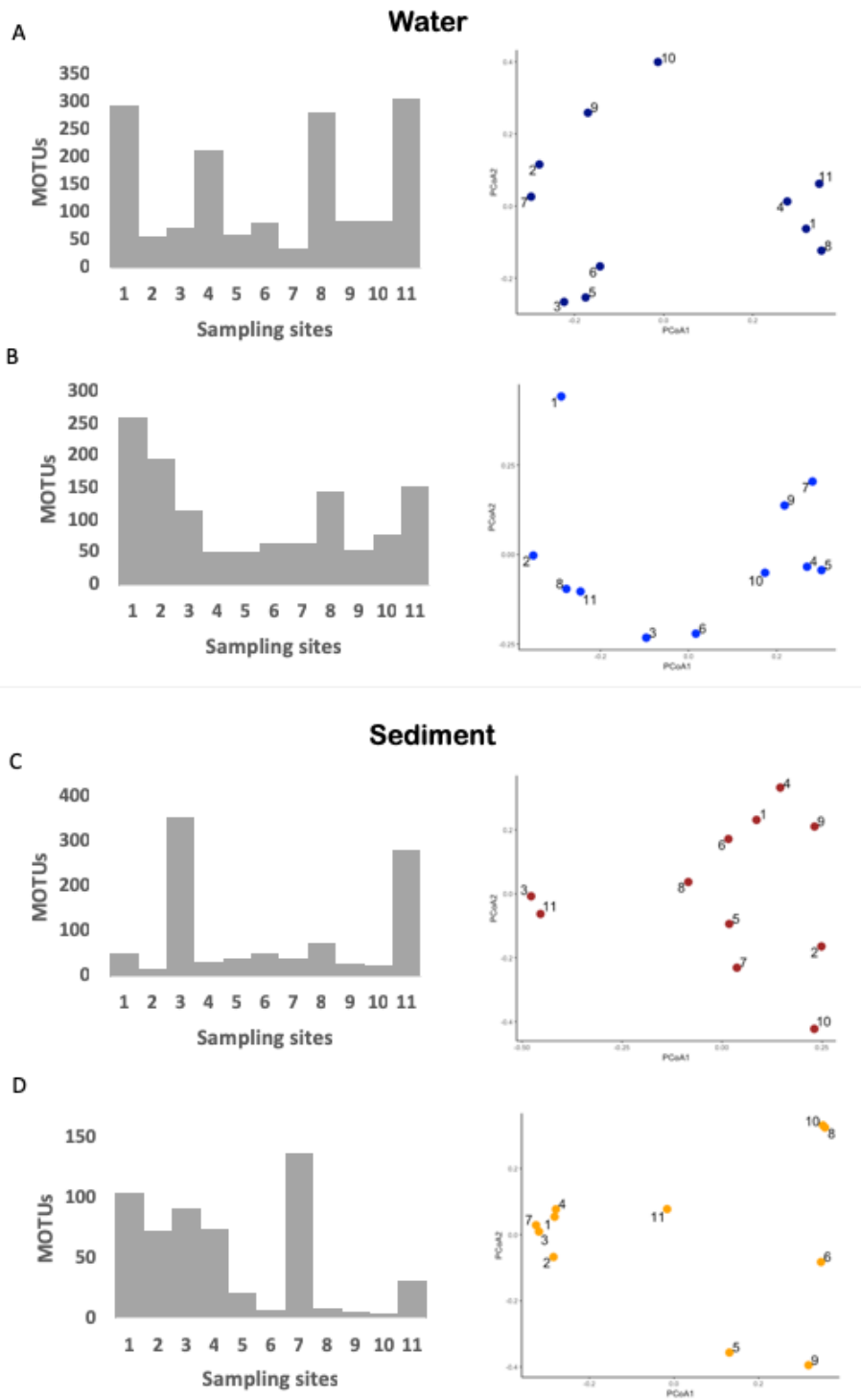


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930 FIGURE 6 | Filtered dataset, showing the species richness distribution along the  
 931 Jequitinhonha River Basin and Principal Coordinates Analysis (PCoA) of  $\beta$ -diversity of  
 932 sampling locations (Jaccard distance). A) Water samples obtained in the first campaign; B)  
 933 Water samples obtained in the second campaign; C) Sediment samples obtained in the first  
 934 campaign; D) Sediment samples obtained in the second campaign.

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FIGURE 7 | Unfiltered dataset, showing the species richness distribution along the Jequitinhonha River Basin and Principal Coordinates Analysis (PCoA) of  $\beta$ -diversity of sampling locations (Jaccard distance). A) Water samples obtained in the first campaign; B) Water samples obtained in the second campaign; C) Sediment samples obtained in the first campaign; D) Sediment samples obtained in the second campaign.

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**TABLE 1** | Mantel *r* and *p*-values (in parentheses) for all the pairwise comparisons between datasets, sampling media, geographic distance and presence of barriers (dams).

		First campaign				Second campaign				
		Water		Sediment		Water		Sediment		
		Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered	
1	W	Unfiltered	1							
		Filtered	0.689 (p=0.001)	1						
	S	Unfiltered	0.050 (p=0.359)	-0.268 (p=0.939)	1					
		Filtered	0.219 (p=0.162)	0.134 (p=0.250)	0.534 (p=0.005)	1				
2	W	Unfiltered	0.193 (p=0.445)	-0.142 (p=0.815)	0.110(p=0.221)	0.029 (p=0.386)	1			
		Filtered	0.011 (p=0.444)	-0.017 (p=0.491)	0.055(p=0.309)	-0.034 (p=0.555)	0.572 (p=0.001)	1		
	S	Unfiltered	-0.100 (p=0.656)	-0.235 (p=0.914)	0.017(p=0.389)	-0.047 (p=0.548)	-0.025 (p=0.544)	-0.174 (p=0.870)	1	
		Filtered	-0.121 (p=0.691)	-0.278 (p=0.929)	0.109(p=0.269)	-0.104 (p=0.645)	0.075 (p=0.309)	-0.040 (p=0.528)	0.822 (p=0.001)	1
		Longitudinal distance	-0.213 (p=0.897)	-0.258 (p=0.947)	-0.041(p=0.99)	-0.028 (p=0.561)	0.137 (p=0.154)	-0.043 (p=0.597)	0.189 (p=0.114)	0.290 (p=0.052)
		Presence of dam	-0.102 (p=0.690)	-0.172 (p=0.859)	0.028 (p=0.416)	-0.004 (p=0.514)	-0.018 (p=0.488)	-0.181 (p=0.876)	0.178 (p=0.161)	0.108 (p=0.26)

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