



**HAL**  
open science

## From environmental DNA sequences to ecological conclusions: How strong is the influence of methodological choices?

Irene Calderón-sanou, Tamara Münkemüller, Frédéric Boyer, Lucie Zinger, Wilfried Thuiller

### ► To cite this version:

Irene Calderón-sanou, Tamara Münkemüller, Frédéric Boyer, Lucie Zinger, Wilfried Thuiller. From environmental DNA sequences to ecological conclusions: How strong is the influence of methodological choices?. *Journal of Biogeography*, 2020, 47 (1), pp.193-206. 10.1111/jbi.13681 . hal-02324167

**HAL Id: hal-02324167**

**<https://hal.science/hal-02324167>**

Submitted on 20 Nov 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **From environmental DNA sequences to ecological conclusions: How strong is the influence of**  
2 **methodological choices?**

3  
4 **Running title:** Sensitivity of eDNA-based ecological results

5  
6 Irene Calderón-Sanou<sup>1\*</sup>, Tamara Münkemüller<sup>1</sup>, Frederic Boyer<sup>1</sup>, Lucie Zinger<sup>2</sup> & Wilfried Thuiller<sup>1</sup>

7  
8 <sup>1</sup>Univ. Grenoble Alpes, Univ. Savoie Mont Blanc, CNRS, LECA, Laboratoire d'Ecologie Alpine, F-  
9 38000, Grenoble, France

10 <sup>2</sup>Ecole Normale Supérieure, PSL Research University, CNRS, Inserm, Institut de Biologie de l'Ecole  
11 Normale Supérieure (IBENS), F-75005, Paris, France

12 \*Correspondance: E-mail: [irecalsa@gmail.com](mailto:irecalsa@gmail.com)

13  
14 **ACKNOWLEDGMENTS:** We thank the large team of researchers and students that helped collect  
15 the data. The research received funding from the French Agence Nationale de la Recherche (ANR)  
16 through the GlobNets (ANR-16-CE02-0009) project, and from “Investissement d’Avenir” grants  
17 managed by the ANR (Trajectories: ANR-15-IDEX-02; Montane: OSUG@2020: ANR-10-LAB-56).  
18 All computations were performed using the GRICAD infrastructure ([https://gricad.univ-grenoble-  
alpes.fr](https://gricad.univ-grenoble-<br/>19 alpes.fr)).

20 **ABSTRACT**

21 **Aim**

22 Environmental DNA (eDNA) is increasingly used for analysing and modelling all-inclusive  
23 biodiversity patterns. However, the reliability of eDNA-based diversity estimates is commonly  
24 compromised by arbitrary decisions for curating the data from molecular artefacts. Here, we test the  
25 sensitivity of common ecological analyses to these curation steps, and identify the crucial ones to  
26 draw sound ecological conclusions.

27 **Location**

28 Valloire, French Alps.

29 **Taxon**

30 Vascular plants and Fungi.

31 **Methods**

32 Using soil eDNA metabarcoding data for plants and fungi from twenty plots sampled along a 1000-m  
33 elevation gradient, we tested how the conclusions from three types of ecological analyses: (i) the  
34 spatial partitioning of diversity, (ii) the diversity-environment relationship, and (iii) the distance-decay  
35 relationship, are robust to data curation steps. Since eDNA metabarcoding data also comprise  
36 erroneous sequences with low frequencies, diversity estimates were further calculated using  
37 abundance-based Hill numbers, which penalize rare sequences through a scaling parameter, namely  
38 the order of diversity  $q$  (Richness with  $q=0$ , Shannon diversity with  $q=1$ , Simpson diversity with  $q=2$ ).

39 **Results**

40 We showed that results from different ecological analyses had varying degrees of sensitivity to data  
41 curation strategies and that the use of Shannon and Simpson diversities led to more reliable results.  
42 We demonstrated that MOTU clustering, removal of PCR errors and of cross-sample contaminations  
43 had major impacts on ecological analyses.

44 **Main conclusions**

45 In the Era of Big Data, eDNA metabarcoding is going to be one of the major tools to describe, model  
46 and predict biodiversity in space and time. However, ignoring crucial data curation steps will impede  
47 the robustness of several ecological conclusions. Here, we propose a roadmap of crucial curation steps  
48 for different types of ecological analyses.

49

50 **KEYWORDS:** Data curation strategies, distance-decay, environmental DNA, Hill numbers,  
51 metabarcoding, sensitivity analysis, spatial partitioning of diversity.

## 52 **1 INTRODUCTION**

53 Understanding the structure and distribution of biodiversity across space and time is a critical  
54 goal in ecology. The development of environmental DNA (eDNA) metabarcoding approaches now  
55 facilitates the monitoring of species at biogeographical scales and across the whole tree of life  
56 (Drummond et al., 2015; Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). It is now  
57 possible to tackle unresolved questions that could not be addressed with traditional biodiversity  
58 surveys so far. For example, eDNA-based biodiversity studies have enabled the spatial partitioning of  
59 diversity (i.e. gamma, alpha and beta diversity) of so far elusive taxa in both terrestrial and marine  
60 environments (e.g. marine viruses and protists, soil fungi and bacteria), thereby improving our  
61 understanding of their community assembly processes and of their role in structuring communities  
62 and networks at global scales (e.g. Lima-Mendez et al., 2015; Tedersoo et al., 2014). However, while  
63 the eDNA metabarcoding approach promises substantial advances in macroecology and multi-taxa  
64 studies, it requires an appropriate and careful processing of the tremendous amount of sequences  
65 generated to draw robust and ecologically meaningful conclusions.

66  
67 Indeed, the analyses of diversity patterns (e.g. alpha- and beta-diversity; Whittaker, 1960) across  
68 space and of the processes generating these patterns are traditionally based on community matrices  
69 representing the presence/abundance of species across samples. In eDNA metabarcoding surveys, the  
70 data consist of hundreds to millions of DNA sequencing reads from the hundreds to thousands of  
71 species co-occurring within samples. Using bioinformatics, these data are then transformed in  
72 community matrices, but with species replaced by DNA sequences, and species abundance replaced  
73 by a number of sequencing reads. While, in an ideal world, one sequence should correspond to a  
74 single species, in practice, it can correspond to several species if the DNA region has a low taxonomic  
75 resolution, and more critically, one species can be represented by tens to thousands of variant  
76 sequences. Amongst those variants, a few are biologically meaningful (e.g. intraspecific variability),  
77 but the large majority of them are technical errors produced at the different stages of the lab  
78 treatments, from DNA extraction to sequencing (see Table 1 and Appendix S1, Bálint et al., 2016;  
79 Taberlet, Bonin, Zinger, & Coissac, 2018). These errors can represent more than 70% of the  
80 sequences in raw metabarcoding datasets, and have usually low frequencies (e.g. singletons; S. P.  
81 Brown et al., 2015). If interpreted as genuine, these sequences can therefore inflate diversity by  
82 several orders of magnitude and lead to flawed ecological interpretations (Kunin, Engelbrekton,  
83 Ochman, & Hugenholtz, 2010). Molecular protocols are thus applied to reduce and/or control specific  
84 technical errors accumulated during the data production. For example, replicated PCR amplification  
85 and use of negative controls allow identifying artefactual sequences resulting from random errors  
86 introduced by DNA polymerases or sequencers, as well as reagent contaminants (de Barba et al.,  
87 2014). However, error rates remain high even with the most stringent molecular protocols (Bálint et  
88 al., 2016; Taberlet et al., 2018), which has led to the development of bioinformatics algorithms aiming

89 at detecting errors known to occur during data generation (e.g. PCR errors or chimeric sequences).  
90 Also, most of these tools require specifying thresholds and parameter values, which are usually based  
91 on arbitrary decisions and visual assessments. An example is the classification of sequence variants  
92 into MOTUs (Molecular Operational Taxonomic Units) based on the similarity of sequences. While  
93 this step is critical because MOTUs are used as a proxy for species in the majority of DNA  
94 metabarcoding studies (Appendix S1), MOTUs are commonly defined using a 97% sequence  
95 similarity threshold, a value historically defined as the similarity level of full-length 16S rRNA  
96 barcodes below which bacterial strains necessarily belong to different species (Stackebrandt &  
97 Goebel, 1994). However, the optimal threshold value to define MOTUs depends on the focal taxa and  
98 polymorphism/length of the DNA marker used (e.g. E. A. Brown, Chain, Crease, MacIsaac, &  
99 Cristescu, 2015; Kunin et al., 2010). It also depends on the PCR/sequencing error rate, which varies  
100 across molecular protocols, and depends on the amount of target DNA: when it is low, each genuine  
101 DNA fragment has a higher probability of being amplified at each PCR cycle (Taberlet et al., 2018).

102

103 Hence, using DNA metabarcoding requires taking several methodological choices. Beyond those  
104 related to molecular protocols and bioinformatics software, one of the most critical choice is to decide  
105 which data curation steps to include in the curation procedure. Indeed, each step directly affects the  
106 community matrix obtained, by influencing the final list of MOTUs and/or their frequencies within  
107 samples. Previous methodological studies have thus underlined the importance of data curation steps  
108 on the reliability of ecological analyses and provided guidelines for bioinformatics decision-making  
109 (e.g. Alberdi, Aizpurua, Gilbert, & Bohmann, 2018; Schloss, 2010). However, most of these studies  
110 tested the influence of data curation procedures on a single metric or ecological question. However,  
111 questions related to local community richness can be very sensitive to errors (Flynn, Brown, Chain,  
112 MacIsaac, & Cristescu, 2015), while comparisons of communities' composition might be less affected  
113 (Leray & Knowlton, 2015; Taberlet et al., 2018). In addition, most studies have focused on microbial  
114 communities (bacteria or fungi), and few have addressed such questions to macro-organisms. Finally,  
115 most published tests have so far relied on mock communities (i.e. positive controls) usually made of  
116 DNA extracts for few known species. While mock communities are useful to identify errors and  
117 estimate error rates, the conclusions cannot easily be translated to realistic environments with rich and  
118 complex communities (Alberdi et al., 2018).

119

120 Here, we address how methodological choices related to the DNA metabarcoding data curation  
121 strategy influence the results for different types of ecological analyses and their related diversity  
122 metrics. We used soil eDNA data from an elevation gradient in the French Alps, and focused on  
123 plants and soil fungi to represent both macro- and microorganisms, as well as DNA markers with  
124 different length (Table 2). Patterns of plant diversity have been extensively studied in this area (e.g.  
125 Chalmandrier, Münkemüller, Lavergne, & Thuiller, 2015) and serve as a good reference to evaluate

126 the results estimated from eDNA metabarcoding data. We subjected these data to 256 different data  
127 curation strategies, which correspond to all possible combinations of seven critical data curation steps.  
128 We then tested how the curation strategies influence the inferences drawn from three different  
129 ecological analyses: 1) a spatial partitioning of diversity (i.e. gamma, alpha and beta diversities) to  
130 estimate the regional and local diversity of the gradient, 2) a diversity-environment relationship, to  
131 analyse the influence of environment on the local community diversity (alpha), and 3) a distance-  
132 decay analysis, to evaluate if similarities between communities (beta) decrease with increasing  
133 geographic distances. To this end, we first checked the accuracy of eDNA metabarcoding data in  
134 detecting ecological patterns by comparing the eDNA-based diversity patterns with the expected  
135 values based on mock communities and traditional botanical surveys (only available for plants).  
136 Second, we did an overall sensitivity analysis to test the sensitivity of ecological results to the data  
137 curation strategy. Finally, with a variance partitioning analysis we identified the crucial curation steps  
138 (i.e. those that introduced more variance to the results) to include or consider in the curation  
139 procedure.

140

141 To achieve these objectives, we built on Hill numbers (Hill, 1973) to estimate diversity, which  
142 unifies mathematically the best known diversity measures in ecology through a unique parameter  $q$   
143 (i.e. Richness at  $q=0$ , the exponential of Shannon entropy at  $q=1$  and the inverse of Simpson at  $q=2$ ).  
144 In this framework, the weight of rare species decreases when increasing the value of the parameter  $q$ .  
145 This feature is particularly relevant for DNA metabarcoding data, since artefactual sequences are  
146 usually rare compared to the genuine ones (Bálint et al., 2016; Taberlet et al., 2018). Hill numbers  
147 can thus penalize these rare sequences at different degrees:  $q=1$  is the order of diversity that levels the  
148 MOTUs exactly according to their relative abundances, while  $q<1$  overweigh rare MOTUs and  $q>1$   
149 overweight abundant MOTUs. As a result, we could expect that diversity measures that give less  
150 importance to rare sequences (i.e.  $q>0$ ) are less sensitive to the data curation strategy, because they  
151 penalize the artefactual sequences targeted by the curation steps.

152

## 153 **2 MATERIAL AND METHODS**

### 154 **2.1 Sample data**

155 Soil cores were sampled at 10 different elevations equally distributed across an elevation gradient in  
156 the northern French Alps (from 1748 m to 2725 m a.s.l.) in 2012. At each elevation, two 10m × 10m  
157 plots were selected (20 plots in total). In each plot, 21 soil cores distributed along the two diagonals  
158 were sampled. Soil corers were cleaned and sterilized between each sample collection. Extracellular  
159 DNA was then extracted twice, from 15 g as described in Taberlet, Prud'Homme, et al., (2012).  
160 Aboveground plant community information (hereafter observed plant diversity) was obtained in each  
161 plot with a botanical survey conducted during the annual productivity peak (mid-July) using the  
162 Braun-Blanquet cover-abundance scale (Braun-Blanquet, 1946).

163

## 164 **2.2 Molecular analyses**

165 eDNA-based plant diversity was estimated by targeting a vascular plant specific marker (P6 loop of  
166 chloroplast trnL, Table 2). It targets highly conserved priming sites across vascular plants and  
167 amplifies a short region, which is desired when working with degraded DNA. eDNA-based fungal  
168 diversity was assessed using the nuclear ribosomal Internal Transcribed Spacer 1 (ITS1, Table 2). For  
169 each DNA extract, PCRs were run in duplicate leading to four technical replicates per core sample  
170 and DNA marker. PCR thermocycling conditions and mixes composition and purification can be  
171 found in Table S2.1 in Appendix S2. To control for potential contaminants, extraction and PCR blank  
172 controls were included in the experiment. To control for false positives caused by tag-switching  
173 events, we also defined “sequencing blank controls”, i.e. tag combinations not used in our  
174 experimental design, but that could be formed at the library preparation or sequencing stage (See  
175 Appendix S1). We also included positive controls in this experiment, which consisted of a mix of  
176 DNA extracted from 16 plant species. For this, genomic DNA was extracted from leaf tissue using the  
177 DNeasy Plant Kit (Qiagen GmbH, Hilden, Germany), quantified, diluted at different concentrations  
178 for each species and mixed to form a mock community (species composition provided in Table S2.2,  
179 Appendix S2). Positive controls allow for quantification of technical biases introduced by PCR and  
180 sequencing. Illumina sequencing was performed on a HiSeq platform (2x100bp paired-end reads) for  
181 plant amplicons and on a MiSeq (2x250bp paired-end reads) for fungi amplicons, both using the  
182 paired-end technology.

183

## 184 **2.3 Bioinformatics analyses**

185 The Illumina sequencing paired-end reads (Table S2.3) were pre-processed for each marker with three  
186 procedures: (i) assembling forward and reverse paired-end reads based on their overlapping 3'-end  
187 sequences, (ii) assigning each read to its respective sample (demultiplexing) and (iii) combining  
188 strictly identical sequences into unique DNA sequences while keeping information on their abundance  
189 (number of sequencing reads) in each sample (dereplication). Then we systematically processed the  
190 dereplicated sequences following common data curation procedures that included removal of  
191 sequences with low paired-end alignment scores, removal of singletons, removal of short sequences  
192 and removal of sequences containing ambiguous bases (not to be confounded with a phred-quality  
193 filtering; Fig.1a, Table 1 and Table S2.4). Singletons are sequences that occur only once in the whole  
194 dataset and many studies agree that their removal is necessary to reduce data  
195 complexity/computational time and because they mostly correspond to molecular artefacts that may  
196 inflate disproportionately diversity indices (S. P. Brown et al., 2015; Kunin et al., 2010). In our data,  
197 they represented 70-80% of the total number of sequences but only 1-15% of the total number of  
198 sequencing reads for plants and fungi respectively (Table S2.3 in Appendix S2). We finally assigned  
199 each remaining sequence to a taxonomic clade with the *ecotag* command from the OBITOOLS

200 software package (Boyer et al., 2016) that uses a lowest common ancestor algorithm for the  
201 assignment, and the EMBL database version 133 as a reference.

202

203 Next, data from each marker were processed following a range of different data curation  
204 strategies to test the sensitivity of ecological analyses to different methodological choices (Fig. 1b).  
205 To do so, we selected seven important steps: (i) removal of PCR errors, (ii) filtering of highly  
206 spurious sequences, (iii) removal of chimeras, (iv) sequence classification into MOTUs (MOTU  
207 clustering), (v) removal of reagent contaminants, (vi) cross-sample contamination cleaning and (vii)  
208 dysfunctional PCRs filtering (see Table 1, Appendix S1 and Table S2.4 in Appendix S2 for target  
209 errors and step descriptions). Curation steps were either kept or excluded, and were always performed  
210 in the same order in each data curation strategy. For the MOTU clustering step, when kept, three  
211 clustering thresholds were tested (1, 2 or 3 mismatches allowed between pairwise aligned sequences).  
212 We used here raw mismatches rather than percentages of dissimilarities because the DNA markers  
213 used are short (<100 bp) and/or highly polymorphic in length. Using the percentages of dissimilarity  
214 in this case would penalize more little differences when alignments are short than when they are long.

215

216 All different possible combinations of these curation strategies were implemented (Fig.1b). Most  
217 of the curation steps were done using the software OBITOOLS (Boyer et al., 2016). Chimera detection  
218 was performed with UCHIME (Edgar, Haas, Clemente, Quince, & Knight, 2011) and we used  
219 SUMACLUSt (Mercier, Boyer, Bonin, & Coissac, 2013) for MOTU clustering due to its ability in  
220 handling large datasets and its flexibility for defining the clustering threshold (see Table S2.4 for  
221 more details on the algorithm). After data curation, PCR replicates were summed and standardized by  
222 the total number of reads in each core sample. We then pooled the samples for each of the 20 plots to  
223 obtain a single community per plot. For this, MOTUs abundance (already standardized by the number  
224 of reads) were summed and standardized by the number of samples in each plot. For each of the data  
225 curation strategies, we obtained a community matrix with rows representing plots and columns  
226 representing all the MOTUs obtained after curation, which we used here as a proxy for species.  
227 Therefore, our sensitivity analysis was conducted on a total of 256 matrices for each DNA marker  
228 (Fig.1c).

229

## 230 **2.4 Ecological questions**

231 We tested the sensitivity of the results for three common ecological analyses to the above-  
232 mentioned data curation strategies using MOTUs as equivalent of species:

233

234 *Spatial partitioning of diversity* - We used the multiplicative diversity partitioning approach  
235 (Whittaker, 1960) to analyse gamma (here the diversity across the entire gradient), alpha (diversity of  
236 local communities) and beta diversity (diversity between communities). In the Hill numbers

237 framework, gamma diversity is the effective number of species in the pooled meta-community (i.e.  
238 across all plots), alpha diversity is the effective number of species per community (i.e. plot), and beta  
239 diversity is the effective number of communities, calculated as the ratio of gamma diversity to alpha  
240 diversity. We followed Chao, Chiu, & Jost, (2014)'s definition where beta diversity is independent of  
241 alpha and ranges from 1 (all communities are identical) to the total number of communities N (when  
242 N=20 all communities are different). We limited our study to taxonomic diversity, because the DNA  
243 markers we used here are rather short (Table 2) and are highly variable in length, which make them  
244 not suitable for inferring accurate phylogenetic relationships at the scale of the community.

245

246 *Diversity-environment relationship (Alpha~SOM content)* – Diversity is often linked to abiotic  
247 drivers, and a common ecological research question is how alpha diversity changes along an  
248 environmental gradient. Here, we fitted a linear model to determine changes in alpha diversity along a  
249 gradient of soil organic matter content (SOM content), known to be a strong predictor of diversity  
250 changes in the study site (Ohlmann et al., 2018).

251

252 *Distance-decay relationship (Similarity~geographic distance)* – Species' distributions and resulting  
253 diversity patterns are controlled by both species dispersal abilities and spatial turnover of  
254 environmental conditions (Tuomisto, 2003). One hypothesis is thus that spatially distant communities  
255 are more different than close communities ("distance-decay", Green et al., 2004; Tuomisto, 2003).  
256 We used the Jaccard-type overlap ( $U_{qN}$ ) as a measure of similarity (Chao et al., 2014) and we fitted a  
257 linear model using the log transformation of similarity against the geographic distance to evaluate the  
258 distance-decay. The geographic distance between plots was calculated with Euclidean distances using  
259 the elevation values of the plots.

260

261 For each DNA marker (plant and fungi), we calculated the gamma, alpha and beta diversities  
262 (spatial partitioning of diversity) for each of the 256 community matrices obtained from the different  
263 metabarcoding data curation strategies using Hill numbers with values of  $q=\{0,0.5,1,2\}$ . For the  
264 diversity-environment and the distance-decay relationships, we fitted our models to each community  
265 matrix and extracted the slopes and the R-squares of the models. Alpha diversity and community  
266 similarity were calculated using Hill numbers with values of  $q=\{0,1,2\}$ .

267

## 268 **2.5 Sensitivity analyses**

269 *Detectability of ecological patterns* - To test the ability of eDNA metabarcoding data and of the  
270 different data curation strategies to detect ecological patterns we (1) evaluated the completeness of the  
271 sampling unit (plot), and (2) used the observed plant diversity and positive controls as references to  
272 evaluate the accuracy of the ecological results. We acknowledge that eDNA-based diversity is  
273 expected to slightly diverge from observed diversity (see discussion) but they should follow similar

274 trends (Hiiesalu et al., 2012; Träger, Öpik, Vasar, & Wilson, 2019; Yoccoz et al., 2012). The  
275 sampling completeness of each plot was evaluated with rarefaction curves for the different orders of  
276 diversity  $q=\{0,1,2\}$  and for three data curation strategies with varying filtering stringency: a “no data  
277 curation” strategy with no curation step at all; a “basic curation” strategy including only the chimera  
278 removal and a traditional clustering threshold allowing three mismatches between clustered sequences  
279 and, a “rigorous curation” strategy, including all the curation steps considered here and a clustering  
280 threshold allowing two mismatches.

281

282 *Overall sensitivity analyses* - To test the sensitivity of the results for the different ecological analyses  
283 and their related diversity metrics to the data curation strategy, we used the variance of each diversity  
284 estimate, obtained across the 256 community matrices and for each marker (Fig. 1c). For the  
285 diversity-environment and the distance-decay relationships, we looked at the variance in the slope and  
286 the R-square of the linear regression across the 256 models for each marker. In addition, we used ‘the  
287 rigorous’ and ‘the basic’ curation strategies explained above, that correspond to commonly used  
288 pipelines, to exemplify how results can differ between studies.

289

290 *Identifying the crucial steps of the curation procedure* - To identify the crucial steps we did a variance  
291 partitioning analysis for each diversity metric. In respect to spatial partitioning of diversity, the  
292 diversity metrics (gamma, alpha and beta diversities) were used as the response variable in function of  
293 the curation steps. For the diversity-environment and the distance-decay relationships we used the  
294 slope and the R-square of the models as the response variable in function of the curation steps.  
295 Variance partitioning analyses were done with the R package RELAIMPO (Grömping, 2006).

296

297

## 298 **3 RESULTS**

### 299 **3.1 Detectability of ecological patterns with eDNA metabarcoding data**

300 *Sampling completeness of the plots* – For both markers/taxa, the total diversity was well represented  
301 by the number of reads sequenced, when considering the diversity at  $q=\{1,2\}$  (Fig.S2.1 and Fig.S2.2  
302 in Appendix S2). At  $q=\{0\}$ , the rarefaction curve rarely saturated, but we obtained more asymptotic  
303 curves when increasing the stringency of the data curation strategy.

304

305 *Spatial partitioning of diversity* – Overall, we found that alpha diversity estimates at  $q=\{1,2\}$  were  
306 closer to the observed plant diversity (Fig.2b) and to the positive controls composition (Fig. 3) than at  
307  $q=\{0,0.5\}$ . However, diversity at  $q=\{1\}$  slightly underestimated gamma (Fig.2a) and beta (Fig.2c)  
308 while all diversity components were underestimated for most curation strategies at  $q=\{2\}$  (Fig.2a-c).

309 Richness ( $q=0$ ) was always overestimated. While we obtained very accurate results for diversity at  
310  $q=\{0.5\}$  when using a rigorous pipeline, a basic pipeline led to a substantial overestimation.

311

312 *Diversity-environment relationship* – While the expected positive slope was in most cases detected  
313 (Fig.2g) and its value was on average very similar to the one observed for plant diversity, especially  
314 when using a rigorous pipeline, it was highly overestimated for some data curation strategies at  
315  $q=\{0,1\}$ .

316

317 *Distance-decay relationship* – The expected negative slope of the distance-decay curve was always  
318 detected (Fig.2k). However, independently of the data curation strategy, the slope was always  
319 underestimated compared to the curve calculated with observed plant diversity. Also, the R-square of  
320 the distance-decay relationship was reduced at  $q=\{2\}$  (Fig.2l).

321

### 322 **3.2 Overall sensitivity of ecological questions and diversity metrics**

323 The results of different ecological questions had varying degrees of sensitivity to the data curation  
324 strategies. While the estimates in all ecological questions were highly sensitive (width of the boxplots  
325 in Fig. 2), the main signal of the diversity-environment and the distance-decay relationships was  
326 consistent across most curation strategies.

327

328 *Spatial partitioning of diversity* - Sensitivity of gamma, alpha and beta diversity decreased for higher  
329 values of  $q$ , i.e. weighing down rare MOTUs (Fig.2a-f). Diversity estimates at  $q=\{0\}$  were the most  
330 sensitive, with more than two orders of magnitude for both gamma and alpha (Fig.2a & b) diversities  
331 of plants. Likewise, the rigorous and basic curation strategies (circles and triangles in Fig.2) exhibited  
332 a steep difference at  $q=\{0\}$ , which decreased when using higher values of  $q$  in the majority of cases.

333

334 *Diversity-environment relationship* - The interpretation of the alpha-SOM content relationship could  
335 change depending on the data curation strategy used. However, the alpha-SOM content relationship  
336 was more robust when using  $q=\{1,2\}$ , i.e. a positive relation between alpha diversity and SOM  
337 content was detected independently of the data curation strategy used (Fig.2g,h). Patterns in fungi  
338 diversity were more robust, i.e. no relation between fungi diversity and SOM content was detected  
339 across the different pipelines. A very weak positive relation between fungi diversity and SOM content  
340 was observed for  $q=\{1,2\}$ . The rigorous and the basic strategies led to very similar results for both  
341 DNA markers/taxa.

342

343 *Distance-decay relationship* – In contrast, a significant distance-decay relationship was always  
344 detected from eDNA metabarcoding data independently of the data curation strategy, but the rate at  
345 which similarity decays with increasing distance between plots (i.e. slope) slightly changed across

346 strategies. While very similar results were found between the rigorous and the basic strategies for the  
347 distance-decay curve of plants, the slope of the distance-decay curve for fungi was very low when  
348 using a basic instead of a rigorous strategy.

349

### 350 **3.3 Crucial steps of the curation procedure**

351 Overall, we found that two curation steps, the removal of PCR error and the clustering to define  
352 MOTUs, explained most of the variation in diversity estimates across data curation strategies (more  
353 than 15% each and usually more than 40% in total) for most of the diversity metrics in the ecological  
354 analyses and for both markers/taxa (Fig.4 and Fig.S2.3 in Appendix S2). Also, cross-sample  
355 contamination removal explained large parts of the variance of beta diversity in the spatial  
356 partitioning of diversity analyses (Fig.4a,b) and of R-squares and slopes in the diversity-environment  
357 (Fig.4c,d) and distance-decay (Fig.4e,f) relationships analyses.

358

359

## 360 **4 DISCUSSION**

361 Ecologists do now increasingly rely on DNA metabarcoding to measure biodiversity as this approach  
362 holds the promise of allowing testing long-standing hypotheses at spatial, temporal and taxonomic  
363 scales that were hitherto inaccessible with traditional approaches. However, the technique is still  
364 hampered by a substantial amount of technical errors (Table 1, Appendix S1; Bálint et al., 2016;  
365 Taberlet et al., 2018). Here, we sought at testing the sensitivity of the conclusions drawn from  
366 different ecological analyses and diversity metrics to the steps commonly used to curate DNA  
367 metabarcoding data from such errors. We show that ecological conclusions had varying degrees of  
368 sensitivity to the data curation strategies and that the use of metrics that are less sensitive to rare  
369 species/MOTUs (i.e. Shannon and Simpson diversity) leads to more robust diversity estimates. Also,  
370 we demonstrated that MOTU clustering, removal of PCR errors and removal of cross-sample  
371 contaminations have a major influence on ecological results, and must always be carefully included  
372 when curating DNA metabarcoding data.

373

374 The breadth of our study makes our findings generalizable to other systems. Indeed, we found  
375 similar trends in the sensitivity of gamma and alpha diversity estimates for both our observed plant  
376 diversity and the mock community (Figure 2 vs Figure 3). Second, our study focus on both plants and  
377 fungi, that widely differ in their ecological properties and the length of their markers (on average 50  
378 bp for plants vs. 225 bp for fungi). Still, while they do not share the same diversity patterns, their  
379 sensitivity to data curation strategies were comparable. Further, we expect that our study and the  
380 experimental testing design we developed will stimulate further methodological studies (e.g. for

381 tropical or aquatic systems and other markers/taxa) and that they will serve as a guide to prioritize  
382 some curation steps when deciding for a curation strategy.

383

#### 384 **4.1 Linking methodological choices with ecological questions**

385 The ecological question(s) underlying a study should lead the prioritization of the curation steps to be  
386 included in the data curation procedure, as well as the selection of appropriate diversity metrics (Fig.  
387 5). If the aim of the study is to estimate the spatial partitioning of diversity (Fig.5a), it is important to  
388 keep in mind that all diversity components are biased by the data curation steps. Richness is highly  
389 sensitive to error accumulation, and was hence the metric responding the strongest to the data curation  
390 strategy. Consequently, if measuring richness is crucial for the study, and, thus, rare species are  
391 important, the reliability of the results must be confirmed with additional analyses. For example, a  
392 more conservative strategy (i.e. keeping only MOTUs present in more than a certain number of PCR  
393 replicates) can improve the reliability of final results, but with the risk of missing species represented  
394 by few sequences in only a few samples due to the sampling process occurring when preparing  
395 aliquots of one DNA extract (Alberdi et al., 2018). Verifying the pertinence of species detected by  
396 looking in detail into the taxonomic assignments can also improve the reliability of results, even  
397 though this could be problematic for poorly known taxa with incomplete reference databases  
398 (Cristescu, 2014). Also, positive controls (with mock communities) and numerous negative controls  
399 (extraction, PCR) must be included in all the phases of sequence generations to ensure the accuracy of  
400 richness estimates (Bálint et al., 2016). In any cases, a certain degree of uncertainty will always  
401 remain because of the complexity of deciding objectively which sequences are genuine and which are  
402 artefactual.

403

404 We corroborated that richness is a very sensitive metric and is always overestimated (Fig.2a-  
405 c). The intrinsic properties of eDNA can inflate the diversity compared to traditional surveys because  
406 eDNA can persist in the environment or be transported through space depending on the abiotic  
407 conditions (e.g. water transport, temperature, UV, or microbial activity; Barnes & Turner, 2016). This  
408 means that the diversity eDNA estimates not only encompass local and current species, but also  
409 species that are dormant (Hiiesalu et al., 2012), that were present in the recent past (Yoccoz, 2012) or  
410 that are present in the vicinity of the studied area (Taberlet et al., 2018). In other words, the  
411 spatiotemporal window captured by local eDNA diversity estimates may be larger than that captured  
412 by traditional approaches, a property that can be desirable or not depending on the question addressed.  
413 Distinguishing this feature from methodological bias remains at this stage difficult, as it may look like  
414 cross-contamination, and also because the cycle of eDNA in the environment remains poorly  
415 understood (Barnes & Turner 2016). However, it is crucial to account for eDNA properties when  
416 interpreting richness-based studies to avoid meaningless conclusions.

417

418           When the detection of rare species is not of importance, Hill numbers are a promising  
419 solution to increase the robustness of results and to avoid the inflation of diversity estimates. The Hill  
420 numbers approach has been already proposed to better estimate microbial diversity (e.g. Bálint et al.,  
421 2016; Chiu & Chao, 2016), and we corroborate its efficiency for estimating plant diversity and  
422 potentially other macro-organisms from metabarcoding data. Both, Shannon and Simpson diversity  
423 measures led to a satisfying representativeness of the sampling unit diversity and were robust to the  
424 different data curation strategies tested here, but Shannon diversity was less biased. In the same way  
425 that richness overestimated diversity, Simpson diversity tended to underestimate diversity. Diversity  
426 measures, other than richness (i.e.  $q > 0$ ), account for species/MOTUs abundance structure. The factors  
427 determining species' abundances in a community are not the only factors determining the MOTUs'  
428 abundances. These correspond to a pool of DNA fragments from current, dormant, or past populations  
429 (e.g. microbes) down to one (or part of one) single multicellular individual that are besides amplified  
430 by PCR. Consequently, a highly abundant MOTU does not necessarily imply that more individuals of  
431 the corresponding taxon were present, it could also be due to e.g. higher body mass, larger root  
432 systems, or slower DNA decomposition. Besides, given the exponential nature of the PCR  
433 amplification, abundant taxa become even more abundant in this step and this could lead to an  
434 underestimation of Simpson diversity. Hence, interpreting MOTUs frequency directly as species  
435 abundance can be highly misleading, and estimating species abundance in terms of number of  
436 individuals or biomass from eDNA is still a major challenge in the field (Deiner et al., 2017).  
437 However, MOTUs frequency correlates to a certain extent to species relative abundance, and more  
438 importantly, errors are usually rarer than genuine sequences (reviewed in Taberlet et al., 2018).  
439 Accordingly, Shannon diversity from eDNA samples appears here as a balanced diversity measure,  
440 robust to the data curation strategy, and hence, to rare errors. This can be generalized to all ecological  
441 analyses tested in this study. Given these results, we argue that using a complete diversity profile (for  
442 example, with  $q$  values between 0 and 2) may allow improving confidence in diversity estimates from  
443 eDNA data while getting information about MOTUs structure of abundances.

444

445           Another important outcome of our assessment is that despite the above-mentioned limits,  
446 robust conclusions can be obtained from eDNA metabarcoding data if the aim is to link local diversity  
447 (alpha) or community similarity (beta) to environmental or geographic gradients (Fig.5b). Changes in  
448 local diversity across an environmental gradient were more sensitive to the data curation strategies  
449 than the distance-decay relationship. Our results thus corroborate other studies that demonstrated the  
450 robustness of beta diversity to bioinformatics analyses (Botnen, Davey, Halvorsen, & Kauserud,  
451 2018; Deiner et al., 2017). However, the slope of the distance-decay was always underestimated  
452 compared to that obtained from observed plant diversity. On one hand, this could result from a lack of  
453 phylogenetic resolution of the genetic marker used here, which is relatively short. In alpine  
454 ecosystems, it is common to see abundant species replaced by closely related species across an

455 elevation gradient (Chalmandrier et al., 2015). A genetic marker with a low phylogenetic resolution  
456 would not detect these changes and as a consequence, gamma and beta diversities would be  
457 underestimated. However, the underestimation of gamma diversity relative to alpha diversity is not  
458 strong enough, suggesting that other reasons may also explain the lower slope of the distance-decay  
459 curve for eDNA-based plant diversity. Botanical surveys used in this study represent just a local  
460 snapshot of the visible plant diversity at the sampling time, and, unlike the eDNA approach, may miss  
461 species with an offset phenology or present only in the vicinity of the sampling area (Hiiesalu et al.,  
462 2012). We can expect that the larger spatiotemporal window captured by the eDNA metabarcoding  
463 approach would thus result in higher similarity among the sites, which could be tested by increasing  
464 the botanical sampling effort across seasons and years to reduce botanical surveys biases related to the  
465 differentiated phenology of the species.

466

#### 467 **4.2 Crucial steps for designing a careful curation protocol**

468 While we included here curation steps that are common to most bioinformatic tools (e.g. QIIME,  
469 USEARCH), we acknowledge that algorithms within OBITOOLS have their own particularities, as  
470 each of the other packages, and that the results obtained here may not be directly transferable.  
471 However, we expect that the differences from a specific software are minor compared to the  
472 differences caused by the choice of specific curation steps (Bonder, Abeln, Zaura, & Brandt, 2012).  
473 In general, we corroborate past studies concluding that the clustering threshold used for defining  
474 MOTUs leads to significant changes in diversity estimates and that this is especially important for  
475 alpha and gamma diversities, but less so for beta diversity (Botnen et al., 2018; E. A. Brown et al.,  
476 2015; Kunin et al., 2010). Additionally, we found that PCR errors and cross-sample contaminations  
477 are critical steps and that including them leads to more realistic spatial diversity patterns and estimates  
478 of diversity components. These two steps correct the diversity at local levels (i.e. sample level) and  
479 are especially important when comparing communities. To our knowledge, this is the first study  
480 testing in a systematic way the effect of these curation steps on results across different types of  
481 ecological analyses. We recommend carefully choosing the MOTU clustering threshold, e.g.  
482 empirical means can be estimated for each marker or targeted taxa using *in silico* methods with  
483 reference databases (Taberlet et al., 2018) or experimentally, using mock communities (E. A. Brown  
484 et al., 2015), and considering removing PCR errors and cross-sample contaminations when designing  
485 a curation protocol to study biodiversity patterns. Furthermore, a rigorous data curation strategy  
486 including all the curation steps of the present study allowed obtaining accurate diversity estimates and  
487 diversity-environment and distance-decay relationships. This demonstrates that the other curation  
488 steps should not be neglected.

489

#### 490 **5 DATA AVAILABILITY STATEMENT**

491 Pre-filtered sequencing data as well as associated metadata are available on the Dryad Digital  
492 Repository ([doi:10.5061/dryad.0t39970](https://doi.org/10.5061/dryad.0t39970)).

493 **6 REFERENCES**

494

495 Alberdi, A., Aizpurua, O., Gilbert, M. T. P., & Bohmann, K. (2018). Scrutinizing key steps for  
496 reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, 9(1), 134–147.  
497 <https://doi.org/10.1111/2041-210X.12849>

498 Bálint, M., Bahram, M., Eren, A. M., Faust, K., Fuhrman, J. A., Lindahl, B., ... Tedersoo, L. (2016).  
499 Millions of reads, thousands of taxa: microbial community structure and associations analyzed via  
500 marker genes. *FEMS Microbiology Reviews*, 40(5), 686–700. <https://doi.org/10.1093/femsre/fuw017>

501 Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for  
502 conservation genetics. *Conservation Genetics*, 17(1), 1–17. [https://doi.org/10.1007/s10592-015-0775-](https://doi.org/10.1007/s10592-015-0775-4)  
503 4

504 Bonder, M. J., Abeln, S., Zaura, E., & Brandt, B. W. (2012). Comparing clustering and pre-processing  
505 in taxonomy analysis. *Bioinformatics*, 28(22), 2891–2897.  
506 <https://doi.org/10.1093/bioinformatics/bts552>

507 Botnen, S. S., Davey, M. L., Halvorsen, R., & Kausrud, H. (2018). Sequence clustering threshold has  
508 little effect on the recovery of microbial community structure. *Molecular Ecology Resources*.  
509 <https://doi.org/10.1111/1755-0998.12894>

510 Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., & Coissac, E. (2016). OBITOOLS : a UNIX -  
511 inspired software package for DNA metabarcoding. *Molecular Ecology Resources*, 16(1), 176–182.  
512 <https://doi.org/10.1111/1755-0998.12428>

513 Braun-Blanquet, J. (1946). Über den Deckungswert der Arten in den Pflanzengesellschaften der  
514 Ordnung Vaccinio-Piceetalia. *Jahresbericht Der Naturforschenden Gesellschaft Graubünden*, 130,  
515 115–119.

516 Brown, E. A., Chain, F. J. J., Crease, T. J., MacIsaac, H. J., & Cristescu, M. E. (2015). Divergence  
517 thresholds and divergent biodiversity estimates: can metabarcoding reliably describe zooplankton  
518 communities? *Ecology and Evolution*, 5(11), 2234–2251. <https://doi.org/10.1002/ece3.1485>

519 Brown, S. P., Veach, A. M., Rigdon-Huss, A. R., Grond, K., Lickteig, S. K., Lothamer, K., ...  
520 Jumpponen, A. (2015). Scraping the bottom of the barrel: are rare high throughput sequences  
521 artifacts? *Fungal Ecology*, 13, 221–225. <https://doi.org/10.1016/j.funeco.2014.08.006>

522 Chalmandrier, L., Münkemüller, T., Lavergne, S., & Thuiller, W. (2015). Effects of species'  
523 similarity and dominance on the functional and phylogenetic structure of a plant meta-community.  
524 *Ecology*, 96(1), 143–153. <https://doi.org/10.1890/13-2153.1>

525 Chao, A., Chiu, C.-H., & Jost, L. (2014). Unifying species diversity, phylogenetic diversity,  
526 functional diversity, and related similarity and differentiation measures through Hill numbers. *Annual*  
527 *Review of Ecology, Evolution, and Systematics*, 45(1), 297–324. [https://doi.org/10.1146/annurev-](https://doi.org/10.1146/annurev-ecolsys-120213-091540)  
528 [ecolsys-120213-091540](https://doi.org/10.1146/annurev-ecolsys-120213-091540)

529 Chiu, C.-H., & Chao, A. (2016). Estimating and comparing microbial diversity in the presence of  
530 sequencing errors. *PeerJ*, 4, e1634. <https://doi.org/10.7717/peerj.1634>

531 Cristescu, M. E. (2014). From barcoding single individuals to metabarcoding biological communities:  
532 towards an integrative approach to the study of global biodiversity. *Trends in Ecology & Evolution*,  
533 29(10), 566–571. <https://doi.org/10.1016/j.tree.2014.08.001>

534 de Barba, M., Miquel, C., Boyer, F., Mercier, C., Rioux, D., Coissac, E., & Taberlet, P. (2014). DNA  
535 metabarcoding multiplexing and validation of data accuracy for diet assessment: application to  
536 omnivorous diet. *Molecular Ecology Resources*, 14(2), 306–323. [https://doi.org/10.1111/1755-](https://doi.org/10.1111/1755-0998.12188)  
537 0998.12188

538 Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., ...  
539 Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and  
540 plant communities. *Molecular Ecology*, 26(21), 5872–5895. <https://doi.org/10.1111/mec.14350>

541 Drummond, A. J., Newcomb, R. D., Buckley, T. R., Xie, D., Dopheide, A., Potter, B. C., ... Nelson,  
542 N. (2015). Evaluating a multigene environmental DNA approach for biodiversity assessment.  
543 *GigaScience*, 4(1). <https://doi.org/10.1186/s13742-015-0086-1>

544 Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., & Knight, R. (2011). UCHIME improves  
545 sensitivity and speed of chimera detection. *Bioinformatics*, 27(16), 2194–2200.  
546 <https://doi.org/10.1093/bioinformatics/btr381>

547 Flynn, J. M., Brown, E. A., Chain, F. J. J., MacIsaac, H. J., & Cristescu, M. E. (2015). Toward  
548 accurate molecular identification of species in complex environmental samples: testing the  
549 performance of sequence filtering and clustering methods. *Ecology and Evolution*, 5(11), 2252–2266.  
550 <https://doi.org/10.1002/ece3.1497>

551 Green, J. L., Holmes, A. J., Westoby, M., Oliver, I., Briscoe, D., Dangerfield, M., ... Beattie, A. J.  
552 (2004). Spatial scaling of microbial eukaryote diversity. *Nature*, 432(7018), 747–750.  
553 <https://doi.org/10.1038/nature03034>

554 Grömping, U. (2006). Relative Importance for Linear Regression in R: The Package **relaimpo**.  
555 *Journal of Statistical Software*, 17(1). <https://doi.org/10.18637/jss.v017.i01>

556 Haegeman, B., Hamelin, J., Moriarty, J., Neal, P., Dushoff, J., & Weitz, J. S. (2013). Robust  
557 estimation of microbial diversity in theory and in practice. *The ISME Journal*, 7(6), 1092–1101.  
558 <https://doi.org/10.1038/ismej.2013.10>

559 Hiiesalu, I., Öpik, M., Metsis, M., Lilje, L., Davison, J., Vasar, M., ... Pärtel, M. (2012). Plant species  
560 richness belowground: higher richness and new patterns revealed by next-generation sequencing.  
561 *Molecular Ecology*, 21(8), 2004–2016. <https://doi.org/10.1111/j.1365-294X.2011.05390.x>

562 Hill, M. O. (1973). Diversity and Evenness: A Unifying Notation and Its Consequences. *Ecology*,  
563 54(2), 427–432. <https://doi.org/10.2307/1934352>

564 Kunin, V., Engelbrekton, A., Ochman, H., & Hugenholtz, P. (2010). Wrinkles in the rare biosphere:  
565 pyrosequencing errors can lead to artificial inflation of diversity estimates. *Environmental*  
566 *Microbiology*, *12*(1), 118–123. <https://doi.org/10.1111/j.1462-2920.2009.02051.x>

567 Leray, M., & Knowlton, N. (2015). DNA barcoding and metabarcoding of standardized samples  
568 reveal patterns of marine benthic diversity. *Proceedings of the National Academy of Sciences*, *112*(7),  
569 2076–2081. <https://doi.org/10.1073/pnas.1424997112>

570 Lima-Mendez, G., Faust, K., Henry, N., Decelle, J., Colin, S., Carcillo, F., ... Raes, J. (2015).  
571 Determinants of community structure in the global plankton interactome. *Science*, *348*(6237),  
572 1262073–1262073. <https://doi.org/10.1126/science.1262073>

573 Mercier, C., Boyer, F., Bonin, A., & Coissac, E. (2013). SUMATRA and SUMACLUSt: fast and  
574 exact comparison and clustering of sequences. *Programs and Abstracts of the SeqBio 2013 Workshop*.  
575 *Abstract*, 27–29. Citeseer.

576 Ohlmann, M., Mazel, F., Chalmandrier, L., Bec, S., Coissac, E., Gielly, L., ... Thuiller, W. (2018).  
577 Mapping the imprint of biotic interactions on  $\beta$ -diversity. *Ecology Letters*, *21*(11), 1660–1669.  
578 <https://doi.org/10.1111/ele.13143>

579 Schloss, P. D. (2010). The effects of alignment quality, distance calculation method, sequence  
580 filtering, and region on the analysis of 16S rRNA gene-based studies. *PLoS Computational Biology*,  
581 *6*(7), e1000844. <https://doi.org/10.1371/journal.pcbi.1000844>

582 Stackebrandt, E., & Goebel, B. M. (1994). Taxonomic Note: A Place for DNA-DNA Reassociation  
583 and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology. *International*  
584 *Journal of Systematic and Evolutionary Microbiology*, *44*(4), 846–849.  
585 <https://doi.org/10.1099/00207713-44-4-846>

586 Taberlet, P., Bonin, A., Zinger, L., & Coissac, E. (2018). *Environmental DNA: for biodiversity*  
587 *research and monitoring*. New York: Oxford University Press.

588 Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., & Willerslev, E. (2012). Towards next-  
589 generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, *21*(8), 2045–  
590 2050. <https://doi.org/10.1111/j.1365-294X.2012.05470.x>

591 Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., ... Willerslev, E. (2007).  
592 Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. *Nucleic Acids*  
593 *Research*, *35*(3), e14–e14. <https://doi.org/10.1093/nar/gkl938>

594 Taberlet, P., Prud'Homme, S. M., Campione, E., Roy, J., Miquel, C., Shehzad, W., ... Coissac, E.  
595 (2012). Soil sampling and isolation of extracellular DNA from large amount of starting material  
596 suitable for metabarcoding studies. *Molecular Ecology*, *21*(8), 1816–1820.  
597 <https://doi.org/10.1111/j.1365-294X.2011.05317.x>

598 Tedersoo, L., Bahram, M., Polme, S., Koljalg, U., Yorou, N. S., Wijesundera, R., ... Abarenkov, K.  
599 (2014). Global diversity and geography of soil fungi. *Science*, *346*(6213), 1256688–1256688.  
600 <https://doi.org/10.1126/science.1256688>

601 Träger, S., Öpik, M., Vasar, M., & Wilson, S. D. (2019). Belowground plant parts are crucial for  
602 comprehensively estimating total plant richness in herbaceous and woody habitats. *Ecology*, *100*(2),  
603 e02575. <https://doi.org/10.1002/ecy.2575>

604 Tuomisto, H. (2003). Dispersal, Environment, and Floristic Variation of Western Amazonian Forests.  
605 *Science*, *299*(5604), 241–244. <https://doi.org/10.1126/science.1078037>

606 White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal  
607 ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White  
608 (Eds.), *PCR protocols a guide to methods and applications* (pp. 315–322). New York: Academic  
609 Press.

610 Whittaker, R. H. (1960). Vegetation of the Siskiyou Mountains, Oregon and California. *Ecological*  
611 *Monographs*, *30*(3), 279–338. <https://doi.org/10.2307/1943563>

612 Yoccoz, N. G., Bråthen, K. A., Gielly, L., Haile, J., Edwards, M. E., Goslar, T., ... Taberlet, P.  
613 (2012). DNA from soil mirrors plant taxonomic and growth form diversity. *Molecular Ecology*,  
614 *21*(15), 3647–3655. <https://doi.org/10.1111/j.1365-294X.2012.05545.x>

615

616

617 **BIOSKETCH:** Irene Calderon-Sanou is a PhD student aiming at a better understanding of multi-  
618 trophic assemblages through the use of environmental DNA. Author contributions: WT initiated the  
619 overall idea, and together with ICS, LZ and TM conceived the overall analyses. ICS, LZ and FB  
620 conceptualized the data curation strategies, ICS run the curation procedures and analysed all the  
621 results, and led the writing with the significant contribution of all co-authors.

622

623

624 **TABLE CAPTION**

625

626 **TABLE 1** Brief description of classical technical errors occurring in DNA metabarcoding data, the  
627 associated data curation steps tested in the present study and the curation methodology. Target errors  
628 make reference to the errors described further in Appendix S1. See also Table S2.4 for more details on  
629 the curation steps used in this study.

630

631 **TABLE 2** Characteristics of the DNA markers used to estimate eDNA-based diversity in this study.

632

633

634  
635  
636

**TABLES**  
**TABLE 1**

<b>Target error</b>	<b>Definition</b>	<b>Curation step (Abbreviation) and methodology</b>
Mixed	Common obvious molecular/sequencing errors such as mis-paired reads, sequences with ambiguous bases, that are too short or singletons.	Common basic filtering : Removal of sequences meeting these criteria. This step is not tested here and has been applied systematically.
PCR error	Base mis-incorporation by the DNA polymerase during the PCR amplification.	PCR errors removal (PCR error): Identification of PCR errors using a model-based classification of sequences based on their similarities and abundances. The model reflects the accumulation of base mis-incorporation across PCR cycles, where genuine sequences remain more abundant than their respective errors.
Highly spurious sequences	Chimeras from multiple parents, primers dimers, etc. or sequences from highly degraded DNA fragments that largely differ from any known sequence.	Highly spurious sequences removal (Spurious): Removal of sequences of whose similarity with their closest match in public reference databases is below 70% (plants) or 50% (fungi).
Chimeras	Sequences obtained from the recombination of two or more parent sequences	Chimera detection and removal (Chimeras): Removal of sequences that have a high probability to be a subsequence from other, more abundant sequences in the dataset.
Remaining PCR errors/ biological variation	Sequences from the same species either resulting from a PCR error that could not be filtered above, or from intraspecific variability	MOTU clustering (Clustering): Clustering of sequences into MOTUs on the basis of their pairwise similarity. Here done at different sequence similarity thresholds.
External contaminants	DNA coming from an external source other than the biological sample	Reagent contaminants cleaning (Reagent): Removal of sequences that are more abundant in negative controls relative to biological samples because of the absence of other competing DNA fragments during the amplification process.
Cross-contaminations or Tag-jumps	Genuine sequences present in a sample where actually absent, either due to cross-contaminations at the bench, or due to tag-jumps occurring during the library preparation or the sequencing , i.e. switches of nucleotidic labels used to assign the sequencing reads to their samples. These contaminants are usually of much lower abundance than in their sample of origin.	Cross-sample contamination curation (Cross): If the abundance of a given MOTU in a given sample is below 0.03% of the total MOTU abundance in the entire dataset, it is considered as absent in this sample.
Dysfunctional PCRs	PCRs that are too different in comparison with their technical replicates.	Dysfunctional PCR removal (DysPCR): Removal of PCR replicates from a single biological sample that are more dissimilar to each other in MOTUs composition and structure than are the PCR obtained from other biological sample.

637 **TABLE 2**  
 638  
 639

<b>DNA Marker</b>	<b>Target taxa</b>	<b>Forward primer (5'-3')</b>	<b>Reverse primer (5'-3')</b>	<b>Length [range] (bp)</b>	<b>References</b>
P6 loop of the chloroplast <i>trnL</i> intron	Vascular plants	g: GGGCAATCCTGAGCCAA	h: CCATTGAGTCTCTGCACCTATC	48 [10-220]	Taberlet et al., 2007
Nuclear ribosomal DNA Internal Transcribed Spacer 1 (ITS1)	Fungi	ITS5: GGAAGTAAAAGTCGTAACAAGG	Fung02: CCAAGAGATCCGTTGYTGAAAGTK	226 [68-919]	White, Bruns, Lee, & Taylor, 1990; Taberlet et al., 2018

640 **FIGURE CAPTION**

641

642 **FIGURE 1** Workflow of the sensitivity analysis. (a) Raw data are curated with basic filtering steps for  
643 each DNA marker (plants: trnL-P6 loop, fungi: ITS1). (b) Filtered data are processed using seven  
644 curation steps that were varied or removed in each data curation strategy making a total of 256  
645 possible combinations. As a result, 256 community matrices are obtained per DNA marker and used  
646 to (c) conduct three types of ecological analyses. The range of values obtained for each ecological  
647 analysis and diversity metric represents the variance due to the data curation strategy.

648

649 **FIGURE 2** Estimated values of the spatial partitioning of diversity components (a-f), of the regression  
650 parameters from the diversity-environment (g-j), and of distance-decay (k-n) relationships across the  
651 256 curation strategies for different diversity metrics (Hill numbers,  $q=\{0,0.5,1,2\}$ ). The top row (a-c,  
652 g-j, k-l) corresponds to the plant DNA marker (trnL-P6 loop) and bottom row (d-f, i-j, m-n) to the  
653 fungi DNA marker (ITS1). Size of each box (including whiskers) represents the sensitivity of the  
654 diversity metrics or the model parameters to the data curation strategy. The circle and the triangle  
655 symbols indicate the values obtained from a rigorous and a basic curation strategy, respectively. The  
656 star symbol indicates the values calculated from botanical survey (only represented for plants, top  
657 row).

658

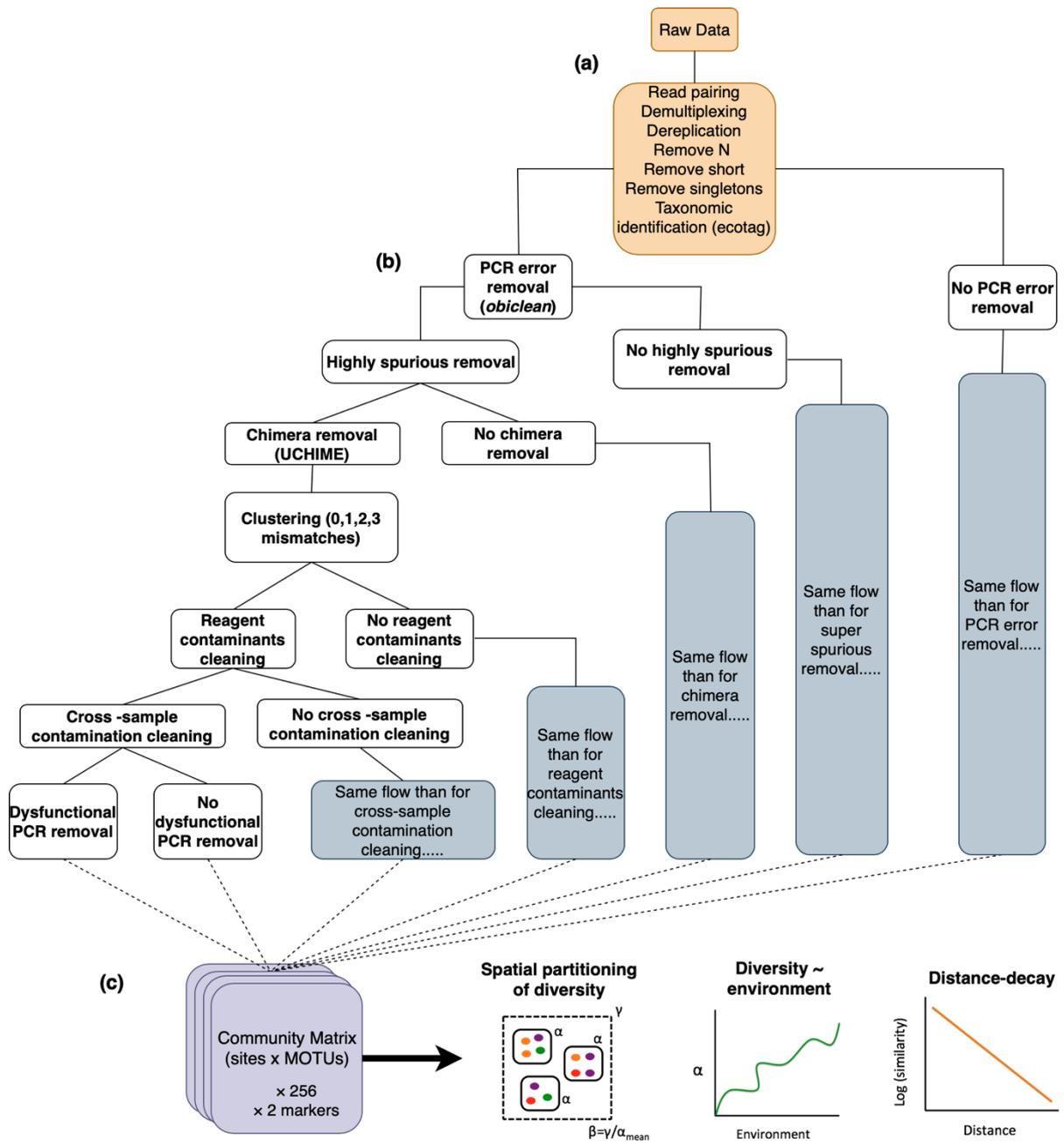
659 **FIGURE 3** Mean diversity estimated in positive controls across the 256 data curation strategies for  
660 different diversity metrics (Hill numbers,  $q=\{0,0.5,1,2\}$ ). Size of each box (including whiskers)  
661 represents the sensitivity of the diversity metrics to the data curation strategy. The star symbol  
662 indicates the values calculated from the known species composition in positive controls, the other  
663 symbols are as in Fig. 2.

664

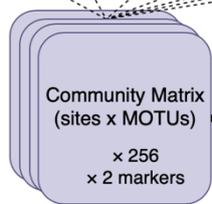
665 **FIGURE 4** Relative importance (% of variance explained) of the data curation steps on the variability  
666 of estimated values of the spatial partitioning of diversity components (a,b) and of the parameters  
667 from the diversity-environment (c,d) and distance-decay (e,f) relationships, using Hill numbers at  
668  $q=\{1\}$  (see Fig.S2.3 for the other  $q$  values). The top row (a,c,e) corresponds to the plant DNA marker  
669 (trnL-P6 loop) and bottom row (b,d,f) to the fungi DNA marker (ITS1). A model was fitted  
670 independently for each diversity component (a,b) or model parameter (c-f) as response variable, with  
671 curation steps as main effects.

672

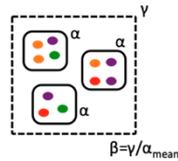
673 **FIGURE 5** Guidelines to improve the reliability of ecological results when analysing eDNA  
674 metabarcoding data.



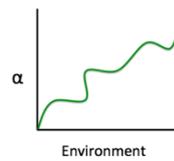
**(c)**



Spatial partitioning of diversity



Diversity ~ environment



Distance-decay

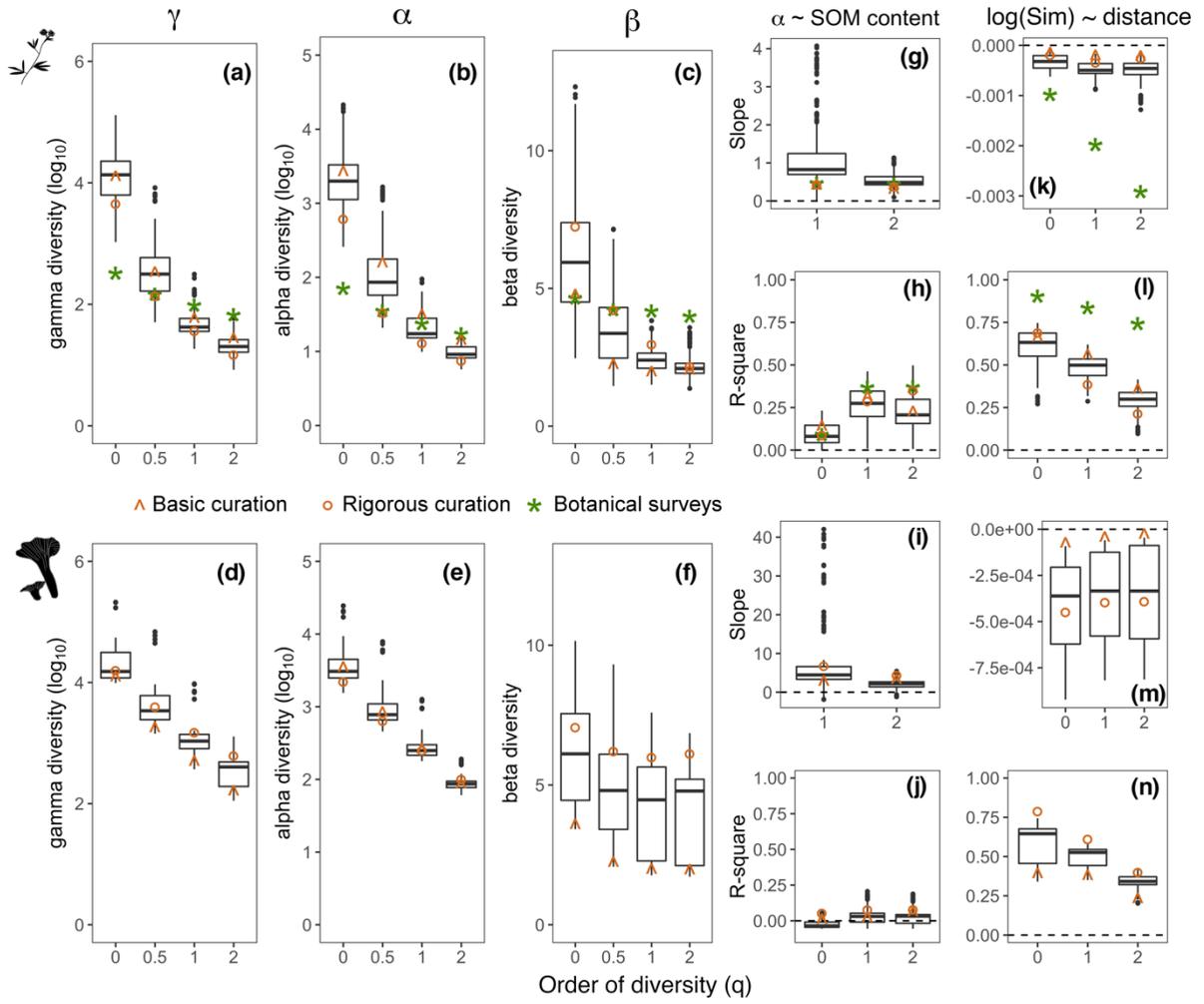


675  
676  
677

FIGURE 1

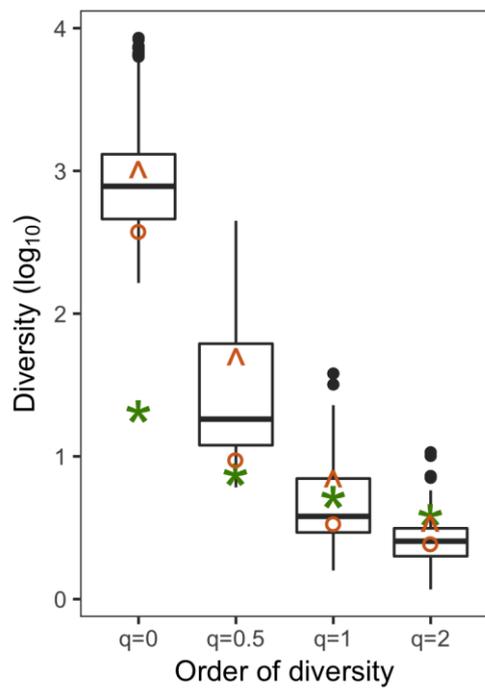
Spatial partitioning of diversity

Diversity ~ environment    Distance-decay



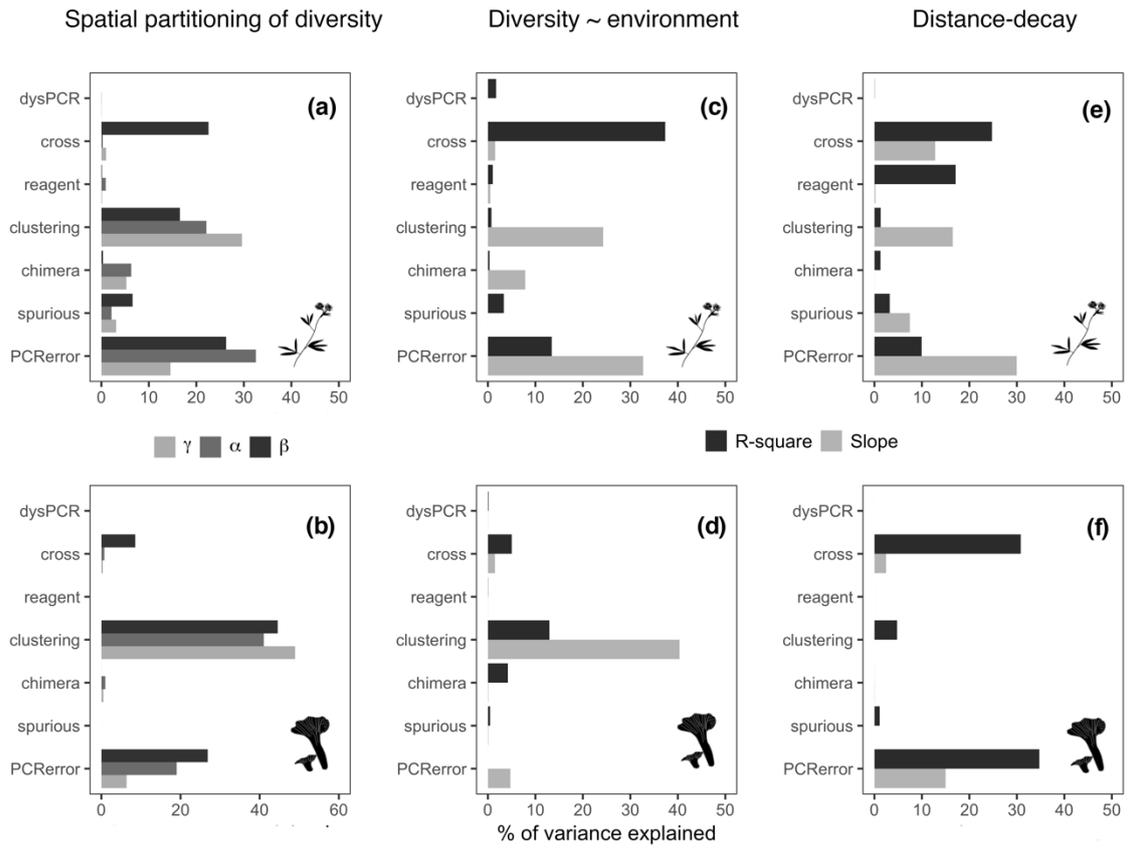
678  
 679  
 680

FIGURE 2



681  
682  
683

FIGURE 3



684  
685  
686

FIGURE 4

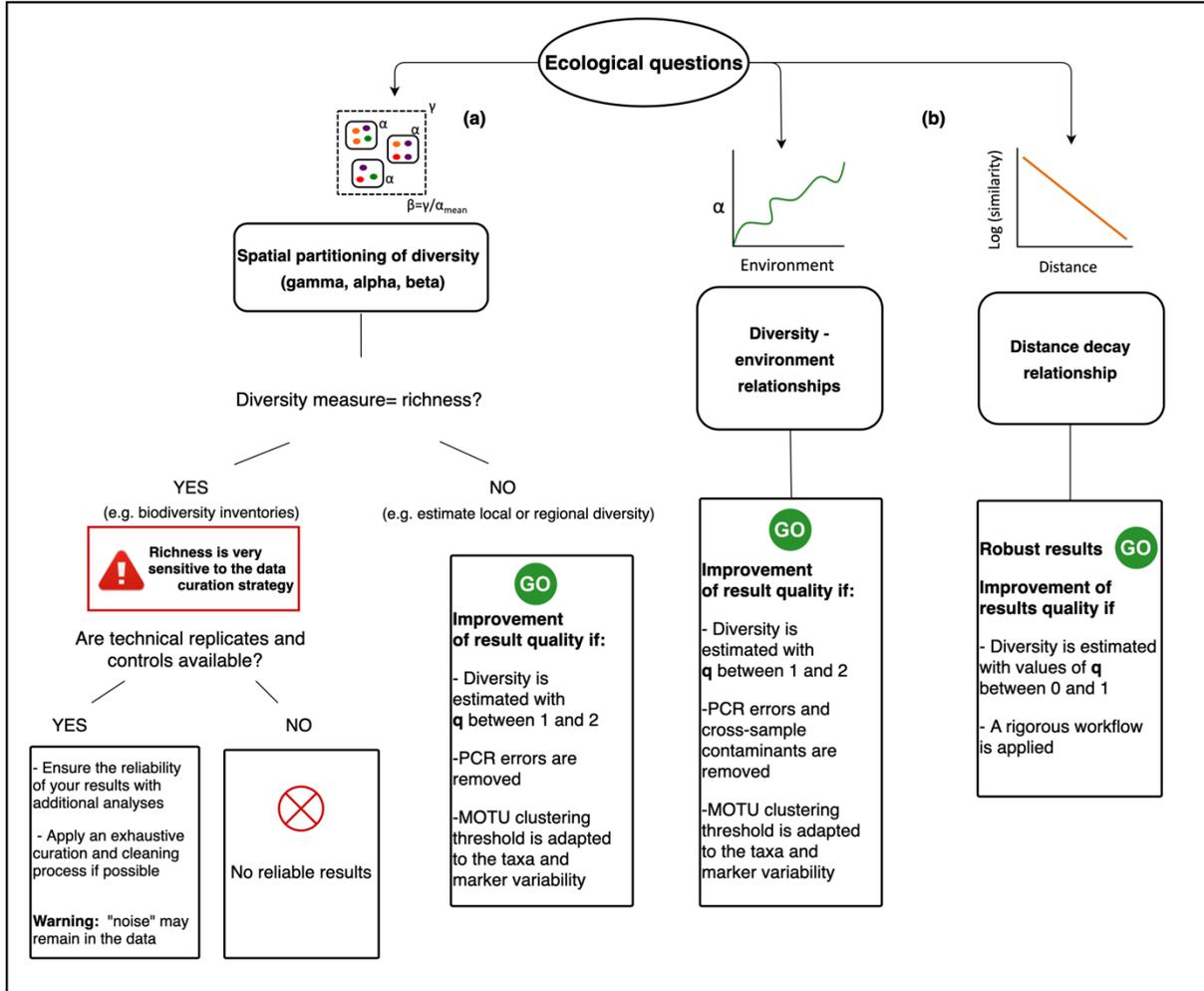


FIGURE 5