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## **Conservation assessment based on large-scale monitoring of eDNA: Application to freshwater mussels**

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## TITLE PAGE

Conservation assessment based on large-scale monitoring of freshwater bivalves: an eDNA point of view

*Short running title:*

Freshwater bivalves monitoring with eDNA

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## **COMPETING INTEREST STATEMENT**

The authors declare no competing interest statement. The first author is now working in a private company providing eDNA services.

## **DATA AVAILABILITY**

All the data presented here is integrated (or is intended to integrate in a near future, for the OFB data) in the national data base of the National Museum of Natural History, [www.inpn.mnhn.fr](http://www.inpn.mnhn.fr).

## **AUTHOR CONTRIBUTIONS**

Drafting of the manuscript: Prié. Critical revision of the manuscript for important intellectual content: Taberlet and Lopes-Lima. Protocol elaboration, bioinformatics and data analyses: Prié, Danet, Valentini, Besnard and Gargominy. Obtained funding: Prié, Lopes-Lima and Déjean. Field sampling: Prié, Breugnot, Couprie, Jardin, Lamand, Roset, Vigneron. Study design and supervision: Prié, Lopes-Lima, Taberlet and Dejean.

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1 TITLE PAGE

2 Conservation assessment based on large-scale monitoring of eDNA: application to freshwater  
3 mussels.

4 *Short running title:*

5 Freshwater bivalves monitoring with eDNA

6

7 ABSTRACT

8 Environmental DNA (eDNA) analysis allows efficient surveys of freshwater species and is  
9 being increasingly used. However, because different authors use different methods, eDNA  
10 data cannot easily be summarised for a global overview. One promising approach is to  
11 standardise methods for long-term monitoring. Standardised eDNA metabarcoding methods  
12 have been implemented in France for an elusive group: freshwater bivalves. All French  
13 bivalve species can be detected and identified at each sampling site. Here, we analyse this  
14 comprehensive dataset (280 sites across all France; 40 species) to investigate detection  
15 probabilities, infer species distributions and reassess conservation status, and finally examine  
16 community patterns (richness) and their determinants.

17 Our method gave over 95% probability of detection for most species. Species distributions  
18 inferred from eDNA sampling were compared with currently known distributions. Four  
19 invasive species and one species of conservation concern were found outside their known  
20 range. Others were found in a surprisingly small number of localities, although they are  
21 currently thought to be widespread and not of conservation concern. Indeed, the databases  
22 currently used to determine conservation status are biased by the inclusion of old data, mainly  
23 based on shell records only, and may include misidentified species. The main drivers of  
24 species richness were latitude and distance from the spring. Co-occurrence of species was

25 investigated for the first time. The results highlight the valuable data that eDNA analysis can  
26 provide when used on a large scale, for monitoring and ecological studies, as well as  
27 providing improved species distributions, which are essential for conservation policymaking.

28

29 **KEYWORDS**

30 freshwater bivalves, environmental DNA, detection probability, invasive species, distribution,  
31 conservation

32

33

## 34 **1 INTRODUCTION**

35 Freshwater ecosystems support significant biodiversity relative to their size: 10% of all  
36 known species are supported by wetlands, which cover less than 1% of the Earth's surface  
37 (Strayer and Dudgeon, 2010). They are the most threatened ecosystems in the world, mainly  
38 due to the impact of human activities (Carpenter et al., 2011). As a result, a significant  
39 proportion of freshwater species are threatened (Albert et al., 2021). However, quantifying  
40 these impacts is difficult because freshwater species are often difficult to detect and  
41 sometimes difficult to identify. Both sampling and identification of freshwater species require  
42 special skills, and sometimes the use of DNA barcoding (species identification using a  
43 specific gene fragment) to ensure the identification. The use of environmental DNA (eDNA)  
44 analysis improves both detection and identification problems and is being increasingly used.  
45 Environmental DNA analysis can include metabarcoding (an extension of barcoding through  
46 the use of NGS technology that allows blind identification of all species present in a sample).  
47 This is particularly true for freshwater molluscs, especially freshwater bivalves, which are  
48 highly threatened (Böhm et al., 2020). Freshwater bivalves are threatened by pollution, poor  
49 river management, dams, water abstraction and climate change. In addition, the spread of  
50 invasive species could become a major threat in the future. Recent studies (Ollard and  
51 Aldridge, 2023; Nakamura et al., 2023) have come to the same conclusion: there is an  
52 alarming decline in numbers across Europe.

53 Freshwater bivalves are very difficult to detect in the field, especially small species or those  
54 that spend most of their lives buried. They are difficult to identify based on shell morphology  
55 (e.g. Prié et al., 2012; Prié and Puillandre, 2014; Riccardi et al., 2019). DNA barcoding  
56 provides more reliable species identification than shell morphology, but is an invasive method  
57 that requires taking a sample or sacrificing the animal. Recently, eDNA primers have been

58 developed for the metabarcoding of freshwater bivalves (Prié et al., 2021). Initial results  
59 showed that the method, optimised from field sampling to the laboratory protocols, was  
60 reliable for the detection and identification of all freshwater bivalve species in France, with no  
61 cases of false positives or false negatives. In addition, the detection probability was much  
62 better than with traditional surveys. Based on these results, we provide here a detailed re-  
63 analysis of an updated and more comprehensive dataset from a conservation perspective.  
64 Traditionally, most freshwater mollusc inventories have been based on shell records (e.g.  
65 Duncan, 2008). Shell-only surveys are a source of misidentifications. The durability of shells  
66 can be an advantage for species detection, but can also lead to potential biases. There are  
67 many examples of species being extirpated from a site where shells can still be found for  
68 many years (e.g. Prié et al., 2018). Shell surveys may therefore overestimate the actual  
69 distribution of species and, consequently, population trends. Therefore, most freshwater  
70 mollusc atlases based on these classic surveys are likely to be biased (Obermeyer, 1998;  
71 Smith et al., 2003). Misidentified specimens, old data, and shell-only data are combined,  
72 generally leading to inflated species distributions and overestimates of species conservation  
73 status. Finally, the methods used to survey large species and micro-bivalves of the order  
74 Sphaeriida are very different (Strayer and Smith, 2003). Surveys of large and small species  
75 are very rarely conducted together. Consequently, although data on community composition  
76 are essential for ecology and conservation, studies very rarely include both taxa, even though  
77 they share similar biological traits and habitats, including shell production, filter feeding  
78 behaviour and a benthic life.

79 The use of eDNA can compensate for these biases and limitations. Here, we analyse a large  
80 dataset of eDNA surveys conducted across France over 5 years to test whether large-scale  
81 eDNA surveys can provide a different picture of freshwater mussel conservation.

82 We first examine the probability of detection of each species to test the efficiency of the  
83 method in providing accurate and reliable data on species occurrence. We then infer species  
84 distribution from the eDNA results and compare our results with available data from  
85 traditional methods. Finally, we examine species richness and its drivers, species  
86 composition, and co-occurrence.

87

## 88 2 MATERIAL AND METHODS

### 89 2.1 Environmental DNA sampling and site database

90 Sampling was carried out between 2015 and 2020, mainly in the summer months between  
91 May and October, as low flow periods are recommended at least for fish eDNA surveys  
92 (Milhau et al., 2019). At one site, bimonthly monitoring was carried out in the year 2017  
93 (Online Appendix 1).

94 Sampling protocols followed the standard protocol described by Prié et al. (2021), i.e., 30  
95 litres of water filtered through a 0.45 µm filtration capsule and repeated twice at each site, 12  
96 PCR replicates and a sequencing depth of 300,000 sequences for each primer pair. We used  
97 two primer pairs, 'Unio' (designed for the species of the order Unionida) and 'Vene'  
98 (designed for the species of the orders Venerida, Myida, Sphaeriida), targeting a short  
99 fragment (150bp) of the 16S ribosomal RNA gene (see Prié et al., 2021 for further details on  
100 the buffer, primers, DNA extraction, and amplification, sequencing and bioinformatics).

101 The dataset used in Prié et al. (2021) was extended to include 118 additional sites where the  
102 sampling protocol was strictly followed, i.e. (i) at least two eDNA samples were collected  
103 (sites with only one sample were withdrawn, if more than two replicates were available from  
104 the same site, the results were concatenated to establish the presence/absence matrix); (ii)  
105 both Unio and Vene primers were used; (iii) the sampling was satisfactory (samples with  
106 water too turbid for efficient filtration, small stream almost dry when sampled and winter  
107 samples were discarded). This resulted in a dataset of 260 sites, plus 20 standing water bodies  
108 (**Figure 1**). Sampling in standing water bodies was carried out in the same way as in flowing  
109 rivers, but the lack of flow is likely to bias the results. These were therefore only been  
110 included in some of the analyses.

111 **Detection probability** was estimated using site occupancy statistics based on the two  
112 replicates (in terms of presence/absence). As the eDNA reads per field replicate were not  
113 available for each site, the analyses are based on a subset of 224 samples. For **community**  
114 **composition** and **co-occurrence**, only river samples were included in the analysis (standing  
115 waters were excluded because they are not suitable for all species, and this was thought to  
116 bias the analysis of species composition and co-occurrence). For **species distribution maps**  
117 (Online Appendix 2), the entire database was used, regardless of sampling type.

118

## 119 **2.2 Statistical analysis**

### 120 **2.2.1 Site occupancy**

121 We modelled the species detection probabilities using a site occupancy modelling framework  
122 (Mackenzie et al., 2002) using the two replicates as temporal replicates and sampling sites as  
123 spatial units. Each species was modelled separately. We excluded the Italian Swan Mussel  
124 *Anodonta exulcerata* Porro, 1838, the Asiatic Clam *Corbicula fluminalis* (O.F. Müller, 1774),  
125 the Witham Orb Mussel *Sphaerium solidum* (Normand, 1844), the False Orb Peaclam  
126 *Euglesa pseudosphaerium* Favre, 1927, the Northern Peaclam *E. globularis* (Clessin, 1876)  
127 and Lilljeborg's Peaclam *E. lilljeborgii* (Clessin, 1886) from this analysis because the number  
128 of detections was extremely low (less than 5 detections). We fitted models with constant  
129 detection and occupancy probabilities. Using the estimated probability of detection for one  
130 replicate, we then calculated the expected probability of detection for 2, 3, or 4 spatial  
131 replicates to provide concrete guidance on the sampling effort required to achieve a given  
132 level of detection (i.e., 95%). All models were fitted using the R environment (R core team,  
133 2020) and the *unmarked* library (Fiske and Chandler, 2011). All estimates are presented with  
134 their 95% confidence interval.

### 135 **2.3 Available freshwater bivalves' data for France**

136 In order to compare the eDNA data collected with the current knowledge on the biogeography  
137 of freshwater bivalves, data provided by the INPN (Inventaire national du Patrimoine naturel,  
138 National Inventory of the Natural Heritage) were used. The INPN is a system set up to  
139 provide, in a standardised manner, the restitution of synthetic data needed for the expertise,  
140 the development of conservation strategies, and the dissemination of information and national  
141 and international reports on the French natural heritage (plant and animal species, natural  
142 habitats and geological heritage, see <https://inpn.mnhn.fr/accueil/presentation-inpn?lg=en>, and  
143 Gargominy, et al. 2016). The information comes from many national programmes and data  
144 provided by various partners. INPN ensures the validation of these data at three levels (1)  
145 conformity, e.g., the species is referenced in the French taxonomic register (Gargominy et al.,  
146 2019) and the observation is located in France; (2) consistency, e.g., observation is not dated  
147 after data integration; (3) scientific validation, e.g., marine species are not recorded on land.  
148 The INPN therefore includes data provided in the literature (both grey and academic), in  
149 museum collections and observations from various programs. Approximately 32 900  
150 freshwater bivalve data were available on the INPN database in 2020 (Online Appendix 2).  
151 For some species of conservation concern (the Giant freshwater pearl mussel *Pseudunio*  
152 *auricularius* (Spengler, 1793), the Depressed river mussel *Pseudanodonta complanata*  
153 (Rossmässler, 1835), the Nut Orb Mussel *Sphaerium rivicola* (Lamarck, 1818), and the  
154 Witham orb mussel *S. solidum*), a distinction could be made between old data (i.e, data  
155 collected before the year 2000) and recent data to give a more accurate picture of the decline.  
156 Shell-only data were considered old data for these three species.

#### 157 **2.2.2 Environmental variables**

158 Site coordinates, latitude and longitude were recorded during sampling events, allowing *a*  
159 *posteriori* qualification of the environmental variables. Classification of river size followed  
160 the national repository ([www.sandre.eaufrance.fr](http://www.sandre.eaufrance.fr), Online Appendix 3A). Distance from  
161 sampling sites to the spring was used as a proxy of the size of the river. Distance from  
162 sampling sites to the spring and the site altitude were extracted via a stream network  
163 reconstruction, performed using the R packages openStars and SSN (Kattwinkel et al. 2020;  
164 Hoef et al. 2014). Geology was chosen as a proxy for water pH, as most naiad species prefer  
165 calcareous waters. The only available database covering the whole of France is the European  
166 Soil Database (<http://eusoils.jrc.ec.europa.eu/>). A single variable was used: siliceous vs non-  
167 siliceous substrate. These parameters were chosen because they were highlighted by Prié et al.  
168 (2014) as important factors in the distribution of freshwater bivalves. The distribution of each  
169 environmental variable is provided in **Online Appendix 3B**.

### 170 **2.2.3 Community composition**

171 Community composition (**Online Appendix 3 C-D**) was analysed using Non-Metric  
172 Dimensional Scaling (NMDS). The distance between community compositions was computed  
173 with Bray-Curtis distance. The community composition was well represented in the two-  
174 dimensional space, with a stress value below 0.2 (Dexter et al. 2018). To explore how much  
175 the community composition was driven by the environment, we fitted environmental variables  
176 to the ordination (envfit function in the vegan package). The significance of the fitted  
177 environmental variables was assessed through 999 permutations of the community  
178 coordinates.

179 Species richness was modelled as a function of latitude, longitude, altitude, distance from  
180 spring, and substrate type using a linear model with a Poisson error. We found significant  
181 spatial autocorrelation in species richness (Moran test:  $I=0.19$ ,  $P=0.005$ , **Online Appendix**

182 **3E**). We then modelled spatial correlation with an exponential correlation structure using the  
183 `glmmPQL` function in the `MASS` package. The validity of the model was assessed with the  
184 visual inspection of the residuals. We also checked for the absence of multicollinearity in the  
185 predictor variables with the Generalised Variance Inflation Factor which were less than two  
186 (Online Appendix 3C).

187 In order to identify the assembly rules that drive mussel's community composition, we  
188 computed species co-occurrence. The expected co-occurrence between species pairs was  
189 compared with the observed co-occurrence. Expected co-occurrence between species pairs is  
190 simply the product of the probability of occurrence of the two species multiplied by the total  
191 number of sites (Griffith et al. 2016). The significance of the species co-occurrence was  
192 assessed using the probabilistic model of Veech (2013), with  $\alpha=0.1$ . Of the 780 species pair  
193 combinations, 247 pairs (31.67 %) were removed because they had an expected cooccurrence  
194 below 1. Analyses of species co-occurrences were performed using the `cooccur` R package  
195 (Griffith et al. 2016).

196 All the analyses were performed in R v3.6.3 (R Core Team, 2020) and the scripts used for the  
197 analysis are available online at [https://github.com/alaindanet/prie\\_mussels](https://github.com/alaindanet/prie_mussels).

198

199

## 200 **3 RESULTS**

### 201 **3.1 Detection probabilities and seasonal variations**

202 According to the model used here, species detection probabilities varied from 0.37 to 0.93  
203 with only one replicate, from 0.60 to 0.99 with two replicates, and reached 0.84 to 1 with four  
204 replicates (**Figure 2**). Sphaerids generally had the lowest detection probabilities, and  
205 introduced and invasive species generally had the highest. Two species of conservation  
206 interest, the Giant freshwater pearl mussel and the Depressed river mussel, have lower, also  
207 acceptable, detection probabilities with two replicates, 0.86 and 0.77 respectively.

208 One-year of monitoring was conducted in the Orb River (Online Appendix 1) to test for  
209 seasonal variation in species detection and eDNA yield, with the hypotheses that the amount  
210 of eDNA, and hence the species detection, would increase during reproduction periods, with  
211 gametogenesis, increased metabolic activity and juveniles release and mortality. According to  
212 our results, there is no significant seasonal variation in eDNA yield as measured by the  
213 number of reads. The breeding season does not seem to influence eDNA detection. Rather, we  
214 observed a discrepancy in the species richness depending on water flow conditions: most  
215 species were detected just after a flood, suggesting that strong currents may improve detection  
216 distance. Species richness varied from 4 to 11, depending on the environmental conditions.

### 217 **3.2 Biodiversity and distribution patterns**

218 Each sampled site yielded an average of 8.1 species, ranging from 0 to 24 species. Half of the  
219 sites had between 5 and 11 species. Only three sites had no freshwater mussel at all, all of  
220 which were stagnant waters in small and potentially anoxic ponds. Sites with only one species  
221 were either in ponds, or close to the sea (and therefore possibly brackish), or high up in the  
222 catchment. On the other hand, the richest sites (15 species or more) were always in large  
223 rivers (Online Appendix 3A). The distribution patterns observed with eDNA were globally

224 consistent with the current knowledge of species distribution. However, four invasive species  
225 were found outside their known range, indicating an unnoticed but ongoing colonisation  
226 process (eg. Fig. 3A). Three sphaerid species were also discovered outside their known range,  
227 two boreal relict species with restricted distributions in France, the Globular peaclam *E.*  
228 *globularis*, the Lilljeborg's peaclam *E. lilljeborgi*, and the Iridescent peaclam *E. pulchella*,  
229 which was found to be widespread, albeit rare (Online Appendix 2). Eventually, the Thick-  
230 shelled river mussel, a Habitat Directive species protected in France, was found in places  
231 where it had not been noticed before, except from very few old data (Fig. 3B). On the other  
232 hand, some species were found in a surprisingly small number of localities, although they are  
233 currently thought to be widespread and not of conservation concern (Fig. 3C-D), suggesting  
234 an unnoticed but drastic decline of their conservation status.

### 235 **3.2.1 What are the main drivers of species richness?**

236 The variables latitude, longitude, substrate, and distance to the spring were significantly  
237 associated with the ordination axis, but not altitude (Fig. 4). Latitude and longitude were the  
238 variables the most strongly associated with community composition ( $r^2=0.24$  and  $r^2=0.1$   
239 respectively, Online Appendix 3C). We found that species richness increased by 0.062 log-  
240 units (0.017 se) per degree of latitude and increased by  $4 \times 10^{-7}$  log-units ( $2 \times 10^{-7}$  se) per meter  
241 of distance from the spring (Fig. 4). Species richness increased from 7.5 in the south to 12.5  
242 in the north of France, and from 9 near the springs to 12 at 800 km from the springs.  
243 However, longitude and altitude had no significant effect on species richness. We also found  
244 that species richness was higher in calcareous and mixed substrates (9.5 species) than in  
245 acidic substrates (7.25 species; the effects were of 0.26 log-units, 0.12 se and 0.27 log-units,  
246 0.13 se, respectively).

247

### 248 **3.3 Species co-occurrence patterns**

249 Of the 533 species pairs analysed, 260 pairs were classified as random, 206 as positive, and  
250 only 67 as negative (Fig. 5). The invasive Chinese pond mussel *Sinanodonta woodiana* (Lea,  
251 1834) had the highest number of significant negative co-occurrences with the other species.  
252 Henslow's peaclam *E. henslowana* (Sheppard, 1813), Horny orb mussel *S. corneum*  
253 (Linnaeus, 1758) and Hump-backed peaclam *E. supina* (A. Schmidt, 1851) had the highest  
254 number of significant positive co-occurrences with the other species (Fig. 5).

255

## 256 **4 DISCUSSION**

257 Our sampling protocols and methods have previously been challenged by the global  
258 distribution of freshwater bivalve species and one-off surveys (Prié et al. 2021). The results  
259 presented here confirm their reliability in terms of detection probability. Because detection  
260 probability was high, and therefore eDNA data sufficiently reliable, we were able to redraw  
261 the species distribution maps, re-evaluate their conservation status and investigate community  
262 patterns and their determinants.

### 263 **4.1 Species detection probabilities and implication for sampling protocols**

264 The sampling protocol used here was based on Pont et al. (2018) for fish surveys, assuming  
265 that two replicates were sufficient to detect 96.9% of the fish species in the Rhône River.  
266 However, the analogy with freshwater mussels has never been tested. Here, we used the  
267 freshwater mussel eDNA data collected with two replicates to test for the accuracy of the  
268 protocol to detect each species. The results (Fig. 2) show that the two replicates were  
269 acceptable for detecting some species (i.e., the probability of detection was higher than 95%  
270 with two replicates), but not all. For example, the threatened and much studied Freshwater  
271 pearl mussel *M. margaritifera* (Linnaeus, 1758) is detected with a 95% probability with two

272 replicates, whereas the highly endangered Giant freshwater pearl mussel *P. auricularius*  
273 would require at least three replicates for a satisfactory detection probability.  
274 Unfortunately, the Giant freshwater pearl mussel and the Depressed river mussel, which are  
275 among the most endangered species in France (UICN et al., 2021), appear to have a low  
276 detection probability. For studies focusing on these species, if our standard protocol is used,  
277 we would recommend using at least three replicates to limit the risk of false negatives.  
278 However, this has to be balanced with financial issues.

279 All but one of the invasive species (the Ridgedbeak peaclam *E. compressa* (Prime, 1852), the  
280 only invasive Sphaerid species) have a very good detection rate. This is probably due to their  
281 high abundance in the environment once they are there. Conversely, almost all sphaerids had  
282 a detection probability of less than 90% with two replicates. This can be explained by their  
283 smaller size, but also by the influence of the widespread and abundant *Corbicula* sp. on the  
284 analyses. The most abundant taxa (e.g. *Corbicula*) may out sequence rarer specimens  
285 (Sphaerids).

286 Our two-replicate protocol is good but not optimal, as some species suffer from a lower  
287 probability of detection (60% at worst). However, the detection probability is never 100%.  
288 Although not perfect, our protocol is still better for species detection than traditional methods  
289 (Prié et al. 2021). We can therefore rely on our results to assess species distribution, and  
290 community composition.

### 291 **4.3 Distribution of species of special interest**

292 Our results are largely consistent with the known distribution of the French freshwater bivalve  
293 species (Prié 2017). However, some species were detected outside of their known distribution  
294 range, including introduced and invasive species. Others were not detected where they were  
295 expected, and valuable data were collected on poorly known species.

296 **4.3.1 Out-of-range species**

297 For species of conservation concern, such as the Thick-shelled river mussel *U. crassus*  
298 (categorised EN by the IUCN and listed in the European habitat directive annex II), eDNA  
299 has provided important data. The Thick-shelled River Mussel is widespread in the basins of  
300 the Loire, Saône, Seine, and the north-eastern half of France as shown in Fig. 3B. Although  
301 its populations have largely declined over the last century (Lopes-Lima et al. 2014), there are  
302 many recent records of living populations throughout its range. On the other hand, the species  
303 was not known south of the Loire basin area on the Atlantic coast (except for a few specimens  
304 in museum collections with doubtful vouchers, unpublished data). Historical records exist  
305 downstream of the Rhône basin, but these have not been updated despite intensive research in  
306 the Camargue wetlands and Ardèche River. Data collected by eDNA analysis confirmed the  
307 presence of the species throughout the north-eastern quarter of its range and revealed its  
308 presence (i) upstream of the Dordogne and Charente basin, south of the Loire basin, where  
309 field surveys confirmed its presence by the observation of a few live individuals in 2018 and  
310 2019, and (ii) in several sectors of the Rhône River mainstream, particularly downstream,  
311 where it was not known from recent data.

312 The Rhône River is one of the largest rivers in Europe. The lower Rhône, not surprisingly,  
313 remains a *terra incognita* for malacology. It is a large, fast-flowing, deep, turbid, navigable  
314 river that is very difficult to sample. Neither scuba-diving nor extensive dredging has been  
315 undertaken to study freshwater mussels. The lower Rhône flows in the Mediterranean region,  
316 and its valley forms a northward extension of the northwestern Mediterranean subregion (e.g.  
317 Darwall et al. 2014). Its tributaries in the lower right bank are typically Mediterranean, while  
318 those on the left bank are more alpine. Hence its tributaries, which are easier to sample, are  
319 very different ecosystems from the Rhône itself. While the Rhône valley allows a northward

320 extension of the Mediterranean biome, the Rhône itself provides a southward path for species  
321 with more northern affinities such as the Thick-shelled river mussel and the Swollen river  
322 mussel. These new data could justify the implementation of conservation measures such as  
323 the extension of the Natura 2000 sites FR8201677 “Alluvial environment of the Rhône” or  
324 FR8201749 “Alluvial and aquatic environments of the Ile de la Platière” to include the Thick-  
325 shelled River Mussel, which is in Annex II of the Habitat Directive.

326 Species of the genus *Anodonta* are sometimes difficult to identify and are likely to be  
327 misidentified in databases. Because these species are widespread and common, databases  
328 validation processes tend to ignore these potential misidentifications. In many cases, a  
329 barcoding approach is needed to clarify the taxonomic status of *Anodonta* populations. As an  
330 example, the Italian swan mussel *A. exulcerata* was recently redescribed in Italy based on  
331 molecular data (Froufe et al. 2017). There are no reliable diagnostic morphological characters  
332 to identify it in the field (Riccardi et al. 2019). It was thought to be endemic to Italy and  
333 Switzerland. In this study, eDNA sequence fragments attributable to this species were  
334 detected at a few sites in the upper Loire River. The Italian Swan Mussel appear to be a new  
335 species for France, possibly introduced from Italy. It would certainly have been overlooked  
336 without the eDNA detection.

#### 337 **4.3.2 Invasive species**

338 The Chinese pond mussel *S. woodiana* is a large species that was introduced to France in  
339 2007 and has spread rapidly in the southern France in the Rhone basin (Adam, 2010), and is  
340 currently colonising the Loire, Garonne and Seine basins (Prié in press.). The data collected  
341 by eDNA analysis confirm the expansion of this easily identifiable species in the field and  
342 add some previously unknown localities. These localities include the downstream sections of  
343 the Tarn, confirming the rapid colonisation of the Garonne basin, the Sèvre niortaise, from

344 where it has since been observed using traditional methods, the Saône, where it had not been  
345 detected during the freshwater bivalves surveys carried out by scuba-diving in 2016, and the  
346 Moselle. Conversely, eDNA analyses show that it is probably still absent from the Meuse and  
347 Dordogne basins, where the eDNA monitoring pressure is high, and apparently from the  
348 Adour, Seine, and Charente basins. For all these basins, it would be urgent to implement  
349 protection measures to avoid the colonisation of the Chinese pond mussel. Indeed, it is now  
350 known that this species represents a significant threat to native species (Donrovich et al. 2017;  
351 Huber and Geist 2019). Our results show that this species has the highest number of  
352 significant negative co-occurrences with the other species, which could be the result of its  
353 negative impact on the other species. A closer look at Fig. 5 shows that the negative  
354 correlations are only for Sphaerids (which are detected by a different set of primers, thus  
355 ruling out the hypothesis that too much invasive species DNA might mask the detection of  
356 native species). This invasive species may represent a serious threat for Sphaerids that was  
357 not previously known.

358 The Quagga mussel *Dreissena rostriformis bugensis* (Andrusov, 1897) is a smaller species,  
359 similar in shell shape to the widespread Zebra mussel *Dreissena polymorpha* (Pallas, 1771),  
360 and therefore likely to go unnoticed due to confusion or negligence. It has only been known in  
361 France since 2010 when it was first detected in the Meuse and Moselle basins (Bij de Vaate  
362 and Beisel 2011). According to previously available data shown in Fig. 3A, it was thought to  
363 have colonised only the Meuse and Moselle Rivers, with scattered data in the Rhine and  
364 Rhône Rivers (Prié and Fruget 2017). The eDNA data show that the species has already  
365 spread considerably and has also been introduced into the Mediterranean coastal rivers (Lez  
366 River in Montpellier, probably via the Bas-Rhône-Languedoc canal), and into the Seine basin  
367 (probably via the Canal de Bourgogne), where it had never been observed by traditional

368 surveys. None of our samples from the Atlantic drainages contained Quagga mussel eDNA.  
369 These results highlight the value of eDNA for early detection and monitoring of invasive  
370 species.

371 The Ridgedbeak peaclam *E. compressa* has only recently been formally discovered in France  
372 (Mouthon and Forcellini, 2017), although the first data on its distribution in France date back  
373 to 1989 (Mouthon and Tair Abacci, 2012). Analysis of the eDNA has extended its current  
374 range, providing new records for the Meuse, upstream of the Seine, the Saône, the Ardèche,  
375 and the entire lower Rhône River.

#### 376 **4.3.3 Rare and threatened species**

377 Large-scale sampling in France revealed the worrying conservation status of some species  
378 that are not currently considered to be endangered. It must be acknowledged that the  
379 probability of detection is lower for two endangered species, the Giant Freshwater Pearl  
380 Mussel and the Depressed River Mussel. However, the Depressed River Mussel historically  
381 had a wide distribution in France, as evidenced by museum collections (notably those of the  
382 National Museum of Natural History in Paris, unpublished data), where three subspecies were  
383 recognised (Bouchet, 2002). Unfortunately, this wide historical distribution is not well  
384 documented. To date, only about one hundred records are available for France (Fig.3C), of  
385 which only 46 are recent observations of living individuals. These data confirm the presence  
386 of the species in the Seine (about 15 sites), the Somme (one site), the Meuse (two sites), the  
387 Moselle (one site), and the Rhine (one site) basins. There are no recent records of live  
388 specimens elsewhere in France. Environmental DNA data confirm the current presence of the  
389 species in the Seine, the Meuse (9 sites out of the 52 sampled), the Moselle (only one site  
390 sampled), but also in the Saône (one site out of the two studied), the lower Rhône (only one  
391 site out of the 65 sampled) and the Charente (only one site out of the 7 analysed) rivers.

392 Depressed River Mussel eDNA was not detected in any of the sites sampled in potentially  
393 favourable locations (downstream ecosystems on limestone substrate) in the Garonne basin.  
394 Similarly, although a few suitable sites were studied on the Loire basin, none detected the  
395 species. The Depressed river mussel appear to have been extirpated from most of its range in  
396 France. The only record on the lower Rhône is remarkable, as no historical data are known for  
397 this sector.

398 The Witham orb mussel *S. solidum* is a typical downstream species, living in large rivers, at  
399 depth, in well-oxygenated water, preferably on sandy substrates. This type of environment  
400 was sampled extensively in our study (an estimated 250 sampling sites suitable for the  
401 Witham orb mussel), but the Witham orb mussel was only detected once in our samples, near  
402 one of the three sites where it was last observed alive. Extensive data are available because of  
403 its easily identifiable thick shell, which can be preserved in riverbanks for years, as shown in  
404 Fig. 5D. But this data, based mainly on shell observations, probably give an outdated picture  
405 of reality. According to the eDNA results, the Witham orb mussel appears to have been  
406 extirpated from the Aisne site by now (where sampling took place about 10 years after it was  
407 observed). This would leave only a single population in the lower Seine, making the Witham  
408 orb mussel the most threatened freshwater bivalve in France.

409 The Nut orb mussel *S. rivicola* is also a species characteristic of the lower reaches of large  
410 rivers and is probably less demanding in terms of substrate than the Witham orb mussel,  
411 preferring more muddy sediments. It also appears to be less threatened, but from our eDNA  
412 data, its situation is alarming. About a hundred records are available, spread over most of the  
413 major French catchments. However, very few living populations are currently known. At least  
414 one population appears to subsist downstream of the Loire, in a sector that we did not sample  
415 in the framework of this work. Its eDNA was only detected at only one site in the Rhône

416 basin (out of 65 sampled sites) suggesting that Nut Orb Mussels still occur there, but probably  
417 in a very poor conservation status, as they are not known elsewhere in the basin. Live  
418 individuals have recently been collected from the Meuse River and the eDNA data confirmed  
419 this. Finally, there are several recent records of living individuals in the Seine basin, but the  
420 eDNA sampling revealed the species only once in the Seine basin, close to a previously  
421 reported living population.

422 The False orb pea mussel *E. pseudosphaerium* is a lowland river species that lives in stable  
423 swampy areas, in clear and clean waters and is intolerant of pollution and water level  
424 fluctuations (Welter-Schultes 2012). This type of habitat has become very rare in France, and  
425 so has the False orb pea mussel. Welter-Schultes (2012) gives it a wide distribution range that  
426 covers most of France, although it is rare and localised everywhere. Mouthon and Kuiper  
427 (1987) recorded it from only four sites in the Rhone Valley and report an additional historical  
428 record near Toulouse (south-west France). Despite extensive sampling, we have not detected  
429 its eDNA in the Rhone Valley. It has only been detected by eDNA analyses in two occasions,  
430 one in a small tributary of the Charente River and the other in Brittany (north-west France).  
431 Although suitable habitats for this species are under-sampled, the eDNA data confirm the  
432 worrying conservation status of the False Orb Pea Mussel.

433

#### 434 **4.2 Biodiversity distribution patterns**

435 The main drivers of species richness in France were latitude and longitude. Indeed, most of  
436 the diversity and abundance of freshwater mussels is found in the north-east of France.

437 The longitude effect decreases towards the east of France, which can be explained by the  
438 Rhône River, which acts as a corridor for more continental and northern species such as the  
439 Thick-shelled river mussel *U. crassus*, the Swollen river mussel *U. tumidus* Philipsson, 1788

440 or the River orb mussel *S. rivicola*, species restricted to the northern half of France. This  
441 pattern of species richness distribution can be explained by the post-glacial recolonisation  
442 routes. It has been hypothesised that glacial refugia for European freshwater bivalves were  
443 probably in the Balkans, south-eastern of Europe and the Iberian Peninsula (Lopes-Lima et al.  
444 2021). France is isolated from the Iberian Peninsula by the sea and the Pyrenees mountains. It  
445 is also isolated from the Balkans by the sea and the Alps. Therefore, the most likely  
446 colonisation route is through the north-east of France, along the coast and/or along the  
447 Danube, Rhine, and Rhône rivers connections. An ongoing post-glacial colonisation process  
448 could explain the lower richness and diversity in southern and western France.  
449 Not surprisingly, the distance to the spring, a proxy for river size and the trophic condition  
450 was also an important driver of species richness. Indeed, upstream ecosystems are home to a  
451 few specialists, such as *M. margaritifera*, or ubiquitous species, such as the Caserta peaclam  
452 *E. casertana* (Poli, 1791), but most species are mainly, if not exclusively, found in  
453 downstream ecosystems and lowland rivers.  
454 The correlation with altitude was not significant, although we know that bivalves are rare in  
455 mountain rivers (due to torrential regimes and oligotrophic conditions). This may be due to  
456 the limited number of sampling sites at high altitudes, as 90% of the samples were taken in  
457 lowland rivers at altitudes between sea level and 700 m (Fig. 4E).

458

#### 459 **4.4 Standardized eDNA sampling at a large scale**

460 For the first time, we have implemented eDNA surveys at a large scale using standardised  
461 sampling protocols and analyses. The results of each sample can be compared in both space  
462 and time: it is now possible to reproduce our sampling in the future to monitor the evolution  
463 of bivalve populations. This is particularly important for monitoring invasive species: eDNA

464 analyses are usually used to detect the presence of a given species. However, for invasive  
465 species with a very high probability of detection (Fig. 2), the absence of detection can be a  
466 strong argument for considering a species to be absent at a given time. Our large-scale eDNA  
467 sampling revealed the occurrence of invasive species in their previously known range and in  
468 places where they were expected to colonise, given the potential colonisation routes (i.e.  
469 mainly artificial channels connecting main watersheds, Prié, in press.). Conversely, eDNA  
470 samples corroborates the absence of invasive species in places where they were not (yet)  
471 expected. Regular re-sampling of these sites could allow the documentation of colonisation.  
472 Our results also confirm that some introduced species are not invasive. For example, the Long  
473 fingernail clam *S. transversum* (Say, 1829) has only been recorded twice in France using  
474 traditional methods, and eDNA has only added one isolated record.  
475 On the other hand, large-scale eDNA sampling was valuable in detecting rare and threatened  
476 species. The False Orb pea mussel was detected at only two sites in France, confirming its  
477 critical conservation status. For the very well-known Giant freshwater pearl mussel (Prié et  
478 al., 2018), only one new site could be added by our eDNA samples. Furthermore, some  
479 species such as the Depressed river mussel, the Nut orb mussel, and especially the Witham  
480 orb mussel were rarely detected despite their presumed wide distribution in France, raising  
481 red flags about their conservation status.

482

#### 483 **4.5 Key results and limits**

484 Provided that optimised methods are used, we have shown that eDNA analyses are more  
485 efficient and less costly than traditional surveys (Prié et al., 2021). Admittedly, the method  
486 provides imprecise results (unknown and probably variable detection distance between sites,  
487 lack of information on the state of the detected species populations, etc.). However, this

488 degree of imprecision is negligible for the study of aquatic fauna on a large scale. The  
489 multiplication of sampling sites tends to eliminate the biases associated with the risk of false  
490 negatives.

491 Our results show that the use of eDNA surveys on a large scale is a valuable tool for the  
492 accurate mapping of freshwater biodiversity, for example here at the scale of a whole country  
493 (France is the largest country in Europe). The sampling effort can be deployed in a limited  
494 time frame (here five years was sufficient to cover the whole country and its different  
495 ecoregions and habitats), taking advantage of the multiple actors that can contribute to the  
496 sampling and funding. Here, most of the sampling and funding was provided by NGOs,  
497 wildlife managers, environmental consultancies, developers and their impact studies (see  
498 Acknowledgements). Because the method is standardised, all the results can be easily collated  
499 at a national level.

500 Our eDNA atlas has the advantage of being based on live mussel populations, whereas most  
501 surveys pool live and shell-only data, leading to optimistic mapping and consequently  
502 optimistic views of population trends. Here we show that species not considered to be of  
503 conservation concern are in fact endangered and, *a contrario*, that species of conservation  
504 concern are more widespread than previously thought. We are also addressing the threats to  
505 native bivalves by measuring the progress of invasive species.

506 This paradigm shift has been very fruitful for the establishment of the red list of continental  
507 molluscs of France (UICN et al., 2021). As a result, much more attention is now being paid to  
508 threatened species that had previously been overlooked, such as the Depressed river mussel *P.*  
509 *complanata* (EN), the Witham orb mussel *S. solidum* (EN), the River orb mussel *S. rivicola*  
510 (EN) and the False orb peaclam *E. pseudosphaerium* (EN).

511 Our results are still potentially biased by the uncertainties of molecular taxonomy. While most  
512 taxonomic studies are based on a limited number of specimens, eDNA captures the whole  
513 genetic diversity of the sampled populations, which is mostly undescribed. Our identifications  
514 are based on a few reference sequences, and then on the similarity of the eDNA haplotypes  
515 we collect with those reference sequences. But some of the haplotypes amplified by our  
516 primers may be shared between different species. For example, the Iridescent peaclam is  
517 known from only one 16S sequence, which is closely related to that of the Short-ended  
518 peaclam *E. subtruncata* (Malm, 1855) (which has seven known 16S haplotypes of the short  
519 fragment we use). If the haplotype referenced as *E. pulchella* in our reference library was also  
520 a rare haplotype of *E. subtruncata*, the distribution given here for *E. pulchella* could be  
521 strongly biased. What we present here is the distribution of haplotypes.

522

## 523 **5 Conclusion**

524 The large-scale use of eDNA analysis challenges the current knowledge of freshwater bivalve  
525 distributions and highlight unnoticed rarefactions. Our results are promising for monitoring,  
526 particularly for documenting changes in distribution: e.g. early detection of invasive species  
527 and decline of threatened species. It also provides information on poorly known ecological  
528 parameters such as species co-occurrence, species richness, and biogeography. Environmental  
529 DNA surveys remain dependent on detection probabilities, which were examined here with  
530 respect to the protocol, but which depend on many more parameters including distance and  
531 transport (Deiner et al., 2014; Shogren et al., 2019), flow (Pont et al., 2018), humic substances  
532 and sediment (Stoeckle et al., 2017). Contrary to other studies (e.g. Milhau et al., 2019), our  
533 results do not support variation in detection probability with seasonality and breeding period  
534 for freshwater bivalves.

535 Conservation efforts are often unfocused and biased towards charismatic species and  
536 ecosystems (Mammola et al., 2020). Here, we highlight conservation priorities for under-  
537 recognised threatened species, but also for neglected ecosystems. While conservation efforts  
538 focus on upstream ecosystems (pristine rivers with Freshwater Pearl Mussels), eDNA data  
539 show that downstream ecosystems are the most species-rich. They are also the most  
540 threatened, facing diffuse and cumulative threats from upstream, and are located in the most  
541 human-influenced areas (populated areas, agricultural plains, industrial effluents...). Our  
542 eDNA results highlight particularly important conservation issues for species downstream of  
543 major rivers. In a context of biodiversity erosion and declining population sizes, it seems  
544 essential to implement consistent conservation policies for downstream species, which are  
545 currently insufficiently considered by naturalists and managers alike due to the difficulty of  
546 access to these environments.

547 Using optimized and standardised methods, it is now possible to reliably document both the  
548 spread of invasive species and range contractions of endangered species. Large-scale and  
549 long-term eDNA metabarcoding surveys are a valuable tool for conservation and monitoring  
550 of freshwater bivalves. For conservation issues, large-scale, long-term data are needed to  
551 derive information based on detection patterns. However, regarding the poorly known species,  
552 it should be borne in mind that what we present here is 16S haplotypes distribution. They may  
553 not reflect the true distribution of species as further studies may compromise the current state-  
554 of-the-art of molecular taxonomy. We give here only an eDNA point of view on the  
555 conservation status of freshwater bivalves.

556  
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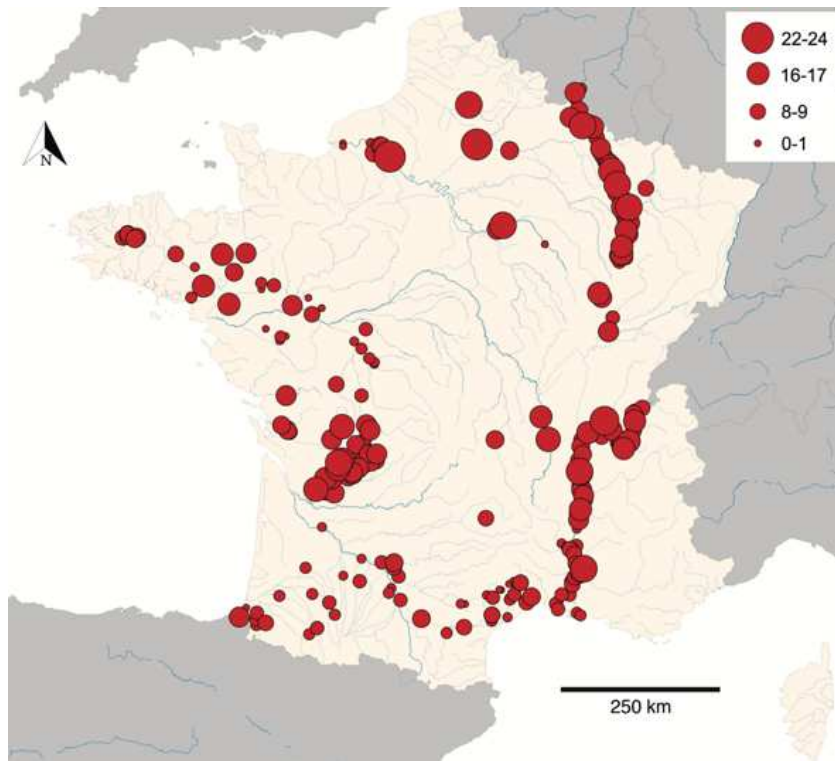
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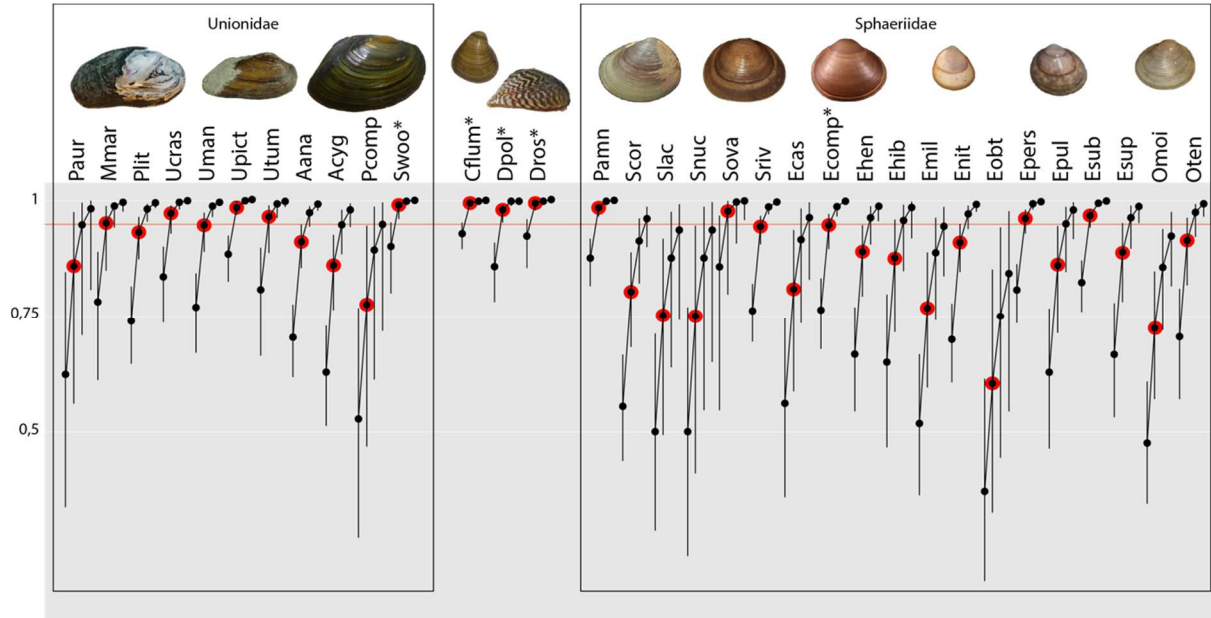
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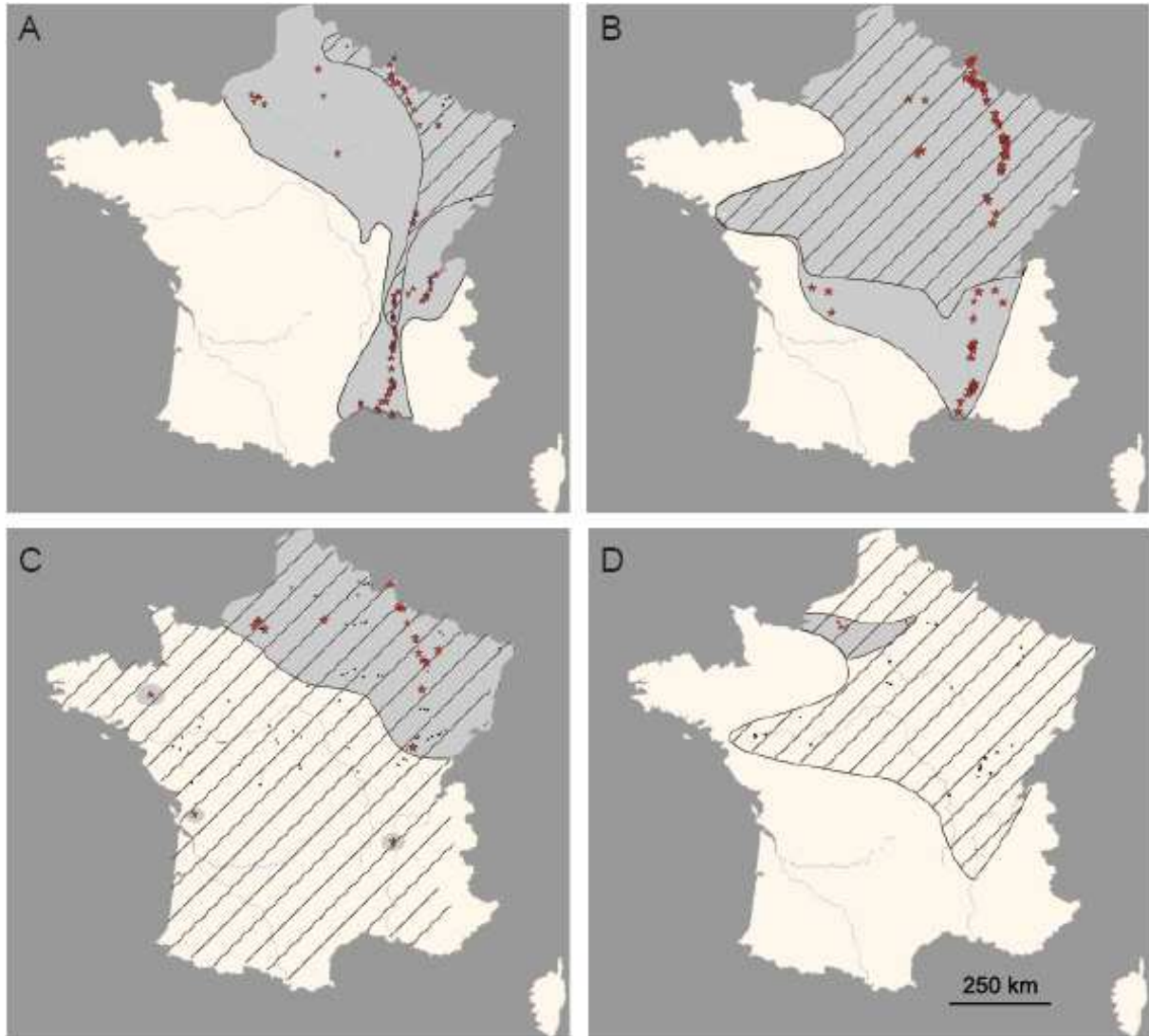


727  
728 **FIGURE 1:** Sampling and species' richness in France. The size of the dots corresponds to the  
729 number of species.  
730

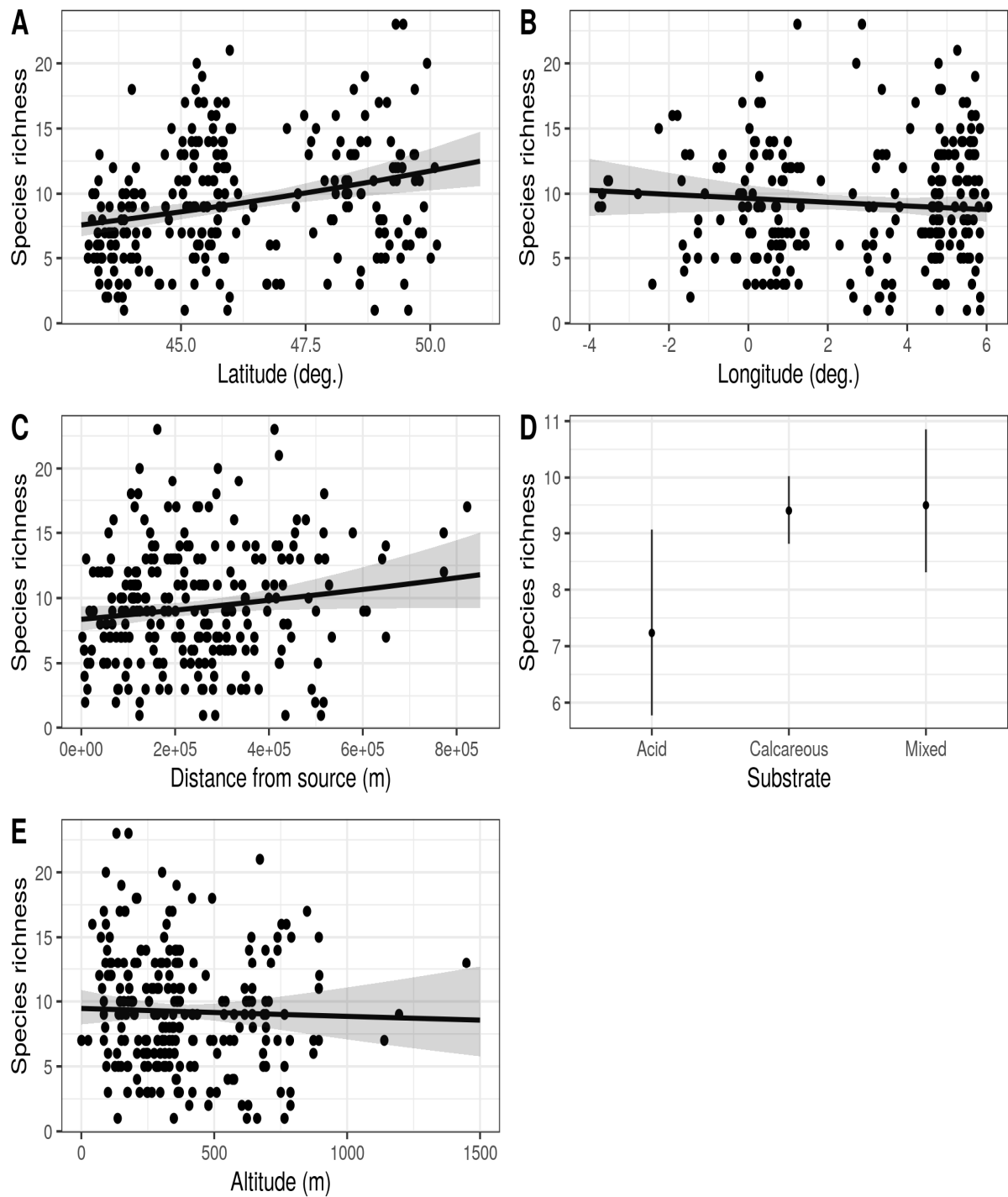
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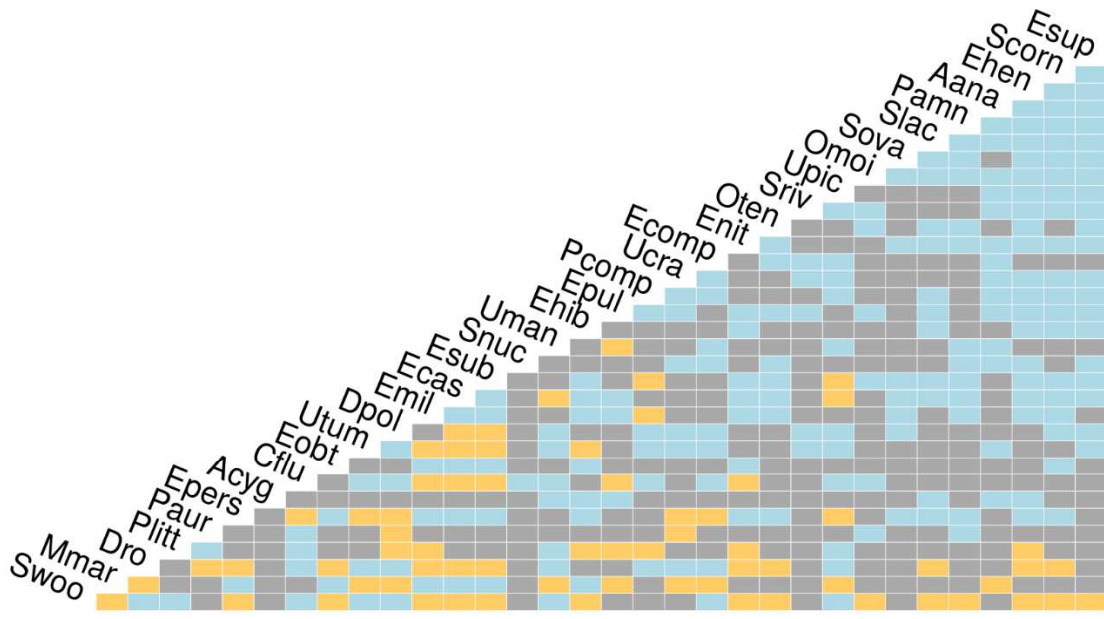
732  
733 **FIGURE 2:** Detection probabilities per species depending on one, two, three or four  
734 replicates, based on presence/absence data in the first two replicates. Red line: 95%  
735 confidence. Red dots: probability with two replicates (this study). \* Invasive species.



736  
 737 **FIGURE 3:** Examples of expanded (A: Quagga mussel *D. r. bugensis*, B: Thick-shelled river  
 738 mussel *U. crassus*) and contracted (C: Depressed river mussel *P. complanata*, D: Witham Orb  
 739 mussel *S. solidum*) distributions inferred from our eDNA samples. Black dots: available data;  
 740 red stars: eDNA data; hatched area: presumed distribution prior to eDNA sampling; shaded  
 741 area: actual distribution range inferred from eDNA samples. Data for absence of  
 742 environmental DNA not shown here, see Supplementary Information for more detailed maps.



743  
 744 **FIGURE 4:** Relationships between species richness and environmental variables, namely (A)  
 745 latitude, (B) longitude, (C) distance from spring, (D) substrate type and (E) elevation. Lines  
 746 and bands represent the mean prediction and its 95% confidence interval, respectively.



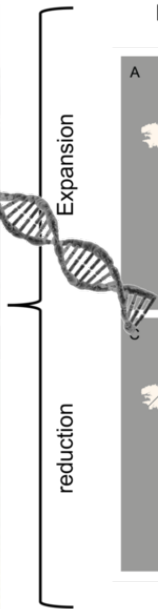
negative
  random
  positive

747

**FIGURE 5:** Co-occurrence of species pairs. 247 pairs (32 %) were removed because they had an expected co-occurrence less than 1.

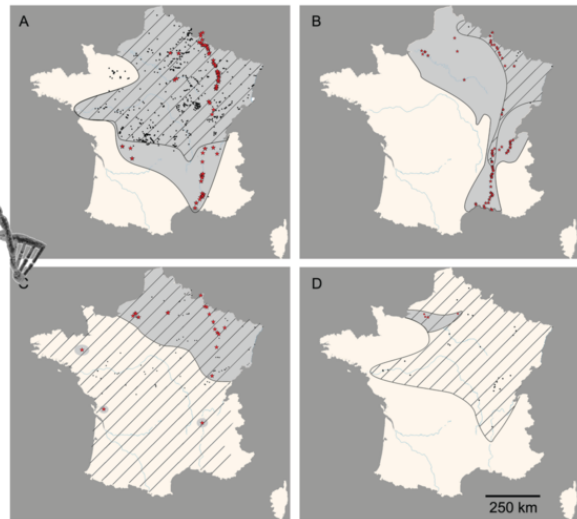
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EX	Extinct
EW	Extinct in the Wild
CR	Critically Endangered
EN	Endangered
VU	Vulnerable
NT	Near Threatened
LC	Least Concern
DD	Data Deficient
NE	Not Evaluated



Protected species

Invasive species



Neglected: overlooked decline