1  **Title:**

2  Mapping differences in mammalian distributions and diversity using environmental

3  DNA from rivers

4

5  **Running title:** Mapping mammals using eDNA

6

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26    **Abstract**

27

28    **Aim:** Finding more efficient ways to monitor, and estimate the diversity of,
29    mammalian communities is a major step towards their management and
30    conservation. Environmental DNA (eDNA) from river water has recently been shown
31    to be a viable method for biomonitoring mammalian communities. Yet, most of the
32    studies to date have focused on the potential for eDNA to detect individual species,
33    with little focus on describing patterns of community diversity and structure. In this
34    study, we focus on the sampling effort required to reliably map the diversity and
35    distribution of semi-aquatic and terrestrial mammals and allow inferences of
36    community structure surrounding rivers.

37

38    **Location:** Southeastern England

39

40    **Methods:** We used eDNA metabarcoding on water samples collected along two
41    rivers and a beaver enclosure over two days, targeting terrestrial and semi-aquatic
42    mammals. Mammalian community diversity and composition was assessed based
43    on species richness and β-diversity. Differences between river communities were
44    calculated and partitioned into nestedness and turnover, and the sampling effort
45    required to rapidly detect semi-aquatic and terrestrial species was evaluated based
46    on species accumulation curves and occupancy modelling.

47

48    **Results:** eDNA metabarcoding efficiently detected 25 wild mammal species from
49    five orders in two days of sampling, representing the vast majority (82%) of the
50    species expected in the area. The required sampling effort varied between orders,
51    with common species (generally rodents, deer and lagomorph species) more readily
52    detected, with carnivores detected less frequently. Measures of species richness
53    differed between rivers (both overall and within each mammalian order) and patterns
54    of β-diversity revealed the importance of species replacement in sites within each
55    river, against a pattern of species loss between the two rivers.

56

57    **Main conclusions:**
58    eDNA metabarcoding demonstrated its capability to rapidly detect mammal species,
59    allowing inferences of community composition that will better inform future sampling

60    strategies for this Class. Importantly, this study highlights the potential use of eDNA

61    data for investigating mammalian community dynamics over different spatial scales.

62

63    **Keywords:**

64    Community; Mammals; Metabarcoding; Occupancy modelling; Semi-aquatic;

65    Terrestrial

## 1. Introduction

Mammalian populations have suffered significant declines globally, with one in four species believed to be threatened (defined as critically endangered, endangered or vulnerable; IUCN, 2021). Information on species' distributions is therefore critical to support effective evidence-based management (Mathews et al., 2018). However, undertaking surveys to capture a broad range of mammals within a particular area or region can be logistically challenging in terms of effort, cost and time (Garden et al., 2007). This is especially evident for species that are difficult to visually encounter or which occur at low densities. Mammals are traditionally surveyed using a variety of methods including camera trapping, live-trapping and/or field sign surveys (Sales, McKenzie et al., 2020) with the accuracy of these methods heavily reliant on the intensity of sampling efforts and the susceptibility of species and individuals to capture/detection by each method. Each method may have additional concerns or limitations, such as ethical considerations in live-trapping (Sikes et al., 2016), surveyor expertise in correctly identifying field signs (Harrington et al., 2010) and camera trap placement (Littlewood et al., 2021; Kaizer et al., 2021). Given the wide variety of ecologies exhibited within mammals, there is clearly no 'one size fits all' method for monitoring either the entire or a significant component of the overall mammalian community.

The emergence of environmental DNA (eDNA) as both a viable and reliable method for biomonitoring is rapidly transforming how species and community-wide surveys are undertaken (Deiner et al., 2017; Fediajevaite et al., 2021). eDNA is any genetic material that has been shed into the environment by macro-organisms through sloughed skin cells, blood, faeces/urine and saliva with no obvious signs of biological source material (Pawlowski, Apotheloz-Perret-Gentil & Altermatt, 2020). When eDNA is combined with next-generation sequencing (NGS) technology via DNA metabarcoding with universal primers, it has the potential to facilitate rapid biodiversity assessments in diverse and complex ecosystems as it can identify multiple species simultaneously from one environmental sample (Deiner et al., 2017). Since water has been shown to be a reliable source of eDNA (Deiner et al., 2017), most eDNA metabarcoding applications to date on vertebrates have been focused on monitoring fishes and amphibians (e.g. McDevitt et al., 2019; Valentini et al., 2016). However, recent studies have demonstrated that eDNA retrieved from

100 water from both lotic (Sales, McKenzie et al., 2020; Sales, Kaizer et al., 2020;

101 Lozano & Caballero, 2021; Macher et al., 2021; Mariani et al., 2021) and lentic

102 (Ushio et al., 2017; Harper et al., 2019) systems can detect components of the

103 overall terrestrial and semi-aquatic mammalian community.

104

105 Mammals frequently come into contact with water through behaviours such as

106 drinking, bathing, foraging, urinating and defecating either in or near the water

107 system (Rodgers & Mock, 2015; Williams et al., 2018) and even mammalian species

108 that display limited interactions with water have been shown to be effectively

109 detected by eDNA (Williams et al., 2018; Sales, Kaizer et al., 2020). Comparisons

110 between eDNA and camera trapping surveys have revealed considerable overlap

111 between the methods in terms of the species detected (Sales, McKenzie et al., 2020;

112 Sales, Kaizer et al., 2020; Harper et al., 2019; Leempoel et al., 2020) or found

113 comparable detection probabilities from eDNA and field surveys (Sales, McKenzie et

114 al., 2020; Lugg et al., 2018). Most of the studies to date have focused on the

115 potential for eDNA to detect individual mammalian species (Ushio et al., 2017; Sales,

116 McKenzie et al., 2020), with little to no focus on describing patterns of community

117 diversity and structure. This is perhaps unsurprising given the infancy of eDNA in

118 terms of its application for monitoring/surveying mammals (Sales, McKenzie et al.,

119 2020; Sales, Kaizer et al., 2020).

120

121 Biodiversity estimates obtained from eDNA metabarcoding are known to be

122 influenced by sampling effort, with some taxonomic groups requiring greater spatio-

123 temporal coverage of eDNA sampling (e.g. carnivores; Harper et al., 2019; Leempoel

124 et al., 2020; Sales, McKenzie et al., 2020). Quantifying the differences between

125 mammalian communities is a major step in understanding the factors that shape

126 communities. The number of species in a local assemblage can help indicate the

127 health of a local ecosystem, with the presence of certain species, such as otters

128 (*Lutra lutra*; Esposito et al., 2020), providing critical insights into factors that affect

129 environmental health (e.g., pollution). Riverine ecosystems are among the most

130 dynamic habitats which support a rich diversity of species but are also exposed to

131 multiple threats including pollution, the spread of invasive species, habitat

132 fragmentation and degradation. Due to the connectivity of these freshwater systems,

133 these threats are easily transported and have profound effects on the distribution of

134     biodiversity (Collen et al., 2009; Dudgeon et al., 2019). As a result of rivers' roles as

135     'conveyor belts of biodiversity information' (Deiner et al., 2016), they represent

136     suitable sampling points for inferring the distribution of mammalian communities with

137     highly divergent functional adaptations (Sales, McKenzie et al., 2020).

138

139     To understand processes responsible for shaping community assembly, estimating

140     species richness and β-diversity (also referred to as inter-community structure) is of

141     paramount importance. β-diversity can be partitioned and used to determine if

142     communities are subsets of sites with higher species richness (nestedness), or if the

143     dissimilarity between sites is driven by species replacement (i.e., spatial turnover;

144     Baselga, 2010). In this context, an eDNA-based ecological assessment can

145     contribute to the identification of locations that require protection and direct future

146     conservation management (Socolar et al., 2016). The main aim of this study is to

147     explore the use of eDNA metabarcoding for assessing the distribution and diversity

148     of terrestrial and semi-aquatic mammals around two adjacent rivers. eDNA sampling

149     was conducted in transects along the Rivers Colne and Blackwater in Essex (Fig.

150     1A) where the Essex Wildlife Trust (EWT) have implemented species management

151     and habitat improvements, including both re-introduction and eradication

152     programmes for critically endangered and invasive mammals, respectively.

153     Additionally, eDNA sampling was conducted in and downstream of a nearby and

154     newly established beaver (*Castor fiber*) enclosure (Fig. 1B) to determine its

155     efficiency for detecting the focal species and surrounding mammalian community.

156     Aiming to expand upon the application of eDNA for monitoring mammalian

157     communities, our objectives are to determine the optimal sampling effort to (1)

158     adequately describe overall mammalian species diversity, and within each

159     mammalian order identified, from eDNA recovered from river water, and to (2)

160     quantify the differences among mammalian communities through analysing spatial

161     patterns of β-diversity within and between rivers.

162 **2. Methods**

163 **2.1 Field sampling**

164 Samples were collected in Essex, England along the Rivers Colne and Blackwater

165 (Figs. 1A and S1A). Five 500 ml water replicates were collected from 15 sites along

166 the River Colne and 10 sites along the River Blackwater on 29th–30th July 2019 at

167 roughly equal intervals within each river and accounting for access. In this area, the

168 EWT implemented the Essex Water Vole Recovery Project (EWVRP) in 2007,

169 focusing on reintroducing water voles (*Arvicola amphibius*) to rivers in Essex,

170 targeted intensive control of the invasive American mink (*Neovison vison*) and

171 habitat improvements (McGuire & Whitfield, 2018). Mink removal has resulted in the

172 natural recolonization of water voles across over 500km$^2$ of North East Essex and

173 the River Colne Water Vole Translocation Project (RCWVTP) was implemented in

174 2009, with 600 water voles released on the Colne between 2010-2012. Mink control

175 has occurred on the river Blackwater but no water vole releases have taken place. In

176 addition to the aforementioned sites, eDNA sampling was conducted at Spains Hall

177 Estate (Figs 1B and S1B), where a pair of Eurasian beavers (*C. fiber*) are housed in

178 a fenced enclosure outdoors. The beavers were released into the enclosure in March

179 2019. For the 'beaver experiment', sampling deviated slightly from the two main

180 rivers described above in that four water replicates were collected from each of the

181 three sampled locations in the beaver enclosure (B1-B3; where B1 was a pond

182 within the enclosure and B2-3 along the stream adjacent to it), five replicates were

183 collected from downstream of the beaver enclosure (B4) and eight replicates were

184 collected from a large pond inlet of a brook (B5; Fig. S1B).

185

186 Water samples were collected using sterile 500 ml water bottles on the shoreline at a

187 reachable distance with complete submersion beneath the surface (Fig. S2). Four

188 field controls consisting of a bottle of distilled water (500 ml) were opened briefly at

189 the beginning and end of each of the two sampling days to test for cross-

190 contamination during sampling. Samples and field blanks were transported in cool

191 boxes, sterilised with 10% bleach and 70% ethanol. Samples were filtered on the

192 same day as collection using sterilised single-use syringes and 0.45 μm Sterivex

193 filter (Merck Millipore, Darmstadt, Germany). The filters were stored at 4°C for 2 days

194 prior to transportation to the laboratory, transported in cool boxes with ice packs and

195  then stored at -20°C until DNA extraction. Water sampling and filtration followed the
196  same procedures as described above.

197

**2.2 eDNA extraction and metabarcoding**

199  DNA was extracted from the filters in a dedicated eDNA clean room following the
200  Mu-DNA protocol (Sellers et al., 2018). Field controls were extracted first, followed
201  by the eDNA samples. Five DNA extraction negative controls (one for each day of
202  extractions) containing only extraction buffers were also included. All surfaces were
203  sterilised with 10% bleach and then washed with 70% ethanol. Small tools were
204  placed in a UV Stratalinker® before, in-between and after extracting each sample to
205  reduce the risk of cross-contamination.

206

207  DNA extracts were stored at -20°C until PCR amplification. Eluted eDNA was
208  amplified using the MiMammal 12S primer set (MiMammal-U-F, 5′-
209  GGGTTGGTAAATTTCGTGCCAGC-3′;         MiMammal-U-R,        5′-
210  CATAGTGGGGTATCTAATCCCAGTTTG-3′; Ushio et al., 2017) targeting a ~170bp
211  amplicon from a variable region of the 12S rRNA mitochondrial gene with sample-
212  specific multiplex identifier (MIDs) tags. PCR amplification protocols followed Sales,
213  McKenzie et al. (2020). PCRs were conducted in triplicates to reduce bias in
214  individual reactions and the replicates were pooled prior to library preparation.
215  Amplification was validated using 1.2% agarose gel electrophoresis stained with
216  GelRed (Cambridge Bioscience). In total, 185 samples were analysed, including 158
217  eDNA samples, four field collection blanks, five extraction blanks, 10 PCR negative
218  controls and eight PCR positive controls (i.e. DNA extraction from a non-target
219  species that is not locally present, the northern muriqui *Brachyteles hypoxanthus*
220  from Brazil, at a concentration of 0.05 ng/µL). eDNA samples were equally
221  distributed into two sequencing libraries, with replicated extraction controls in each
222  library. A left-sided size selection was performed using 1.1x Agencourt AMPure XP
223  (Beckman Coulter) and Dual-Index adapters (Illumina) were added to each library
224  using KAPA HyperPrep kit (Roche). Each library was then quantified by qPCR using
225  NEBNext qPCR quantification kit (New England Biolabs) and pooled in equimolar
226  concentrations. Libraries were sequenced using an Illumina MiSeq v2 Reagent Kit
227  for 2×150 bp paired-end reads (Illumina, San Diego, CA, USA).

228

229 **2.3 Bioinformatic analysis**

230 The bioinformatic analysis was conducted using OBITools metabarcoding package

231 (Boyer et al., 2016) following the protocol described in in Sales, McKenzie et al.

232 (2020). Briefly, the quality of the reads were assessed using FASTQC (Andrews,

233 2015), a filter was used to select fragments of 140-190bp and to remove reads with

234 ambiguous bases using obigrep, followed by a sequence clustering using SWARM

235 (Mahé et al., 2015) and a taxonomic assignment conducted using ecotag against a

236 custom database (Sales, McKenzie et al., 2020). An additional conservative filtering

237 procedure was conducted to exclude MOTUs/reads originating from putative

238 sequencing errors or contamination in order to avoid false positives (Table S1). First,

239 to account for the occurrence of tag-jumps between tagged amplicons (Schnell et al.,

240 2015), the frequency of tag-jumping was calculated for each sequencing library by

241 dividing the total number of reads of the positive control (PC, *B. hypoxanthus*)

242 recorded in the actual eDNA samples and negative controls by the total number of

243 reads of the PC in the PC samples. The frequency was taken off all MOTUs and the

244 PC was removed. Then, to remove putative contaminants, the maximum number of

245 reads recorded for a MOTU in one of the negative controls (whether this be a field

246 collection blanks, extraction blanks or PCR negative controls) was removed from all

247 samples for each MOTU. Finally, non-target MOTUs (non-mammal species, human

248 and domestic species) and MOTUs that were likely to have been carried over from

249 contamination were discarded from the dataset by removing MOTU's with <5 total

250 reads, and only MOTUs that were identified at species level with a best identity of >

251 0.98 were included (Sales, McKenzie et al., 2020).

252

253 **2.4 Statistical analysis**

254 All statistical analyses were performed using R v4.0.0 (R Core Team, 2020). After

255 bioinformatic filtering, bubble charts were created using *ggplot2* (Wickham & Chang,

256 2016) showing the proportional read count of each species identified at each

257 sampling site along each river and around the beaver experiment. Read counts in a

258 water replicate at a sampling site were then converted into binary presence-absence

259 data for downstream analyses. As we aimed to compare the mammalian diversity

260 present in the two river systems (Colne and Blackwater), for subsequent analyses, a

261 dataset comprising only the 15 sites from the River Colne and 10 sites from the River

262 Blackwater (i.e., excluding sites B1-B5 representing the beaver experiment as these

263    included ponds) was used. Species accumulation curves were created, using the R

264    package *iNext* (Hsieh & Chao, 2020)*,* to determine if the number of sample sites

265    sampled was adequate to represent the overall species diversity along both rivers

266    and when observing the systems as a whole, and to estimate the sample effort

267    needed to fully determine the species richness (Hsieh, Ma, & Chao, 2016). Mathews

268    et al. (2018) was used to infer known mammalian species (excluding bats)

269    distributions in the region from 1995-2016.

270

271    In order to determine detection probabilities of each species' eDNA, a single season

272    occupancy model (MacKenzie et al., 2002) was applied to the data where detection

273    histories were created using each of the five water replicates taken at a site as

274    sampling occasions (MacKenzie et al., 2017), following Sales, McKenzie et al.

275    (2020). The core assumption here is that the underlying occupancy state (i.e.

276    occupied or empty) is constant over the sampling period, and therefore, every

277    sampling occasion is an imperfect observation of the true occupancy status of a

278    species' eDNA at that site. Our primary aim was to compare eDNA detectability

279    across different species within our sampling effort, so we did not consider any other

280    competing models (Sales, McKenzie et al., 2020). These analyses were conducted

281    separately for each species, overall and within each river (excluding when a species'

282    eDNA was not detected in a particular river), using the R package *unmarked* (Fiske

283    & Chandler, 2011).

284

285    To illustrate the differences in the average species richness in sample sites between

286    the two river systems, box and jitter plots were created using the *tidyverse* R

287    package (Wickham et al., 2019). A non-metric multidimensional scaling (NMDS)

288    analysis was completed to visualise the differences in community composition

289    between both rivers using the metaMDS function from the *vegan* R package

290    (Oksanen et al., 2019). Jaccard index distances were utilised to create the NMDS

291    plot with a stress value being calculated to verify the goodness-of-fit between the

292    NMDS ordinations and a commonly accepted set of guidelines (Dexter,

293    Rollwagen‑Bollens & Bollens, 2018). Differences between the two rivers were

294    calculated using a permutational multivariate analysis of variance (PERMANOVA)

295    with 1000 permutations being performed using the adonis function in the *vegan* R

296    package (Oksanen et al., 2019) applying Jaccard index distances.

297

298    The *betapart* package (Baselga et al., 2012) was used to assess the spatial patterns

299    of β-diversity using multiple-site dissimilarity measures across all analysed sites. β-

300    diversity was calculated and partitioned into nestedness (i.e., species loss) and

301    turnover (i.e., species replacement) components using the Sørensen index, following

302    Baselga (2010). Three multiple-site dissimilarities were estimated including βsor

303    (Sørensen dissimilarity), βsim (Simpson dissimilarity, turnover component of

304    Sørensen dissimilarity) and βsne (nestedness component of Sørensen dissimilarity)

305    using three different datasets (one for each river - Blackwater and Colne, and one

306    combining the data for both rivers).

307　**3. Results**

308　**3.1. Species detections using eDNA metabarcoding**

309　The MiSeq sequencing run yielded a total of 12,021,106 raw reads. Following

310　filtering criteria, 2,447,689 reads were retained (Table S1). In total, 25 wild mammal

311　species were detected across all sampling locations (Table S2), 23 species on the

312　River Colne (Figs 2 and S3), 12 species on the River Blackwater (Figs 2 and S4) and

313　12 species in and downstream of the beaver enclosure (Fig. S5). Overall, mammals

314　were detected from five orders: Artiodactyla (3 species), Carnivora (7 species),

315　Eulipotyphla (4 species), Lagomorpha (2 species) and Rodentia (9 species). This

316　comprised ten families and twenty genera (Figs 1 and 2; Table S2). This list included

317　15 species designated as Least Concern, two Endangered, one Critically

318　Endangered, three naturalised and four non-native (Table S2; Crawley et al., 2020).

319　The Chao II estimation based on the eDNA results predicted 27 species (95% CI:

320　24-41; Table S3) overall and this is a close representation of the 28 terrestrial and

321　semi-aquatic mammal species expected in Essex (Mathews et al., 2018). On the

322　River Colne 23 species were detected, with 28 predicted (95% CI: 24-49; Table S3)

323　according to the Chao II estimate, whereas 12 species were predicted (95% CI: 12-

324　20; Table S3) on the river Blackwater which represents the same number of species

325　observed. Of the 25 species identified overall with eDNA, 23 are known from the

326　region from 1995-2016 records (Mathews et al., 2018), with the beaver being

327　reintroduced after this period. One species, the red squirrel (*Sciurus vulgaris*), was

328　last detected in the region in 1971 but the species was reintroduced to Mersea Island

329　in 2012 (Dobson & Tansley, 2014), approximately 10km from where it was detected

330　using eDNA. For completeness, this detection was retained in the downstream

331　analyses.

332

333　Species from the order Rodentia were the most prevalent, with at least one species

334　from this order being detected at all sampling sites (Fig. 1). Ten unique species were

335　detected on the river Colne including five carnivores: European otter (*Lutra lutra*),

336　European badger (*Meles meles*), stoat (*Mustela erminea*), least weasel (*Mustela*

337　*nivalis*) and American mink (*N. vison*); two species from the order Artiodactyla: fallow

338　deer (*Dama dama*) and roe deer (*Capreolus capreolus*); common shrew (*Sorex*

339　*araneus*) from the order Eulipotyphla; red squirrel (*S. vulgaris*) from Rodentia and

340　brown hare (*Lepus europaeus*) from Lagomorpha (Table S2). One unique carnivore

341    was detected at one site on the river Blackwater (B13): European polecat (*Mustela*

342    *putorius;* Figs 2 and S4). Eurasian beaver (*C. fiber*) was detected at all sampling

343    sites inside the beaver enclosure (B1-B3) and approximately 300m downstream of

344    the enclosure (B4) but was not detected at the large pond inlet (B5; Figs 1B and

345    S1B; Fig. S5).

346

347    **3.2. Occupancy and detection probabilities in the Rivers Colne and Blackwater**

348    Based on the five water replicates taken at each sampling site, site occupancy and

349    detection probabilities based on eDNA varied markedly between species. Six

350    species (three from the order Carnivora, two from Rodentia and one from

351    Artiodactyla) had detection probabilities close to zero given that they were detected

352    in only a single water replicate at either one or two sampling sites (Figs 2 and 3;

353    Table S4). Despite being detected at eight sampling sites (Fig. 2), the bank vole

354    (*Myodes glareolus*) had a low detection probability of only 0.07 because it was

355    generally only detected in a single water replicate at each site it was found. For the

356    Carnivora, American mink (*N. vison*) and stoat (*M. erminea*) had high detection

357    probabilities despite only being found at 1-2 sampling sites along the River Colne.

358    This is due to both being detected in 2-4 replicates when found at a site. The badger

359    (*M. meles*) was the most frequently detected carnivore (seven sites in the River

360    Colne; Fig. 2), but had a similar detection probability (0.24 compared to 0.20) to the

361    otter (*L. lutra*; Fig. 3; Table S4), which was detected at only two sites on the Colne.

362

363    Species from Rodentia were generally the most frequently detected species at

364    sampling sites overall (e.g. field vole *Microtus agrestis* at 21/25 sites; brown rat

365    *Rattus norvegicus* at 18/25 sites; water vole *A. amphibius* at 13/25 sites; grey

366    squirrel *S. carolinensis* at 12/25 sites; *M. glareolus* at 8/25 sites and woodmouse

367    *Apodemus sylvaticus* at 6/25 sites; Fig. 2; Table S2). These species were frequently

368    detected in the beaver experiment also (Table S2; Fig. S5). Detection probabilities in

369    the Colne and Blackwater combined ranged from 0.26 to 0.49 (with the exception of

370    the aforementioned *M. glareolus*) for these frequently occurring species (Fig. 3;

371    Table S4). From the order Artiodactyla, *C. capreolus* was only detected on a single

372    occasion but *Muntiacus reevesi* and *D. dama* were detected at seven and five

373    sampling sites, respectively. Detection probabilities were 0.46 and 0.30 for *M.*

374    *reevesi* and *D. dama*, respectively. Of the species from Eulipotyphla, the water

375  shrew (*Neomys fodiens*) was found at 7/25 sites, common shrew (*S. araneus*) and

376  mole (*Talpa europaea*) at four sites and pygmy shrew (*S. minutus*) at two. Detection

377  probabilities were similar across these species, ranging from 0.20 to 0.28. For

378  Lagomorpha, the brown hare (*L. europaeus*) was found at four sites on the Colne

379  (detection probability of 0.20), with the European rabbit (*Oryctolagus cuniculus*)

380  detected at 10/25 sites and a detection probability of 0.37 (Fig. 3; Table S4). With the

381  notable exceptions of *M. agrestis* and *R. norvegicus*, 95% confidence intervals were

382  generally large however around these estimates for detection probabilities for most

383  species (Fig. 3; Table S4).

384

385

386  **3.3. Sampling site effort**

387  Evaluation of the species accumulation curves and their asymptotes provide insights

388  into the sampling effort needed to achieve a comprehensive view of the mammalian

389  diversity around the sampling area. By visually examining the asymptote of the

390  accumulation curve for the overall diversity of combined sites for the River Colne and

391  Blackwater, the total of 25 sites herein achieved to capture 24 species (85%) of the

392  28 expected semi-aquatic and terrestrial mammals present in the area, and to reach

393  the asymptote a total of 45 sampling sites may be required (Fig. 4). When analysing

394  the rivers individually an increased sampling effort is required for the River Colne as

395  the curve is gradually increasing toward the asymptote, the 15 sites detected 23

396  species (82%) out of the predicted 28 species that are known to inhabit the sampling

397  area. The River Blackwater required less sampling effort due to the lower overall

398  diversity of the river (Fig. 4).

399

400  When visually inspecting the accumulation curves for each mammalian order for the

401  river Blackwater, they indicate that sufficient sampling effort has been acquired for all

402  orders as all the curves have reached an asymptote within our sampled sites. This is

403  in contrast to the species accumulation curves on the river Colne where only the

404  order Lagomorpha, representing two species, has plateaued within our sampled

405  sites. The orders Eulipotypyla and Artiodactyla did not reach a plateau within our

406  sampled sites but only one species of each order was not detected that are known in

407  the sampling area (Fig. 4; Table S2; Mathews et al., 2018). The accumulation curves

408  for Rodentia did not reach an asymptote and three species of the order known in the

409    sampling area were not detected. Although all carnivores that are known in the

410    sampling area have been detected using eDNA metabarcoding, the accumulation

411    curve represents an over-prediction of species richness for this order. When the

412    sample sites are combined for both rivers, the orders Artiodactyla, Eulipotyphla and

413    Lagomorpha are shown to reach an asymptote within our sampled sites (Fig. 4).

414

415    **3.4. Species richness and β-diversity**

416    The River Colne had a higher species richness when compared to the Blackwater,

417    both overall (Fig. 5A) and when analysing the species richness per taxonomic order

418    (Fig. 4). Differences in community compositions between both river systems were

419    initially visualised with NMDS ordination plots which demonstrates the sampling sites

420    for each river grouped in two clusters, with a small overlap (Fig. 5B). Furthermore,

421    this dissimilarity pattern was further corroborated with the PERMANOVA test which

422    determined that communities were significantly different among the rivers, despite

423    the low variance explained ($R^2 = 0.106$, $p = 0.002$).

424

425    Dissimilarity measures of the estimated overall community composition revealed

426    high β-diversity for each river (Colne: βsor = 0.8376; Blackwater: βsor = 0.7690),

427    with a lower dissimilarity when the rivers are compared (βsor = 0.3714). The

428    compositional dissimilarity found in the River Colne was mainly associated with a

429    high rate of species turnover with nestedness contributing a marginal amount (βsim

430    = 0.7276, βsne = 0.1100), demonstrating that the β-diversity patterns are mostly

431    caused by species replacement between sites. A similar pattern was found for the

432    River Blackwater, but including an increase in the contribution of the nestedness

433    component (βsim = 0.5395, βsne = 0.2295), with species replacement and species

434    loss between sites both contributing to the high β-diversity values. In contrast to that,

435    when comparing the assemblages of both rivers, most of the dissimilarity was due to

436    nestedness (βsim = 0.0833, βsne = 0.2881).

## 4. Discussion

Due to the high costs in terms of effort and economics for monitoring entire communities, biodiversity assessments are often confined to very few indicator species and can therefore only provide a reduced representation of overall community dynamics and ecosystem health (Hilty & Merenlender, 2000). eDNA metabarcoding enables large-scale and multi-taxa surveys from material that can be collected rapidly in the field, and this multi-species monitoring could lead to more effective ecosystem-wide biodiversity assessments (Deiner et al., 2017). The mainstream application of eDNA for monitoring mammalian communities is certainly recent and have so far been relatively limited in scale (e.g. Harper et al., 2019; Sales, McKenzie et al., 2020; Sales, Kaizer et al., 2020). Sales, McKenzie et al. (2020) highlighted the potential of eDNA metabarcoding in riverine ecosystems to provide a rapid and rough 'distribution map' for mammalian biodiversity at a landscape scale. Here we expand upon this and previous studies by incorporating more intensive sampling in two major and adjacent rivers in southeastern England to investigate the effort required to capture the mammalian community, elucidate patterns of diversity and quantify differences within and among these sampled waterways.

In terms of providing a reliable snapshot of the mammalian community, this current study has demonstrated the power of eDNA-based monitoring for 'capturing' almost the entire known terrestrial and semi-aquatic mammalian community in the area in ~30 hours of field sampling. 23 of the 28 known mammals in the area were detected in both rivers (Mathews et al., 2018). Considering the species accumulation curves, most of the mammalian diversity present in the surroundings of both rivers has been detected by eDNA (Fig. 4). Despite the potential need for an increased sampling effort to detect all species in the area, only five species expected in the region were not identified in this study (Table S2). The sampling period here represents a much shorter collection period than would be needed to fully estimate species composition by camera trapping, field signs and physical sightings (Roberts, 2011; Abrams et al., 2019). With 82% of the expected species detected here using eDNA (plus two recent additions), this is on the upper end of what camera trapping is expected to capture (57-86% of terrestrial mammals and avian species; Boitani, 2016). It is clear however that considerable effort is still required in terms of the number of sites sampled and

471 replicates taken within a small geographic area for eDNA-based monitoring of

472 mammals (Figs 1 and 2; Macher et al., 2021). A total of eight species were detected

473 in only one or two locations (Fig. 2; Table S2) and most species had estimated

474 detection probabilities of ~0.20 (Fig. 3, Table S4), suggesting that all five water

475 replicates are generally necessary to detect even common species. An alternative

476 approach would be to filter larger volumes of water within a river system (Cantera et

477 al., 2019; Bessey et al., 2020) but this would limit the applications of this method

478 over larger spatial scales (because of the specialized pumps/equipment required;

479 Cantera et al., 2019) and the involvement of citizen scientists to undertake the

480 sampling (something that is now being applied more widely in eDNA-based

481 monitoring; Biggs et al., 2015). The main consideration around the approach used

482 here is one that can be adopted for national-based monitoring schemes with the

483 involvement of citizen scientists and local conservation groups and this study has

484 clearly shown that such an approach is logistically feasible.

485

486 A clear advantage of eDNA-based surveys in contrast to other sampling techniques

487 (e.g. camera traps) is that it does not appear to be affected by animal body mass,

488 since it has been shown to be capable of capturing effectively and simultaneously

489 small to large mammals. With few exceptions, most species within the orders

490 Artiodactyla, Eulipotyphla, Lagomorpha and Rodentia were frequently detected at

491 multiple sites each (Figs 1 and 2) and had more consistent detection probabilities

492 across species (Fig. 3; Table S4). Five species from Rodentia (*A. amphibius, M.*

493 *glareolus, M. agrestis, R. norvegicus* and *S. carolinensis*), and one each from

494 Artiodactyla (*M. reevesi*), Eulipotyphla (*N. fodiens*), Lagomorpha (*O. cuniculus*) were

495 detected at multiple sites in each river system (Fig. 2). This generally reflects species

496 which are known to be abundant in the region and/or group-living, factors which are

497 important for eDNA detections (Sales, McKenzie et al., 2020; Williams et al., 2018).

498 Other species such as the elusive water shrew (*N. fodiens*) is semi-aquatic and is

499 considered challenging to monitor using conventional methods (Churchfield et al.,

500 2000). Yet it is evident that eDNA represents a rapid and viable method for local

501 detections (Yonezawa et al., 2020). Although the grey squirrel (*S. carolinensis*) is

502 considered an arboreal species, it spends a significant proportion of its time foraging

503 on the ground (more so than the red squirrel *S. vulgaris*) and is frequently detected

504 here by eDNA (Figs 2 and 3).

505

506     As with other surveying techniques, behaviour and ecology can influence eDNA

507 detection in natural water bodies (Harper et al., 2019; Williams et al., 2018). As

508 previous studies have highlighted, carnivores are typically difficult to detect from

509 water-based eDNA (Harper et al., 2019; Sales, McKenzie et al., 2020; Sales, Kaizer

510 et al., 2020). It has been proposed that this is due to the fact that they are generally

511 solitary, wide-ranging and may have less frequent contact with water bodies (e.g.,

512 animals might excrete/defecate on land more frequently) which could produce non-

513 detections despite being present in the area/region (leading to potential false

514 negatives from eDNA; Harper et al., 2019; Leempoel et al., 2020; Sales, McKenzie

515 et al., 2020). Even when known or when regularly captured on camera traps in an

516 area, eDNA has either infrequently, or completely failed to, detect species from this

517 order (Harper et al., 2019; Sales, McKenzie et al., 2020). This study in particular has

518 highlighted the intensity of sampling required to detect all the wild carnivores within a

519 given area (Figs 2 and 4), even with semi-aquatic species such as the otter (*L. lutra*).

520 Although all seven species within the order known in this geographic region were

521 detected, three of these were only detected at a single site and no carnivore was

522 detected on both the Colne and Blackwater (the red fox *V. vulpes* was additionally

523 detected in the beaver experiment; Fig. S5; Table S2). Only the stoat (*M. erminea*)

524 and American mink (*N. vison*) had comparatively higher detection probabilities (Fig.

525 3; Table S4).

526

527     This finding in relation to the American mink is note-worthy because the current

528 study area is an active mink eradication zone due to the critical impacts this invasive

529 species has had on local water vole populations. Browett et al. (2020) discussed the

530 potential application of eDNA-based monitoring for the early detection of invasive

531 mammals and given this mink eDNA detection on the periphery of the eradication

532 zone, continuous monitoring using eDNA-based methods could be warranted to aid

533 in keeping this region and others mink-free. The key for both early detection of

534 invasive species and monitoring critically endangered species is the ability to detect

535 species at low abundance (i.e. when a small number of individuals first

536 colonize/invade an area). The number of individuals present in a system is clearly an

537 important factor for eDNA detection (Williams et al., 2018). This may explain why

538 some species listed as vulnerable such as the hedgehog *Erinaceus europaeus* (a

539  species which has been declining in the UK; Mathews & Harrower, 2020) and locally

540  rare hazel dormouse *Muscardinus avellanarius* (Dobson & Tansley, 2014) were not

541  detected by eDNA (Table S2) and may require more targeted eDNA-based

542  approaches (Priestley et al., 2021). The results from the beaver experiment may be

543  particularly informative in this case however for early detection/detections at low

544  abundance using eDNA. It is of course a semi-aquatic species but only two

545  individuals were present in the enclosure and yet the species was still readily

546  detected in multiple water replicates 300-400m downstream of the enclosure (Figs

547  1B and S5). It is clear that further experiments are required for a more complete

548  understanding of the effects of species abundance along with transport and

549  persistence of eDNA in relation to terrestrial and semi-aquatic mammals. This

550  certainly presents unique challenges in comparison to studies involving species

551  which are fully aquatic (Sales, McKenzie et al., 2020).

552

553  In addition to individual species detections, eDNA has the potential to improve our

554  understanding of the dynamics of biodiversity in both time and space (Deiner et al.,

555  2017). This is essential for the effective management of species and their

556  ecosystems. The investigation of species richness and β-diversity patterns obtained

557  from eDNA samples might provide insights into the underlying processes which

558  structure communities (Deiner et al., 2017). Species assemblages can differ in two

559  potential ways: the first, namely species turnover, which is based on the replacement

560  of species between sites (i.e. substitution of one species in one site by a different

561  one in the other site); and the second way, known as nestedness, refers to a pattern

562  where there is species loss or gain, implying that different sites are strict subsets of

563  richer ones (Baselga et al., 2012). In this study, dissimilarity (β-diversity) within both

564  rivers was mainly driven by species turnover. This pattern was more evident in the

565  Colne than Blackwater. This pattern indicates that the landscape surrounding each

566  river presents a fairly distinct subset of species due to the high occurrence of species

567  replacement. Contrasting to the pattern found within each river, a comparison

568  between both river systems revealed a pattern consistent with higher nestedness-

569  resultant dissimilarity. Nestedness occurs when assemblages of sites with fewer

570  species tend to be a subset of the biotas from richer sites (Wright and Reeves,

571  1992). This result corroborates with the difference in species richness and

572  composition between rivers (Fig. 5) and indicates that the Blackwater may represent

573  a subset of the Colne in terms of the mammalian community surrounding it.

574

575  Different processes might be responsible for shaping community structure (Podani

576  and Schmera, 2011, Gutiérrez-Cánovas et al., 2013). Partitioning β-diversity into its

577  spatial turnover and nestedness components contributes to disentangle the

578  processes underlying beta diversity and understand the putative drivers of

579  community change (Baselga et al., 2010, Legendre and De Cáceres, 2013). For

580  conservation purposes, this distinction is paramount for considering that both are

581  antithetic processes that require distinct conservation strategies (Wright and Reeves,

582  1992). In a nestedness scenario, the prioritization of a small number of the richest

583  sites could be considered, whereas for a species turnover scenario, a higher

584  conservation effort of a large number of sites would be advised (Wright and Reeves,

585  1992). This assumption can be extrapolated to an eDNA sampling strategy for

586  monitoring mammals from water taken from riverine systems. Here, by comparing

587  both rivers for the purposes of monitoring mammals at a landscape-level, we

588  demonstrate that the choice of sampling a single system in a nestedness context

589  could lead to two different outcomes: sampling the richest system and obtaining the

590  detection of most of the mammal diversity found in the area (e.g., sampling only the

591  River Colne), or sampling the less diverse system and obtaining an underestimation

592  due to the sampling effort directed towards detecting solely a subset of the biota

593  from a richer river (e.g., sampling only the River Blackwater). Therefore, choosing to

594  only sample a single river could have consequences for the inferred surrounding

595  mammalian community. Just as importantly, the high turnover contribution within

596  each river course might indicate that there is a low contribution of eDNA transport.

597  eDNA longitudinal transport and diffusion would in theory lead to a higher

598  nestedness within sites located upstream, leading to them being subsets from sites

599  located further downstream in the river (because of eDNA accumulation).

600  Preferentially sampling sites downstream (which may represent 'eDNA reservoirs';

601  Sales et al., 2021) does not seem to represent an optimal sampling strategy for

602  species which are not fully aquatic based on the results presented here.

603

604  This study demonstrates that eDNA metabarcoding from river-derived water is an

605  efficient method for mapping mammalian distributions and diversity, and is highly

606　capable of identifying the vast majority of the expected terrestrial and semi-aquatic
607　species from a variety of mammalian orders within a short but intensive sampling
608　period. We quantitatively demonstrate the effort required to capture different species
609　within different orders and that considerations around individual species' ecologies
610　are important for eDNA-based monitoring of mammalian communities. This study
611　provides the scope for using eDNA from rivers to quantify genuine differences in
612　mammalian communities in their vicinity, and allows for the future incorporation of
613　biotic and abiotic variables to understand the underlying factors behind these
614　differences (Mariani et al., 2021). Adopting eDNA-based approaches for mammals
615　would provide a reliable complement to ongoing national surveying efforts and one
616　that could be readily adapted to involve citizen scientists.

617

624

**Author contributions:**

626　ADM, DT and NGS conceived and designed the study. HAB, EKB, JCP, NS, DT and
627　ADM performed the sampling. HAB, EKB, JCP, SSB and NGS performed the
628　laboratory work and HAB, LMG, NGS, JVL, PB and SSB the bioinformatics. LMG,
629　HAB, NGS and ADM analysed the data. HAB, LMG, NGS and ADM wrote the
630　manuscript, with all authors contributing to discussions and editing.

631

**Data Availability Statement**

633　All bioinformatic steps and scripts can be found on github
634　(https://github.com/McDevitt-Lab). Raw sequence data will be made publicly
635　available upon peer-reviewed publication.

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844 **Figures**

845 **Figure 1.** Community structure of each sampling site represented by Order for the

846 Rivers Blackwater (orange) and Colne (blue; A) in Essex, England (approximate

847 location shown in red on the inset map). For the beaver experiment (pink; B), beaver

848 (*Castor fiber*) detections specifically are also shown.

849

850 **Figure 2.** Species detections from eDNA metabarcoding data by sampling site (see

851 Fig. S1A) within the Rivers Colne (blue) and Blackwater (orange), and combined

852 (green). Species are grouped by order (left to right: Carnivora, Rodentia,

853 Artiodactyla, Eulipotyphla and Lagomorpha).

854

855 **Figure 3.** Estimated detection probabilities (yellow) for each species (grouped by

856 order), with vertical lines representing the 95% confidence intervals.

857

858 **Figure 4.** Accumulation curves of species detected according to the number of

859 sampled sites, including data comprising all species together and divided by Order

860 (represented by the symbols depicted in Fig. 1A) for each river (Blackwater in orange

861 and Colne in blue) and both rivers combined (black). The number of species

862 expected according to Mathews et al. (2018) is indicated by the red dotted line. From

863 left to right and top to bottom: Overall, Carnivora, Rodentia, Artiodactyla,

864 Eulipotyphla and Lagomorpha.

865

866 **Figure 5.** Box plot of overall species richness for each river (A) and an NMDS plot

867 representing β-diversity based on Jaccard distances (B) of sampling sites on the
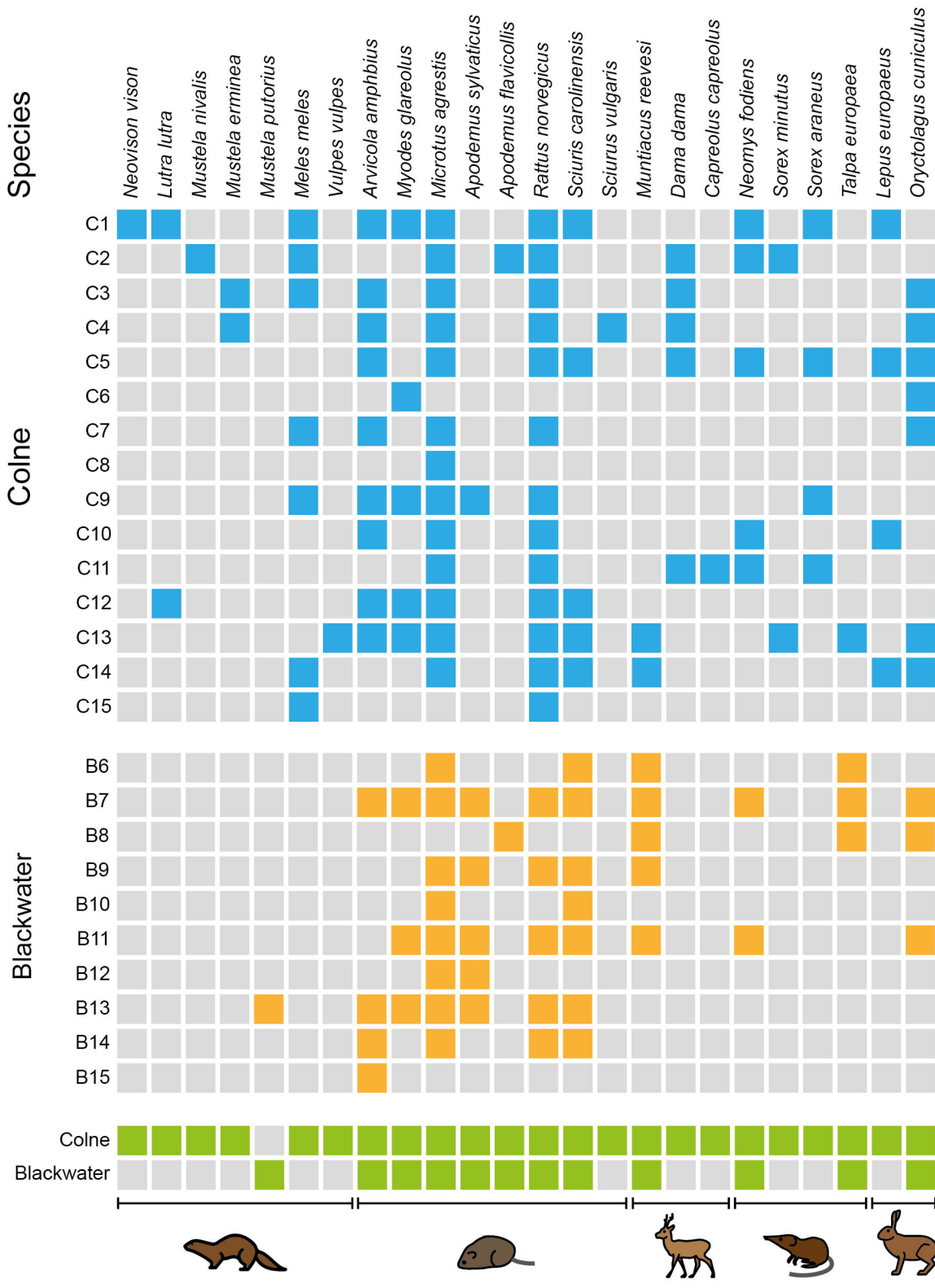
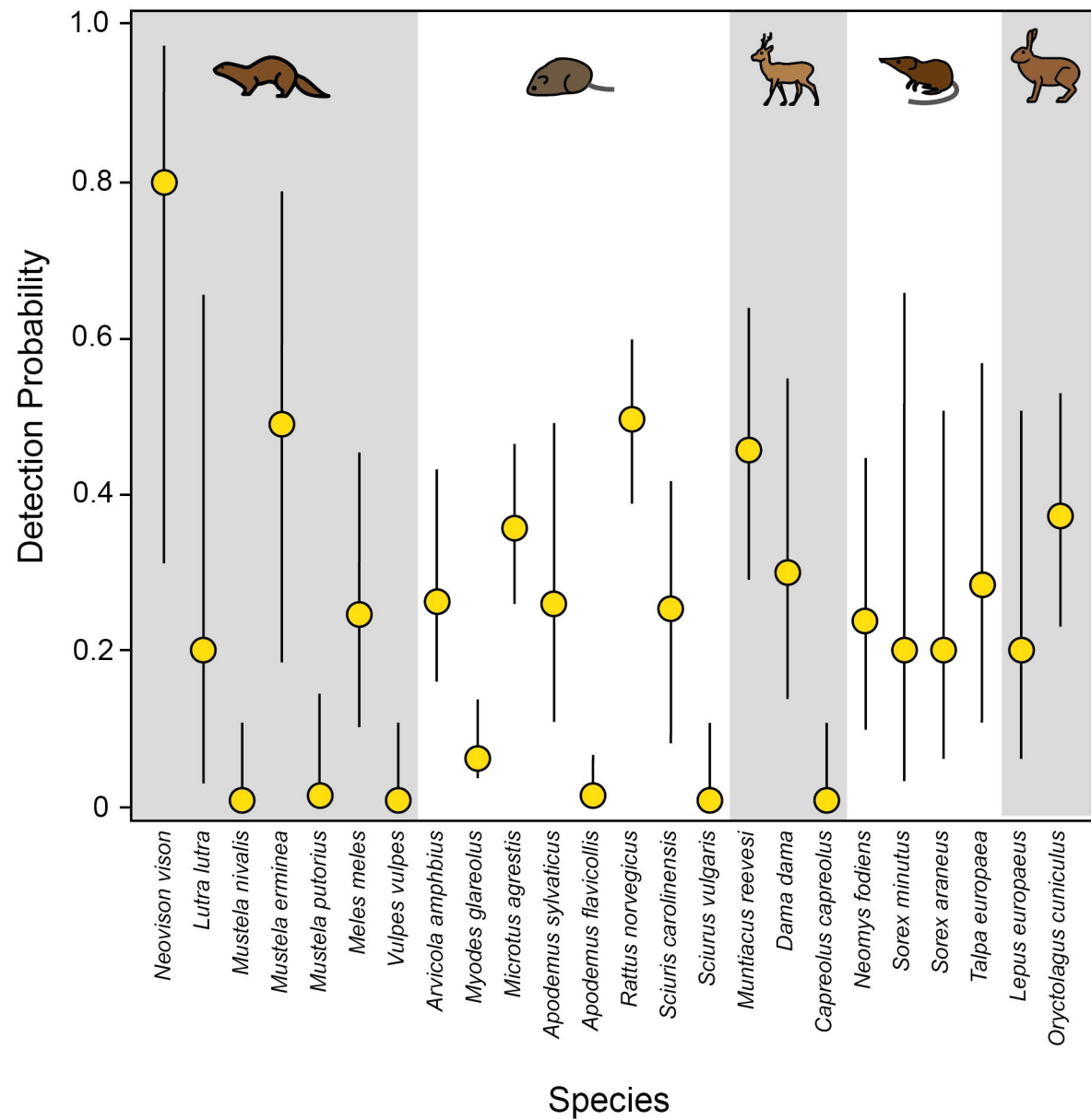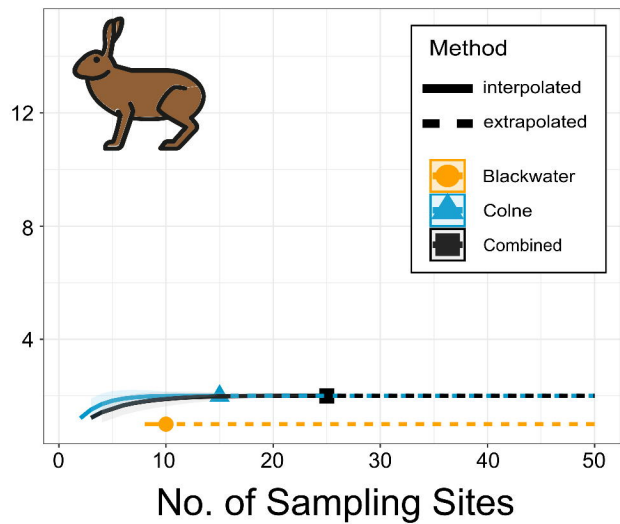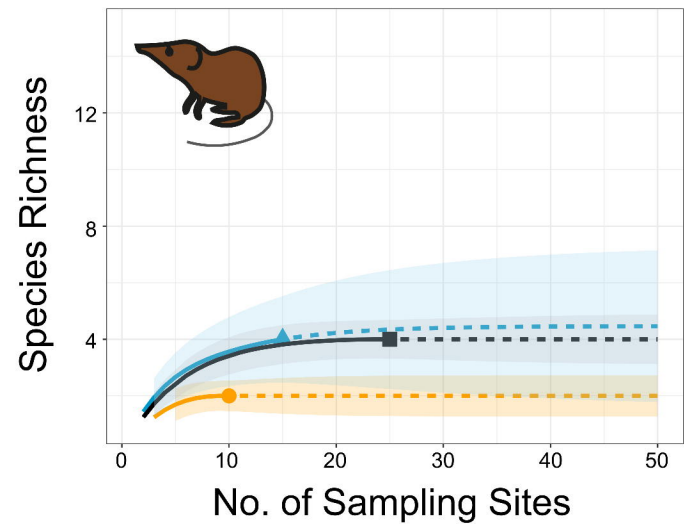868 Rivers Colne (blue) and Blackwater (orange).

A

**Legend (top left):**
- River Blackwater
- River Colne
- Beaver Experiment
- Beaver Enclosure
- Sampling Point

**Legend (bottom left):**
- *Castor fiber*
- Lagomorpha
- Artiodactyla
- Carnivora
- Rodentia
- Eulipotyphla

2km
N

B

50m
N