

1 **Fishing for mammals: landscape-level monitoring of terrestrial and semi-**
2 **aquatic communities using eDNA from lotic ecosystems**

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25 **Abstract**

26 **1.** Environmental DNA (eDNA) metabarcoding has revolutionised biomonitoring in
27 both marine and freshwater ecosystems. However, for semi-aquatic and terrestrial
28 animals, the application of this technique remains relatively untested.

29 **2.** We first assess the efficiency of eDNA metabarcoding in detecting semi-aquatic
30 and terrestrial mammals in natural lotic ecosystems in the UK by comparing sequence
31 data recovered from water and sediment samples to the mammalian communities
32 expected from historical data. Secondly, using occupancy modelling we compared the
33 detection efficiency of eDNA metabarcoding to multiple conventional non-invasive
34 survey methods (latrine surveys and camera trapping).

35 **3.** eDNA metabarcoding detected a large proportion of the expected mammalian
36 community within each area. Common species in the areas were detected at the
37 majority of sites. Several key species of conservation concern in the UK were detected
38 by eDNA sampling in areas where authenticated records do not currently exist, but
39 potential false positives were also identified.

40 **4.** Water-based eDNA metabarcoding provided comparable results to conventional
41 survey methods in per unit of survey effort for three species (water vole, field vole, and
42 red deer) using occupancy models. The comparison between survey 'effort' to reach
43 a detection probability of ≥ 0.95 revealed that 3-6 water replicates would be equivalent
44 to 3-5 latrine surveys and 5-30 weeks of single camera deployment, depending on the
45 species.

46 **5. *Synthesis and Applications.*** eDNA metabarcoding can be used to generate an initial
47 'distribution map' of mammalian diversity at the landscape level. If conducted during
48 times of peak abundance, carefully chosen sampling points along multiple river

49 courses provide a reliable snapshot of the species that are present in a catchment
50 area. In order to fully capture solitary, rare and invasive species, we would currently
51 recommend the use of eDNA metabarcoding alongside other non-invasive surveying
52 methods (i.e. camera traps) to maximize monitoring efforts.

53

54 **Keywords:** biomonitoring, camera trapping, eDNA metabarcoding, latrine surveys,

55 mammals, occupancy modelling, rivers

56 **Introduction**

57 Environmental DNA (eDNA) metabarcoding (the simultaneous identification of multiple
58 taxa using DNA extracted from an environmental sample, e.g. water, soil, based on
59 short amplicon sequences) has revolutionised the way we approach biodiversity
60 monitoring in both marine and freshwater ecosystems (Valentini et al., 2016; Deiner
61 et al. 2017). Successful applications include tracking biological invasions, detecting
62 rare and endangered species and describing entire communities (Holman et al., 2019).
63 Most eDNA metabarcoding applications on vertebrates to date have focused on
64 monitoring fishes and amphibians (Hänfling et al., 2016; Valentini et al., 2016). What
65 has become apparent from studies in lentic systems (ponds and lakes) is that semi-
66 aquatic and terrestrial mammals can also be detected (Hänfling et al., 2016; Harper et
67 al., 2019). As a result, there has been an increasing focus on the use of both vertebrate
68 (Harper et al., 2019) and mammal-specific primer sets (Ushio et al., 2017; Leempoel
69 et al., 2019; Sales et al., 2019) for detecting mammalian communities using eDNA
70 metabarcoding.

71 Mammals include some of the most imperiled taxa, with over one fifth of species
72 considered to be threatened or declining (Visconti et al., 2011). Monitoring of
73 mammalian biodiversity is therefore essential. Given that any optimal survey approach
74 is likely to be species-specific, very few species can be detected at all times when they
75 are present. This imperfect detection (even greater for elusive and rare species) can
76 lead to biased estimates of occurrence and hinder species conservation (Mackenzie
77 et al., 2002). For mammals, repeated surveys using several monitoring methods are
78 usually applied. These include indirect observations such as latrines, faeces, hair, or
79 tracks, or direct observations such as live-trapping or camera trapping surveys over
80 short time intervals such that closure/invariance can be assumed and detectability

81 estimated (Nichols et al., 2008). Each of these methods has associated efficiency, cost
82 and required expertise trade-offs, which become more challenging as the spatial and
83 temporal scales increase.

84 eDNA sampling yields species-specific presence/absence data that are likely
85 to be most valuable for inferring species distributions using well established analytical
86 tools such as occupancy models (MacKenzie et al., 2002). These models resolve
87 concerns around imperfect detection of difficult to observe species. When coupled with
88 location-specific detection histories, these can be used to infer true occurrence states,
89 factors that influence occupancy rates, colonization-extinction probabilities, and
90 estimates of detection probability (MacKenzie et al., 2017). The use of eDNA sampling
91 to generate species-specific detection data has unsurprisingly increased in recent
92 years, and in many cases has outperformed or at least matched conventional survey
93 methods (Lugg et al., 2018; Tingley et al., 2019). Although comparisons between
94 eDNA analysis and conventional surveys for multi-species detection are numerous
95 (see Table S1 in Lugg et al., 2018), studies focusing on detection probability estimates
96 for multiple species identified by metabarcoding are rare (Abrams et al., 2019;
97 Valentini et al., 2016).

98 The aim of this study was to assess the efficiency of eDNA metabarcoding for
99 detecting semi-aquatic and terrestrial mammals in natural lotic systems in the UK. We
100 conducted eDNA sampling in rivers and streams in two areas (Assynt, Scotland and
101 Peak District National Park, England). Together these locations have the majority of
102 UK semi-aquatic and terrestrial mammalian species present (Table S1). Our
103 objectives were two-fold: first, we sought to establish whether eDNA metabarcoding is
104 a viable technique for monitoring semi-aquatic and terrestrial mammals by comparing
105 it to the mammalian communities expected from historical data, a group for which

106 eDNA sampling has rarely been evaluated in a natural setting. Secondly, we evaluate
107 the detection efficiency of water- and sediment-based eDNA sampling in one of these
108 areas (Assynt) for multiple species compared to multiple conventional non-invasive
109 survey methods (latrine surveys and camera trapping).

110 **Material and Methods**

111 ***Latrine surveys***

112 Assynt, a heather-dominated upland landscape in the far northwest of the Scottish
113 Highlands, UK (Fig. 1A), is the location of an ongoing 20-year metapopulation study
114 of water voles (*Arvicola amphibius*) led by the University of Aberdeen (Fig. S1). Here,
115 we mainly focus only on data collected in 2017. The metapopulation is characterized
116 by 116 discrete linear riparian habitat patches (ranging from 90 m to nearly 2.5 km)
117 distributed sparsely (4% of waterway network) throughout the 140 km² study area
118 (Sutherland et al., 2014). Water voles use prominently placed latrines for territory
119 marking (Fig. S2A). Using latrine surveys, a reliable method of detection (Sutherland
120 et al., 2014), water vole occupancy status was determined by the detection of latrines
121 that are used for territory marking (Sutherland et al., 2013). During the breeding
122 season (July and August), latrine surveys were conducted twice at each site. In
123 addition to water vole latrines, field vole (*Microtus agrestis*) pellets are also easily
124 identifiable, and so field vole detections were also recorded along waterways as a
125 formal part of the latrine survey protocol. Live-trapping was then carried out at patches
126 deemed to be occupied by water voles according to latrine surveys to determine their
127 abundances (this was used to determine which sites were sampled for eDNA; Fig.
128 1A).

129

130 ***Camera Trap Data***

131 Camera traps were deployed at the beginning of July and thus overlapped temporally
132 with the latrine survey in Assynt. Data were collected from cameras deployed at seven
133 of these patches. Within each of these patches, cameras were deployed at the
134 midpoint of the areas where active signs (latrines, grass clipping, burrows) were

135 detected, and if no signs were detected, at the midpoint of historical water vole activity
136 (J. Drake, C. Sutherland and X. Lambin, *pers. comm.*). These will also capture images
137 of any species present in the area that come within close proximity of the camera (Fig.
138 S3A-F).

139 Cameras were deployed approximately 1 m above ground on iron 'u-posts' to
140 avoid flooding, prevent knock-down by wind/wildlife, and optimize both depth of field
141 and image clarity. Cameras (Bushnell HD Trophy Cam, Overland Park, KA) were set
142 at normal detection sensitivity (to reduce false-triggers from grass/shadows), low night
143 time LED intensity (to prevent image white out in near depth of field), three shot burst
144 (to increase chance of capturing small, fast moving bodies), and 15 min intervals
145 between bursts (to increase temporal independence of captures and decrease
146 memory burden). The area each camera photographed was approximately 1-2 m².
147 Animals were identified on images and information was stored as metadata tags using
148 the R (R Core Team, 2018) package camtrapR following the procedures described in
149 Niedballa et al. (2018). Independence between detections was based on 60-minute
150 intervals between species-specific detections.

151

152 **eDNA sampling**

153 A total of 18 potential water vole patches were selected for eDNA sampling in Assynt
154 from 25-27th October 2017. The time lag between the latrine/live-trapping and eDNA
155 surveys was because of two main reasons: (i) legitimate concerns around cross-site
156 DNA contamination during latrine/live-trapping where researchers moved on a daily
157 basis between sites as well as regularly handled and processed live animals (for
158 decontamination procedures see the Supplementary Material) and (ii) the selection of
159 eDNA sampling sites was based on the latrine surveys and abundance data provided

160 by live-trapping so could only occur after this was completed by August 6th. Water and
161 sediment samples were collected from patches where water voles were determined to
162 be absent (five sites; A1-A5); with 1-2 individuals present (three sites; A9, A16 and
163 18); 3-5 individuals (five sites; A6, A8, A11, A14 and A17); and 7-11 individuals (five
164 sites; A7, A10, A12, A13 and A15; Fig. 1A). Each of these streams/rivers differed in
165 their characteristics (in terms of width, depth and flow) and a representation of the
166 sites is depicted in Fig. S4A-D. Three water (two litres each) and three sediment
167 (~25mL) replicates were taken at each patch (further details of sample collection are
168 provided in Appendix S1).

169 In addition to Assynt, eDNA sampling was also conducted on a smaller scale in
170 the Peak District National Park, England (Fig. S5) to incorporate additional mammals
171 that are not known to be present in Assynt (Table S1). Here, the occurrence of water
172 vole was identified by the presence of latrines in two sites (P1 and P2) at the time of
173 eDNA sampling (Fig. S2A), whilst no latrines were identified at one site (P3). At site
174 P1, an otter (*Lutra lutra*) spraint was identified at the time of eDNA sampling (Fig. S2B).
175 These three sites were sampled in March 2018 using the same methodology as in
176 Assynt but were taken in close proximity (<50cm) to water vole latrines where present
177 (Fig. S2A).

178

179 **eDNA Laboratory Methods**

180 DNA was extracted from the sediment samples using the DNeasy PowerMax Soil kit
181 and from the water samples using the DNeasy PowerWater Kit (both QIAGEN Ltd.)
182 following the manufacturer's instructions in a dedicated eDNA laboratory in the
183 University of Salford. In order to avoid the risk of contamination during this step, DNA
184 extraction was conducted in increasing order of expected abundance of water voles in

185 the eDNA samples (all field blanks were extracted first, followed by the sites with
186 supposedly zero water vole abundance, up to the highest densities last). Along with
187 field blanks (Assynt = 8, Peak District = 2), six lab extraction blanks were included
188 (one at the end of each daily block of extractions). A decontamination stage using a
189 Phileas 25 Airborne Disinfection Unit (Devea SAS) was undertaken before processing
190 samples from different locations. Additional information regarding decontamination
191 measures and negative controls can be found in the Supplementary Material.

192 A complete description of PCR conditions, library preparation and bioinformatic
193 analyses are provided in Appendix S1. Briefly, eDNA was amplified using the
194 MiMammal 12S primer set (MiMammal-U-F, 5'- GGGTTGGTAAATTCGTGCCAGC-
195 3'; MiMammal-U-R, 5'- CATAGTGGGGTATCTAATCCCAGTTTG-3'; Ushio et al.,
196 2017) targeting a ~170bp amplicon from a variable region of the 12S rRNA
197 mitochondrial gene. A total of 147 samples, including field collection blanks (10) and
198 laboratory negative controls (12, including six DNA extractions blanks and six PCR
199 negative controls), were sequenced in two multiplexed Illumina MiSeq runs. To
200 minimize bias in individual reactions, PCRs were replicated three times for each
201 sample and subsequently pooled. Illumina libraries were built using a NextFlex PCR-
202 free library preparation kit according to the manufacturer's protocols (Bioo Scientific)
203 and pooled in equimolar concentrations along with 1% PhiX (v3, Illumina). The libraries
204 were run at a final molarity of 9pM on an Illumina MiSeq platform using the 2 x 150bp
205 v2 chemistry.

206 Bioinformatic analysis were conducted using OBITools metabarcoding package
207 (Boyer et al., 2016) and the taxonomic assignment was conducted using ecotag
208 against a custom reference database (see Appendix 1). To exclude MOTUs/reads
209 putatively belonging to sequencing errors or contamination, the final dataset included

210 only MOTUs that could be identified to species level (>98%), and MOTUs containing
211 less than 10 reads and with a similarity to a sequence in the reference database lower
212 than 98% were discarded (Cilleros et al., 2019). The maximum number of reads
213 detected in the controls for each MOTU in each sequencing run were removed from
214 all samples (Table S7). For water voles, field voles and red deer (the most abundant
215 wild mammals in terms of sequence reads in our dataset), this equated to a sequence
216 frequency threshold of $\leq 0.17\%$, within the bounds of previous studies on removing
217 sequences to account for contamination and tag jumping (Cilleros et al., 2019;
218 Hänfling et al., 2016; Schnell, Bohmann, & Gilbert, 2015).

219

220 ***Occupancy/Detection Analysis in Assynt***

221 The data collection from the different survey types described above (water-based
222 eDNA, sediment-based eDNA, latrine and camera traps) produced the following site-
223 specific detection/non-detection data:

224

225 (a) Latrine: two latrine surveys at 116 patches.

226 (b) w-eDNA: three water-based eDNA samples at 18 of the 116 patches surveyed.

227 (c) s-eDNA: three sediment-based eDNA samples at 18 of the 116 patches surveyed.

228 (d) Camera: six one-week occasions of camera trapping data at seven of the 18
229 patches surveyed by both Latrine and eDNA (w-eDNA + s-eDNA) surveys.

230

231 We chose to focus on three species that were detected by at least three of the four
232 methods: water voles, field voles and red deer (*Cervus elaphus*). Water voles and field
233 voles were recorded using all four survey methods and had detection histories for 14
234 surveying events ((Latrine \times 2) + (w-eDNA \times 3) + (s-eDNA \times 3) + (Camera \times 6)).

235 Red deer were not recorded during latrine surveys and had detection histories for 12
236 surveying events $((w\text{-eDNA} \times 3) + (s\text{-eDNA} \times 3) + (\text{Camera} \times 6))$. To demonstrate
237 the relative efficacy of the four surveying methods, we restricted the analyses to the
238 18 sites where both latrine surveys were conducted and eDNA samples were taken,
239 seven of which had associated camera trapping data. Although each surveying
240 method differs in terms of effort and effective area surveyed, each are viable surveying
241 methods that are readily applied in practice. A unit of survey effort here is defined as
242 one latrine survey, one w-eDNA replicate, one s-eDNA replicate or one week of
243 camera trapping. So, while the specific units of effort are not directly comparable, the
244 relative detection efficacy per surveying method-specific unit of effort is of interest and
245 will provide important context for designing future monitoring studies and
246 understanding the relative merits of each surveying method. Analyzing the data using
247 occupancy models allowing for method-specific detectability enables such a
248 comparison in per unit effort efficacy between eDNA metabarcoding and multiple
249 conventional survey methods.

250 A single season occupancy model (MacKenzie et al., 2002) was applied to the
251 ensemble data where detection histories were constructed using each of the surveying
252 events as sampling occasions (MacKenzie et al., 2017). The core assumption here is
253 that the underlying occupancy state (i.e. occupied or empty) is constant over the
254 sampling period, and therefore, every sampling occasion is a potentially imperfect
255 observation of the true occupancy status. Because occasions represent method-
256 specific surveying events, we used “surveying method” as an occasion-specific
257 covariate on detection (Latrine, w-eDNA, s-eDNA and Camera). Our primary objective
258 was to quantify and compare method-specific detectability, so we did not consider any

259 other competing models. For comparing the methods, we compute accumulation
260 curves as (MacKenzie & Royle, 2005):

261

$$262 \quad p_{smk}^* = 1 - (1 - \hat{p}_{sm})^k$$

263

264 Where p_{smk}^* is the cumulative probability of detecting species s , when species s is
265 present, using method m after k surveying events based on the estimated surveying
266 method-specific detection probability for each species (\hat{p}_{sm}). We vary k from 1 to a
267 large number and find the value of k that results $p_{smk}^* \geq 0.95$. We conducted the same
268 analysis separately for water voles, field voles, and red deer. Analysis was conducted
269 in R (R Core Team, 2018) using the package unmarked (Fiske & Chandler, 2011).

270 **Results**

271 ***Mammal Detection via eDNA metabarcoding***

272 The two sequencing runs generated 23,276,596 raw sequence reads and a total of
273 15,463,404 sequences remained following trimming, merging, and length filtering.
274 After bioinformatic analysis, the final 'filtered' dataset contained 23 mammals (Tables
275 S2 and S3). For mammals, ~12 million reads were retained after applying all quality
276 filtering steps (see Appendix 1). Reads from humans, cattle (*Bos taurus*), pig (*Sus*
277 *scrofa*), horse (*Equus ferus*), sheep (*Ovis aries*) and dog (*Canis lupus familiaris*), were
278 not considered further as the focus of this study was on wild mammals (Table S4).
279 *Felis* was included because of the potential of it being wildcat (*Felis silvestris*) or
280 domestic cat (*F. catus*)/wildcat hybrids. A final dataset comprising ~5.9 million reads
281 was used for the downstream analyses (Table S4).

282 In Assynt, the wild species identified were the red deer (18/18 sites); water vole
283 (15/18); field vole (13/18); wood mouse (*Apodemus sylvaticus* - 9/18); pygmy shrew
284 (*Sorex minutus* - 4/18); wild/domestic cat (*Felis* spp. - 4/18); mountain hare (*Lepus*
285 *timidus* - 4/18); rabbit (*Oryctolagus cuniculus* - 3/18); water shrew (*Neomys fodiens* -
286 3/18); common shrew (*Sorex araneus* - 2/18); edible dormouse (*Glis glis* - 2/18); grey
287 squirrel (*Sciurus carolinensis* - 1/18); pine marten (*Martes martes* - 1/18); brown rat
288 (*Rattus norvegicus* - 1/18); red fox (*Vulpes vulpes* - 1/18) and badger (*Meles meles* -
289 1/18; Fig. 1B). All of these species are distributed around/within Assynt (Table S1),
290 with the exception of the edible dormouse and the grey squirrel. These are
291 unequivocally absent from the region. The edible dormouse is only present in southern
292 England and the grey squirrel is not distributed that far north in Scotland (Mathews et
293 al., 2018).

294 Of the wild mammals in the Peak District, the water vole, field vole, wood mouse
295 and otter were found in two sites (P1 and P2). The red deer, pygmy shrew, common
296 shrew, water shrew, red squirrel (*Sciurus vulgaris*), grey squirrel, pine marten and
297 badger were each found at a single site (Fig. S5). Only rabbit was found in site P3. All
298 species identified are currently distributed within the Park (Table S1), except the red
299 squirrel and pine marten. The pine marten, which is critically endangered in England,
300 has only two reliable records that have been confirmed in the Park since 2000 and the
301 red squirrel has not been present for over 18 years (Alston et al. 2012).

302 Overall, water samples yielded better results than sediment samples regarding
303 species detection and read count for both areas sampled (Figs 1B and S5). In Assynt,
304 only the wild/domestic cat was exclusively detected in sediment samples (four sites),
305 whereas water samples recovered eDNA for ten additional species not found in the
306 sediment samples. The red deer, water vole, field vole, mountain hare and pygmy
307 shrew were also found in sediment samples in Assynt (Fig. 1B), and water vole and
308 wood mouse in the Peak District sediment samples (Fig. S5).

309

310 **Occupancy Analysis**

311 Of the 18 sites where both latrine and eDNA surveys were conducted, water voles
312 were detected at 13, and field voles were detected at 11. A total of seven wild
313 mammals were recorded at the seven sites with a camera trap from July 10th to
314 October 25th, 2017 (Fig. S3 and Table S5). There were several incidences where a
315 shrew could not be identified to species level using camera traps. For camera traps,
316 water voles were recorded at all sites, red deer at five out of seven, field voles and
317 weasels at three sites, water shrews and otters at two, and a red fox at a single site.

318 For the 18 sites in Assynt, estimated site occupancy (with 95% confidence
319 intervals) from the combined surveying methods was 0.91 (0.63 – 0.98) for water voles
320 and 0.88 (0.57 – 0.98) for field voles. Red deer were observed at every patch by at
321 least one of the methods, and therefore occupancy was 1 (Table 1). For all three
322 species, per sample detection probability was higher for eDNA taken from water than
323 for eDNA taken from sediment (Table 1, Fig. 2). The surveying method specific
324 efficacy pattern was similar for water voles and field voles (Table 1, Fig. 2): latrine
325 surveys had the highest probability of detecting the species (0.77 and 0.52
326 respectively), followed by eDNA from water (0.57 and 0.40 respectively), then camera
327 trapping (0.50 and 0.20 respectively), and finally eDNA from sediment (0.27 and 0.02
328 respectively). Detection probability was higher for water voles than field voles using all
329 four methods (Table 1, Fig. 2). No effort was made to record red deer presence during
330 latrine surveys. Like the water voles and field voles, red deer detection has higher
331 using eDNA from water (0.67, CI: 0.53 – 0.78) compared to eDNA from sediment (0.10,
332 CI: 0.04 – 0.21). Unlike the voles, which were more detectable by cameras than
333 sediment eDNA, red deer detection on cameras was similar to sediment eDNA (0.10,
334 CI: 0.04 – 0.24).

335 The patterns described above detail surveying event-specific detectability. We
336 also computed the cumulative detection probability for each method and each species
337 (\hat{p}_{sm}). The cumulative detection curves over 15 surveying events are shown in Fig. 2.
338 The number of surveying events, k , required to achieve $p_{psm}^* \geq 0.95$ for water voles
339 was 3 surveys, 4 samples, 10 samples, and 5 weeks, for latrines, water eDNA,
340 sediment eDNA, and cameras respectively. The number of surveying events, k ,
341 required to achieve $p_{psm}^* \geq 0.95$ for field voles was 5 surveys, 6 samples, 141 samples,
342 and 14 weeks, for latrines, water eDNA, sediment eDNA, and cameras respectively.

343 The number of surveying events, k , required to achieve $p_{psm}^* \geq 0.95$ for red deer was
344 3 samples, 30 samples, and 29 weeks, for water eDNA, sediment eDNA, and cameras
345 respectively (see also Fig. 2).

346 **Discussion**

347 Despite the increasing potential of eDNA metabarcoding as a biomonitoring tool
348 (Deiner et al., 2017), its application has largely been focused on strictly aquatic or
349 semi-aquatic animals, thus restricting management and conservation efforts of the
350 wider ecosystem (Williams et al., 2018). Here, we demonstrate the ability of eDNA
351 metabarcoding to provide a valuable 'terrestrial dividend' for mammals from freshwater
352 lotic ecosystems, with a large proportion of the expected species from the wider
353 landscape being detected in each of the two study locations. In particular, we have
354 demonstrated that water-based eDNA sampling offers a promising and
355 complementary tool to conventional survey methods for the detection of whole
356 mammalian communities.

357

358 *Detection of mammalian communities using eDNA metabarcoding*

359 Of the species known to be common in both Assynt and the Peak District, eDNA
360 metabarcoding readily detected the water vole, field vole and red deer at the majority
361 of sites surveyed (Figs. 1B and S5). Pygmy, common and water shrews, wood mice
362 and mountain hares were also detected by eDNA metabarcoding at multiple sites in
363 Assynt (Fig. 1B). A higher eDNA detection rate is expected for aquatic and semi-
364 aquatic mammals compared to terrestrial mammals in aquatic environments due to
365 the spatial and temporal stochasticity of opportunities for terrestrial mammals to be in
366 contact with the water (Ushio et al., 2017). The semi-aquatic water vole was generally
367 detected by eDNA metabarcoding where we expected to find it and at relatively high
368 read numbers (Figs. 1B, S1 and S5). This is in line with previous studies in lentic
369 systems (Harper et al., 2019). However, the red deer was the only terrestrial species

370 detected by eDNA sampling at all sites in Assynt, and the terrestrial field vole at over
371 70% of surveyed sites.

372 In addition to lifestyle (semi-aquatic or terrestrial), the number of individuals of
373 each species (i.e. group-living) may be important for eDNA detection (Williams et al.,
374 2018). As a counter example to this, otters and weasels were notably absent in the
375 eDNA samples in Assynt despite being captured by camera traps (Fig. S3 and Table
376 S5). Otters were present in the water eDNA samples at two sites in the Peak District,
377 albeit at a lower number of reads in comparison to most of the other species detected
378 (Fig. S5; Table S2). This mirrors previous studies where eDNA analysis has performed
379 relatively poorly for otter detection in captivity and the wild (Harper et al., 2019;
380 Thomsen et al., 2012). Carnivores were generally detected on fewer occasions (e.g.
381 red foxes, badgers and pine martens; Figs. 1B and S5) or not at all (e.g. stoats and
382 American mink in addition to those discussed above) in comparison to smaller
383 mammals and red deer, and a similar pattern has been shown with North American
384 carnivores in a recent study using eDNA from soil samples (Leempoel et al., 2019).
385 For some of these species, species ecology/behavior such as a relatively large home
386 range and more solitary nature (e.g. red foxes) may go some way towards explaining
387 a lack of, or few, eDNA records. Furthermore, as demonstrated by Ushio et al. (2017)
388 poor efficiency for amplifying some mammal species might be associated to
389 suboptimal experimental conditions (e.g. inadequate primer design, primer bias, DNA
390 concentration, species masking and/or annealing temperatures).

391 Regarding the sampling medium for eDNA, we demonstrated that water is a
392 more effective method for detection of mammal eDNA than sediment (Table 1; Figs.
393 1B and S5). For one of our focal species, the water vole, 75% of sites which were
394 deemed unoccupied by latrine surveys and those with ≤ 2 individuals (8 sites) in

395 Assynt, returned a non-detection for sediment eDNA as opposed to 37.5% of sites for
396 water (Figs. 1A, 1B and S1). Distinct temporal inferences are provided by eDNA
397 recovered from water and sediment samples. DNA bound to sediments can remain
398 detectable for a longer period (i.e. up to hundreds of years) and provide historical data,
399 whereas, eDNA retrieved from water samples provide more contemporary data due to
400 a faster degradation in the water column (Turner et al., 2015). It is worth investigating
401 further if sediment eDNA could indicate the presence of a more 'established'
402 population, where a certain threshold of individuals and long-term occupation (i.e.
403 historical) is required for detection in sediment (Fig. S1; Turner et al., 2015; Leempoel
404 et al., 2019).

405 Importantly, sparse or single eDNA records should be carefully verified. The
406 edible dormouse and grey squirrel sequences identified within the Assynt samples
407 (Fig. 1B) and red squirrel within the Peak District (Fig. S5) highlights the caveats
408 associated with this technique. If management decisions had relied on eDNA evidence
409 alone, false positives for these species could lead to unnecessary resources being
410 allocated for management/eradication programmes as the edible dormouse and grey
411 squirrel are classified as invasive species within Great Britain. These potentially arose
412 due to sample carryover from a previous sequencing run on the same instrument (a
413 known issue with Illumina sequencing platforms; Nelson et al., 2014) which included
414 those species for the reference database construction. Controlling for false positives
415 is certainly a huge challenge in eDNA metabarcoding and the need to standardize and
416 optimize thresholds for doing so is an ongoing debate (Ficetola et al., 2015; Harper et
417 al., 2019).

418 Even with these concerns around false positives highlighted, two records are
419 potentially noteworthy in a conservation context for UK mammals because of the

420 relatively high read number associated with these records (Tables S2 and S3). The
421 first of these is the *Felis* records in sediment samples in multiple sites in Assynt (Fig.
422 1B). Even with 'pure' *F. silvestris* as reference sequences, it was not possible to
423 distinguish between the wild and domesticated species for this 12S fragment (data not
424 shown). Despite ongoing conservation efforts, there may now be no 'pure' Scottish
425 wildcats left in the wild in the UK but isolated populations (perhaps of hybrid origin)
426 may exist in this region (Sainsbury et al., 2019). Given that these eDNA detections
427 were all from sediment samples, it is possible that they may be historical rather than
428 contemporary (see above). The other significant eDNA record was the pine marten in
429 the Peak District. The pine marten (*Martes martes*) is known to occur in the Scottish
430 Highlands but had disappeared from most of the UK and recently has been recovering
431 from historical persecution, including a potential expansion of its range. Still, authentic
432 records from northern England are scarce or lacking altogether (Alston et al., 2012;
433 Sainsbury et al., 2019). However, a record of a recent roadkill exists from just outside
434 the Park's boundary (BBC News, 2018). The high number of reads recovered for the
435 Peak District sample (4293 reads versus 25 in the Assynt sample) adds credence to
436 this positive eDNA detection but further investigations are warranted into the potential
437 presence of this species in the area.

438

439 *Comparisons between surveying methods*

440 Comparisons of species detection by traditional survey approaches and eDNA
441 analysis are now numerous in the literature, and mainly focus on what is and what is
442 not detected within and across different methods (Hänfling et al., 2016; Leempoel et
443 al., 2019). Yet, there has been growing incorporation of occupancy modelling to
444 estimate the probability of detecting the focal species, in comparison to one other

445 survey method, either for a single species (Lugg et al., 2018; Tingley et al., 2019) or
446 multiple species (Valentini, et al., 2016; Abrams et al., 2019). Simultaneous multi-
447 method comparisons for multiple species have been lacking and this study directly
448 addresses this for the first time.

449 The probability of detecting the water vole and field vole was higher for the
450 latrine surveys than eDNA sampling (both water and sediment) and camera traps
451 (Table 1; Fig. 2). However, when considering confidence intervals, there was
452 considerable overlap between latrine, water-based eDNA metabarcoding and camera
453 traps for both species, with only sediment-based eDNA metabarcoding yielding a low
454 probability of detection (Table 1). Detection probabilities for water-based eDNA
455 metabarcoding and camera traps were similar for water voles, with camera traps less
456 likely to detect the field vole than water-based eDNA. For the red deer (for which no
457 latrine survey was undertaken), water-based eDNA metabarcoding had a much higher
458 probability of detection than either sediment-based eDNA metabarcoding or camera
459 traps (which performed similarly; Table 1). Despite the increasing adoption of camera
460 traps in providing non-invasive detections for mammals (Hofmeester et al., 2019),
461 camera traps were outperformed by water-based eDNA metabarcoding for the three
462 focal species in this component of the study. Here, camera traps were deployed so as
463 to sample the habitat of the water vole (see Fig. S3), which may explain lower detection
464 for other terrestrial species in comparison to eDNA metabarcoding (see above).
465 Studies focusing on a single species often report that eDNA analysis outperforms the
466 conventional survey method in terms of detection probabilities (e.g. Lugg et al., 2018).
467 For metabarcoding, there is clearly a need to carefully consider the potential for cross
468 contamination between samples and how false positives (and negatives) could impact
469 detection probabilities using occupancy modelling with eDNA data (Brost et al., 2018;

470 Lahoz-Monfort et al., 2016). Among the recommendations made by Lahoz-Monfort et
471 al. (2016) to account for these uncertainties, one was the simultaneous collection of
472 data from more conventional surveying methods. Here, we have demonstrated
473 general congruence between surveying methods for the water vole (Table S5; Fig. S1)
474 and using certain species to apply a multiple detection methods model would be
475 appropriate in further studies (Lahoz-Monfort et al., 2016). Alternatively, using
476 repeated sampling and known negative controls in occupancy models that fully
477 incorporate false-positive errors could be applied in the absence of other surveying
478 data (Brost et al., 2018). Overall, multi-species metabarcoding studies may trade-off
479 a slightly lower (but comparable) detection probability than other survey methods for
480 individual species (Fig. 2) in favor of a better overall “snapshot” of occupancy of the
481 whole mammalian community (Ushio et al., 2017).

482 The comparison between survey ‘effort’ for the four methods to reach a
483 probability of detection of ≥ 0.95 is highly informative and provides a blueprint for future
484 studies on mammal monitoring. Focusing on the water vole for example, three latrine
485 surveys would be required. A total of four water-based and 10 sediment-based eDNA
486 replicates or five weeks of camera trapping would be required to achieve the same
487 result (Fig. 2). This increases for the field vole in the same habitat, with five latrine
488 surveys and six water-based eDNA replicates. Sediment-based eDNA metabarcoding
489 would be impractical for this species and camera trapping would take 14 weeks. What
490 is important here is the spatial component and the amount of effort involved in the
491 field. Taking 4-6 water-based eDNA replicates from around one location within a patch
492 could provide the same probability of detecting these small mammals with three latrine
493 surveys. In many river catchments, there may be 100s to 1000s of kilometers to survey
494 that would represent suitable habitat, and only a fraction of that may be occupied by

495 any given species. This is particularly relevant in the context of recovery of water vole
496 populations post-translocation or in situations where remnant populations are
497 bouncing back after invasive American mink (*Neovison vison*) control has been
498 instigated. On a local scale, finding signs of water voles through latrine surveys is not
499 necessarily difficult, but monitoring the amount of potential habitat (especially lowland)
500 for a species which has undergone such a massive decline nationally is a huge
501 undertaking (Morgan et al., 2019).

502 The use of eDNA metabarcoding from freshwater systems to generate an initial,
503 coarse and rapid 'distribution map' for vertebrate biodiversity (and at a relatively low
504 cost) could transform biomonitoring at the landscape level. For group-living (i.e. deer)
505 and small mammal species, carefully chosen sampling points (with at least five water-
506 based replicates) along multiple river courses could provide a reliable indication of
507 what species are present in the catchment area if conducted during times of peak
508 abundance (i.e. Summer and Autumn). Then, on the basis of this, practitioners could
509 choose to further investigate specific areas for confirmation of solitary, rare or invasive
510 species (e.g. carnivores) with increased effort in terms of both the number of sampling
511 sites and replicates taken. At present, we would recommend the use of eDNA
512 metabarcoding alongside other non-invasive surveying methods (e.g. camera traps)
513 when monitoring invasive species or species of conservation concern to maximize
514 monitoring efforts (Abrams et al., 2019; Sales et al., 2019).

515 It is clear that eDNA metabarcoding is a promising tool for monitoring semi-
516 aquatic and terrestrial mammals in both lotic (this study) and lentic systems (Harper
517 et al., 2019; Ushio et al., 2017). We detected a large proportion of the expected
518 mammalian community (Table S1). Water-based eDNA metabarcoding is comparable
519 or out-performs other non-invasive survey methods for several species (Fig. 2).

520 However, there remain challenges for the application of this technique over larger
521 spatial and temporal scales. Technical issues of metabarcoding in laboratory and
522 bioinformatic contexts have been dealt with elsewhere (Harper et al., 2019) but
523 understanding the distribution of eDNA transport in the landscape and its entry into
524 natural lotic systems is at an early stage (and incorporating such variables in
525 occupancy modelling approaches). This clearly requires more detailed and systematic
526 eDNA sampling than undertaken here, particularly in an interconnected river/stream
527 network with organisms moving between aquatic and terrestrial environments.
528 Leempoel et al. (2019) recently demonstrated the feasibility for detecting terrestrial
529 mammal eDNA in soil samples but this study has shown that sampling a few key areas
530 in freshwater ecosystems (e.g. larger rivers and lakes) within a catchment area could
531 potentially provide data on a large proportion (if not all) of the mammalian species
532 within it, even when some species are present at low densities (Deiner et al., 2017).
533 In this regard, future studies might also investigate the value of citizen science, where
534 trained volunteers can contribute to data collection at key sites, thus scaling up the
535 reach of research whilst raising public awareness and the significance of mammalian
536 conservation concerns (Parsons et al., 2018).

538 **Data accessibility**

539 Data will be made available in Dryad upon acceptance.

540

541 **Authors contributions**

542 ADM, XL, CS, OSW, IC, SM, NGS, SSB, EO, BH and LLH conceived the study.

543 Monitoring and live-trapping of water voles was part of XL, CS, EB and JD's ongoing

544 work in Assynt. JD analysed the camera trap data. DAD advised on primer set/data

545 validation and provided information and data on mammals in the Peak District. ADM,

546 NGS, SSB and MBM carried out the eDNA sampling. MBM, NGS, SSB, CB and ADM

547 performed the laboratory work. NGS, OSW, LRH, MBM, CB and ADM carried out the

548 bioinformatic analyses. NGS, ADM, IC and MBM analysed the eDNA data. CS and JD

549 conducted the occupancy modelling. ADM, NGS, CS, JD, MBM and LRH wrote the

550 paper, with all authors contributing to editing and discussions.

551

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563 anonymous reviewers for significantly improving the manuscript.

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682

683 **Tables**

684

685 Table 1. Estimated site occupancies and detection probabilities obtained for water-
 686 based eDNA (w-eDNA), sediment-based eDNA (s-eDNA) and conventional survey
 687 methods (Latrine and Camera) in Assynt.

Species	Occupancy	Detection probability			
		<i>Latrine</i>	<i>w-eDNA</i>	<i>s-eDNA</i>	<i>Camera</i>
Water vole	0.91 (0.63 – 0.98)	0.77 (0.59 – 0.89)	0.57 (0.43 – 0.71)	0.27 (0.16 – 0.41)	0.50 (0.35 – 0.65)
Field vole	0.89 (0.57 – 0.98)	0.52 (0.34 – 0.69)	0.40 (0.26 – 0.55)	0.02 (0.00 – 0.14)	0.20 (0.10 – 0.37)
Red deer	1.00 (1.00 – 1.00)	--	0.67 (0.53 – 0.78)	0.10 (0.04 – 0.21)	0.10 (0.09 – 0.24)

688

689

690

FIGURES

Figure 1. Environmental DNA (eDNA) sampling sites in Assynt, Scotland (A). Categorical values for water vole abundance at each site based on live-trapping data. In (B), a bubble graph representing presence-absence and categorical values of the number of reads retained (after bioinformatic filtering) for eDNA (water in blue and sediment in orange) from each wild mammal identified in each site in Assynt (A1-A18).

Figure 2. The detection probabilities of each survey method (sediment-based eDNA, water-based eDNA, latrine and camera) for each of three focal species (from top to bottom on the left); water vole; field vole and red deer. On the right, the accumulation curves for each species for the number of sampling events for each survey method to provide a ≥ 0.95 probability of detection.

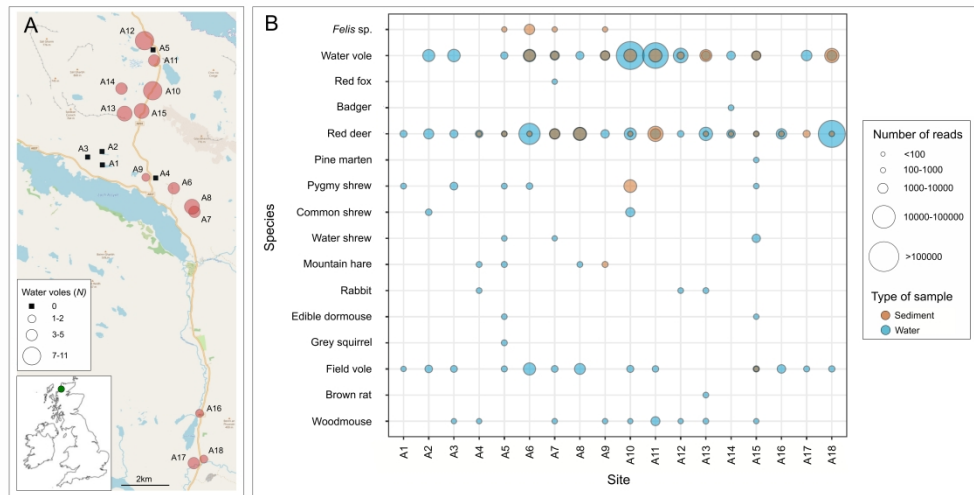


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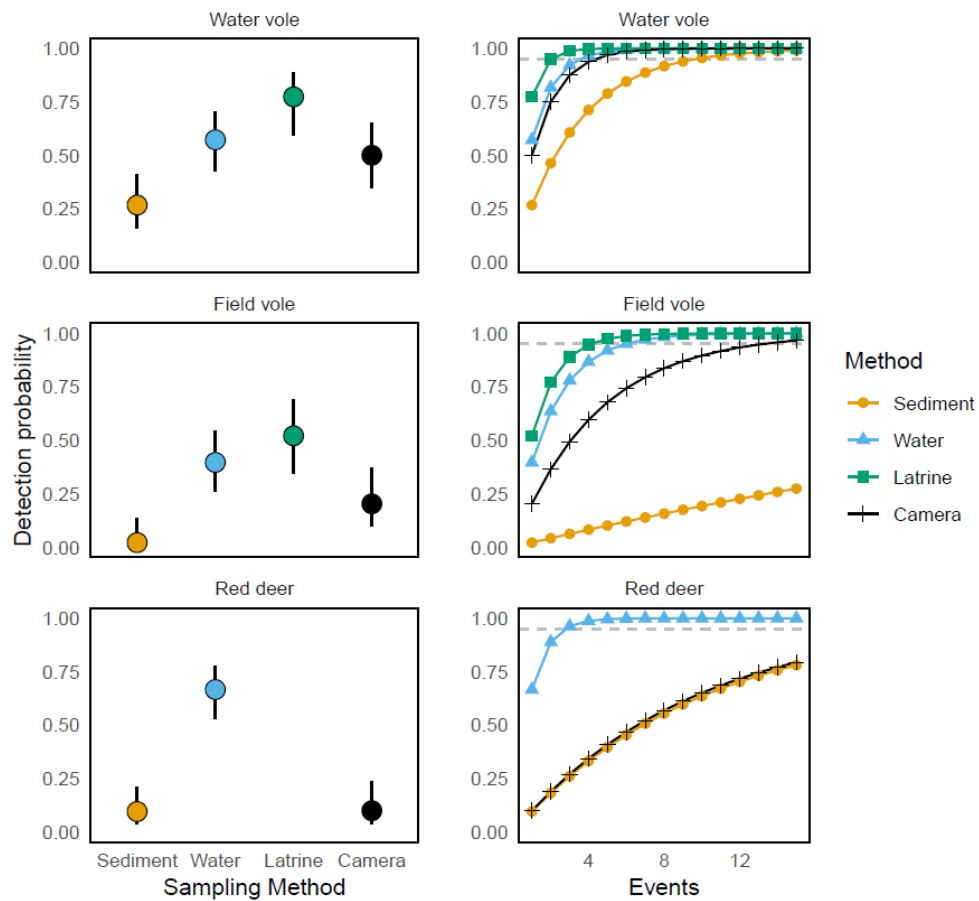


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SUPPLEMENTARY MATERIAL**Fishing for mammals: landscape-level monitoring of terrestrial and semi-aquatic communities using eDNA from lotic ecosystems**

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24 **Appendix 1**

25

26 **eDNA sample collection**

27 Three water sample replicates (two litres each) and three sediment sample replicates
28 (50 ml falcon tube, approximately half-filled) were taken at each site in Assynt, always
29 within a reachable distance from the river's edge and at a depth where sediment
30 samples could be taken (Fig. S4A). Water samples were filtered on site using a
31 Sterivex 0.45 µm filter unit (Merck Millipore) and filters were stored in silica beads in
32 the field (1-3 days; Majaneva et al., 2018) then frozen until DNA extraction. Sediment
33 samples were stored in 100% ethanol. Appropriate decontamination precautions were
34 taken including the use of disposable gloves and decontamination of all equipment
35 and surfaces by using 50% bleach solution). Samples from the Peak District were
36 filtered within 5 hours in the University of Salford laboratory facilities due to its close
37 proximity to the sampling locations. A single filter was used for each replicate in Assynt
38 and the Peak District, and the volume filtered varied between each, ranging from 150
39 ml to 2 L (see Tables S2 and S3). Negative field controls were taken in both Assynt
40 (N= 8) and the Peak District (N= 2) and were obtained by collecting, preserving and
41 processing distilled water in exactly the same way as the field samples. The amount
42 of sediment collected also varied, with 4 to 10g used in the extractions. A Pearson's
43 correlation was performed to determine if the amount of water/sediment influenced the
44 amount of retained reads for mammals after bioinformatic filtering.

45

46 **Reference database**

47 Given that this project proposed to use mammal-specific primers (MiMammal-U, Ushio
48 et al., 2017) to target the same region of 12S as the MiFish primers (Miya et al., 2015),

49 an *in silico* evaluation was first performed using ecoPCR (Ficetola et al., 2010) of the
50 MiMammal-U primer set against a custom, phylogenetically curated reference
51 database for mammals distributed in the UK and Ireland. This database was one of
52 several databases constructed for UK vertebrates and used in an eDNA
53 metabarcoding study of pond biodiversity (see Harper et al. 2019 for details). The
54 mammal database was updated in July 2018 for the purposes of the present study.
55 Parameters were set to allow a fragment size of 50-250 bp and different number of
56 mismatches (0, 1, 2, 3) between each primer and each sequence in the reference
57 database. Reference sequence data was available for 103 mammal species (91.96%)
58 in the UK. The nine species that were not represented were either cetaceans or bats.
59 Of those species with reference sequence data (N = 103), 44 (42.72%), 65 (63.11%),
60 72 (69.90%), and 82 (79.61%) mammals were amplified when 0, 1, 2, and 3 primer-
61 sequence mismatches were allowed respectively. Species that did not amplify under
62 any scenario due to the lack of an appropriate reference sequence for the specific 12S
63 region being targeted for MiMammal (and of relevance to this study) were the
64 European water vole (*Arvicola amphibius*), greater white-toothed shrew (*Crocidura*
65 *russula*), Millet's shrew (*Sorex coronatus*), Eurasian pygmy shrew (*Sorex minutus*),
66 field vole (*Microtus agrestis*), common vole (*Microtus arvalis*), grey squirrel (*Sciurus*
67 *carolinensis*), and European polecat (*Mustela furo*).

68 Because certain focal mammalian species were missing from online reference
69 databases, a new reference database of 32 UK terrestrial mammals targeting this
70 fragment of the 12S gene was created from ethanol-preserved tissues samples
71 obtained from National Museums Scotland (Table S6). DNA was extracted using the
72 ISOLATE II kit according to the manufacturer's protocol. These DNA samples were
73 then included in a large vertebrate barcoding project using the MiFish (Miya et al.,

74 2015) primers (O. Wangensteen et al., *unpublished data*). Although these primers
75 were originally designed to amplify fishes, they are known to amplify mammals also
76 and target the exact same region as the MiMammal primers (Ushio et al., 2017). This
77 was conducted to save on sequencing costs and the prior knowledge that these
78 primers would generate reference sequences for the majority of UK mammals
79 (Hänfling et al., 2016). Of these mammals, only *Sorex araneus* and *Neomys fodiens*
80 failed to generate reference sequences. PCRs were then carried out on a subset of
81 the tissue-extracted DNA (see Table S6) and Sanger-sequenced (Macrogen Inc.)
82 using the MiMammal-U primers (Ushio et al., 2017) to confirm the results obtained
83 with the MiFish primers.

84

85 ***eDNA Laboratory Methods***

86 ***Field and Laboratory controls***

87 In order to avoid the risk of contamination, clean and consistent field and laboratory
88 protocols are paramount. Besides the decontamination measures taken, three types
89 of negative controls (field, extraction and PCR) were included. Field blanks comprised
90 of distilled water which was preserved and processed using exactly the same protocols
91 and equipment as the field samples. These were processed first to ascertain if
92 contaminations arose in the field (either during the water/sediment sampling or during
93 the filtering process). DNA extraction blanks, represented by empty tubes included in
94 the extraction step, were undertaken at the end of each batch of extractions to
95 ascertain the potential for contaminations arising from reagents and the laboratory
96 environment. Finally, no-template amplification controls (NTC) were included during
97 the amplification step (PCR) of the actual samples through the inclusion of several
98 reactions lacking DNA to account for putative contamination during this procedure.

99 The chronology of DNA extraction followed an increasing order of expected
100 abundance in the eDNA samples (all field blanks extracted first, followed by the sites
101 with supposedly zero water vole abundance, up to the highest densities last). Field
102 blanks were processed at the beginning of the DNA extraction to try to tease apart the
103 potential contamination between field and lab contaminations. The implementation of
104 this chronology was due to the fact that it is the first time a study focusing on using
105 eDNA with terrestrial and semi-aquatic mammals has been undertaken like this in
106 multiple sites that were sampled in the same session, with the researchers moving
107 around in the habitat (terrestrial) of the target group of organisms.

108

109 ***eDNA amplification and sequencing***

110 A set of 96 primers pairs with seven-base sample-specific MIDNs and a variable number
111 (2-4) of fully degenerate positions (leading Ns) to increase variability in amplicon
112 sequences were used. PCR amplification was conducted using a single-step protocol
113 and to minimize bias in individual reactions, PCRs were replicated three times for each
114 sample and subsequently pooled. The PCR reaction consisted of a total volume of 20
115 μl including 10 μl Amplitaq; 0.16 μl of BSA; 1.0 μl of each of the two primers (5 μM);
116 5.84 μl of ultra-pure water, and 2 μl of DNA template. The PCR profile included an
117 initial denaturing step of 95°C for 10 min, 40 cycles of 95°C for 30s, 60°C for 45s, and
118 72°C for 30s and a final extension step of 72°C for 5 min. Amplification were checked
119 through electrophoresis in a 1.5% agarose gel stained with GelRed (Cambridge
120 Bioscience). PCR products were pooled in two different sets and a left-sided size
121 selection was performed using 1.1x Agencourt AMPure XP (Beckman Coulter).
122 Illumina libraries were built from each set, using a NextFlex PCR-free library
123 preparation kit according to the manufacturer's protocols (Bioo Scientific). Libraries

124 were then quantified by qPCR using a NEBNext qPCR quantification kit (New England
125 Biolabs) and pooled in equimolar concentrations along with 1% PhiX (v3, Illumina).
126 The libraries were run at a final molarity of 9pM on an Illumina MiSeq platform using
127 the 2 x 150bp v2 chemistry.

128

129 ***Bioinformatic analysis***

130 OBITools metabarcoding package (Boyer et al., 2016) was used for the bioinformatic
131 analysis. Quality of the reads was assessed using FastQC, paired-end reads were
132 aligned using illumina-paired-end and the ngsfilter command was used for dataset
133 demultiplexing. Short fragments originated from library preparation artefacts (primer-
134 dimer, non-specific amplifications) and reads containing ambiguous bases were
135 removed applying a length filter selecting fragments of 140-190bp using obrigrep.
136 Clustering of strictly identical sequences was performed using obiuniq and a chimera
137 removal step was applied in vsearch (Rognes et al., 2016) through the uchime-denovo
138 algorithm (Edgar et al., 2011). The taxonomic assignment was conducted using
139 ecotag.

140 A stringent approach was applied to our analyses to avoid false positives and
141 exclude MOTUs/reads putatively belonging to sequencing errors or contamination.
142 The final dataset included only MOTUs that could be identified to species level (>0.98),
143 and MOTUs containing less than 10 reads and with a similarity to a sequence in the
144 reference database lower than 98% were discarded (Cilleros et al., 2019). Singleton
145 reads within individual replicates were also discarded. The maximum number of reads
146 detected in the controls for each MOTU in each sequencing run were removed from
147 all samples (Table S7). For water voles, field voles and red deer (the most abundant
148 wild mammals in terms of sequence reads in our dataset), this equated to a sequence

149 frequency threshold of $\leq 0.17\%$, within the bounds of previous studies on removing
150 sequences to account for contamination and tag jumping (Cilleros et al., 2018; Schnell,
151 Bohmann, & Gilbert, 2015). The number of retained reads per replicate was not
152 significantly correlated with the volume of water filtered (Pearson's correlation: $r =$
153 0.213 ; $p = 0.094$) or the amount of sediment collected (Pearson's correlation: $r = 0.076$;
154 $p = 0.556$).

155 **TABLES**

156

157 **Table S1.** Species (and the Order to which they belong) that are expected to be found

158 within Assynt (based on Matthews et al. 2018) and the Peak District (Alston et al. 2012)

159 and whether or not they were detected by eDNA. A * indicates species where presence

160 is uncertain from Matthews et al. (2018).

161

Common name	Scientific name	Order	eDNA
Assynt			
Red deer	<i>Cervus elaphus</i>	Artiodactyla	Yes
Sika deer	<i>Cervus nippon</i>	Artiodactyla	No
Roe deer	<i>Capreolus capreolus</i>	Artiodactyla	No
Water vole	<i>Arvicola amphibius</i>	Rodentia	Yes
Field vole	<i>Microtus agrestis</i>	Rodentia	Yes
Wood mouse	<i>Apodemus sylvaticus</i>	Rodentia	Yes
Bank vole*	<i>Myodes glareolus</i>	Rodentia	No
Brown rat	<i>Rattus norvegicus</i>	Rodentia	Yes
Pygmy shrew	<i>Sorex minutus</i>	Eulipotyphla	Yes
Water shrew	<i>Neomys fodiens</i>	Eulipotyphla	Yes
Common shrew	<i>Sorex araneus</i>	Eulipotyphla	Yes
Hedgehog*	<i>Erinaceus europaeus</i>	Eulipotyphla	No
European mole	<i>Talpa europaea</i>	Eulipotyphla	No
Mountain hare	<i>Lepus timidus</i>	Lagomorpha	Yes
European rabbit	<i>Oryctolagus cuniculus</i>	Lagomorpha	Yes
Stoat	<i>Mustela erminea</i>	Carnivora	No
Weasel	<i>Mustela nivalis</i>	Carnivora	No
Badger	<i>Meles meles</i>	Carnivora	Yes
Otter	<i>Lutra lutra</i>	Carnivora	No
Red fox	<i>Vulpes vulpes</i>	Carnivora	Yes
Pine marten	<i>Martes martes</i>	Carnivora	Yes
Wildcat*	<i>Felis silvestris</i>	Carnivora	?
Peak District			
Red deer	<i>Cervus elaphus</i>	Artiodactyla	Yes
Roe deer	<i>Capreolus capreolus</i>	Artiodactyla	No
Fallow deer	<i>Dama dama</i>	Artiodactyla	No
Water vole	<i>Arvicola amphibius</i>	Rodentia	Yes
Field vole	<i>Microtus agrestis</i>	Rodentia	Yes
Wood mouse	<i>Apodemus sylvaticus</i>	Rodentia	Yes
Bank vole	<i>Myodes glareolus</i>	Rodentia	No
Brown rat	<i>Rattus norvegicus</i>	Rodentia	No
House mouse	<i>Mus musculus</i>	Rodentia	No
Grey squirrel	<i>Sciurus carolinensis</i>	Rodentia	Yes
Harvest mouse*	<i>Micromys minutus</i>	Rodentia	No
Pygmy shrew	<i>Sorex minutus</i>	Eulipotyphla	Yes
Water shrew	<i>Neomys fodiens</i>	Eulipotyphla	Yes
Common shrew	<i>Sorex araneus</i>	Eulipotyphla	Yes
Hedgehog	<i>Erinaceus europaeus</i>	Eulipotyphla	No
European mole	<i>Talpa europaea</i>	Eulipotyphla	No

Mountain hare	<i>Lepus timidus</i>	<i>Lagomorpha</i>	No
Brown hare	<i>Lepus europaeus</i>	<i>Lagomorpha</i>	No
European rabbit	<i>Oryctolagus cuniculus</i>	<i>Lagomorpha</i>	Yes
Stoat	<i>Mustela erminea</i>	<i>Carnivora</i>	No
Weasel	<i>Mustela nivalis</i>	<i>Carnivora</i>	No
Badger	<i>Meles meles</i>	<i>Carnivora</i>	Yes
Otter	<i>Lutra lutra</i>	<i>Carnivora</i>	Yes
Red fox	<i>Vulpes vulpes</i>	<i>Carnivora</i>	No
American mink	<i>Neovison vison</i>	<i>Carnivora</i>	No
Pine marten	<i>Martes martes</i>	<i>Carnivora</i>	Yes
Polecat	<i>Mustela putorius</i>	<i>Carnivora</i>	No

162

163

164 **Table S2.** Species identified (with at least 98% identity to the reference database) and
165 their associated number of reads after bioinformatic filtering in each site (Assynt A1-
166 A18 and Peak District P1-P3) and in each of three replicates (_1 to _3) for water-based
167 eDNA. The volume of water filtered is indicated for each replicate.

168 *Additional file: TableS2_Reads_Water.xlsx*

169

170 **Table S3.** Species identified (with at least 98% identity to the reference database) and
171 their associated number of reads after bioinformatic filtering in each site (Assynt A1-
172 A18 and Peak District P1-P3) and in each of three replicates (_1 to _3) for sediment-
173 based eDNA. The weight of sediment used for the DNA extraction is indicated for each
174 replicate.

175 *Additional file: TableS3_Reads_Sediment.xlsx*

176

177 **Table S4.** Number of reads obtained after all filtering steps applied to remove non-
 178 target MOTUs.

WATER	Total
Total Reads	13,336,064
After removing reads from the blanks	10,709,199
After removing non-mammal reads	10,262,851
After removing human reads	8,508,564
After removing domestic animals (<i>Sus</i> , <i>Bos</i> , <i>Equus</i> , <i>Ovis</i> , <i>Canis</i>)	5,544,208
MOTUs with minimum identity of 0.98	5,414,427

SEDIMENT	Total
Total Reads	3,309,866
After removing reads from the blanks	1,684,433
After removing non-mammal reads	1,543,826
After removing human reads	649,499
After removing domestic animals (<i>Sus</i> , <i>Bos</i> , <i>Equus</i> , <i>Ovis</i> , <i>Canis</i>)	500,473
MOTUs with minimum identity of 0.98	465,997

179

180

181

182

183 **Table S5.** Mammalian species recorded at seven camera traps in Assynt. Boxes
 184 shaded in grey represent sites where each species was recorded.

185

Common name	Scientific name	Site						
		A5	A10	A11	A12	A13	A14	A15
Water vole	<i>Arvicola amphibius</i>							
Red deer	<i>Cervus elaphus</i>							
Field vole	<i>Microtus agrestis</i>							
Water shrew	<i>Neomys fodiens</i>							
Weasel	<i>Mustela nivalis</i>							
Otter	<i>Lutra lutra</i>							
Red fox	<i>Vulpes vulpes</i>							
Unidentified Shrew	-							

186

187

188 **Table S6.** List of tissue samples from mammals used for generating a local reference
 189 database using MiFish primers (Miya et al. 2015). All species were tested for
 190 amplification using MiMammal-U primers (Ushio et al. 2017) and those highlighted in
 191 bold were Sanger-sequenced.

192

Common name	Scientific name	ID
Wood mouse	<i>Apodemus sylvaticus</i>	Z.2009.101.1025
Wood mouse	<i>Apodemus sylvaticus</i>	Z.2009.101.1149M
House mouse	<i>Mus domesticus</i>	Z.2009.101.593M
House mouse	<i>Mus domesticus</i>	Z.2009.101.426
Field Vole	<i>Microtus agrestis</i>	Z.2009.101.1045
Field Vole	<i>Microtus agrestis</i>	Z.2009.101.1994M
Bank Vole	<i>Myodes glareolus</i>	Z.2009.101.97M
Bank Vole	<i>Myodes glareolus</i>	Z.2009.101.696M
Weasel	<i>Mustela nivalis</i>	Z.2009.101.664
Weasel	<i>Mustela nivalis</i>	Z.2009.101.363
Yellow-necked mouse	<i>Apodemus flavicollis</i>	Z.2009.101.983M
Yellow-necked mouse	<i>Apodemus flavicollis</i>	Z.2009.101.984M
Water shrew	<i>Neomys fodiens</i>	Z.2009.101.141M
Water shrew	<i>Neomys fodiens</i>	Z.2009.101.1915M
Pygmy shrew	<i>Sorex minutus</i>	Z.2009.101.1162M
Pygmy shrew	<i>Sorex minutus</i>	Z.2009.101.458M
Common shrew	<i>Sorex araneus</i>	Z.2009.101.611M
Common shrew	<i>Sorex araneus</i>	Z.2009.101.126M
Common Vole	<i>Microtus arvalis</i>	Z.2009.101.991
Common Vole	<i>Microtus arvalis</i>	Z.2009.101.917
Brown Rat	<i>Rattus norvegicus</i>	Z.2009.101.931
Brown Rat	<i>Rattus norvegicus</i>	Z.2009.101.1026
Grey Squirrel	<i>Sciurus carolinensis</i>	23/24
Grey Squirrel	<i>Sciurus carolinensis</i>	23/10
Water Vole	<i>Arvicola amphibius</i>	23/15
Water Vole	<i>Arvicola amphibius</i>	23/17
Edible dormouse	<i>Glis glis</i>	23/16
Edible dormouse	<i>Glis glis</i>	23/35
Brown hare	<i>Lepus europaeus</i>	23/22
Mountain hare	<i>Lepus timidus</i>	23/20
Mountain hare	<i>Lepus timidus</i>	23/1
Hedgehog	<i>Erinaceus europaeus</i>	23/19
Mole	<i>Talpa europaea</i>	23/13
Mole	<i>Talpa europaea</i>	23/14
Red fox	<i>Vulpes vulpes</i>	23/25
Badger	<i>Meles meles</i>	23/12
Badger	<i>Meles meles</i>	23/34
Otter	<i>Lutra lutra</i>	23/7
Otter	<i>Lutra lutra</i>	23/33
Polecat	<i>Mustela putorius</i>	23/5
Polecat	<i>Mustela putorius</i>	23/6
Red deer	<i>Cervus elaphus</i>	23/31
Red deer	<i>Cervus elaphus</i>	23/32
Sheep	<i>Ovis aries</i>	23/9
Horse	<i>Equus caballus</i>	24/31
Red Squirrel	<i>Sciurus vulgaris</i>	1/24
Red Squirrel	<i>Sciurus vulgaris</i>	1/31

Pine marten	<i>Martes martes</i>	1/1
Pine marten	<i>Martes martes</i>	1/13
Coypu	<i>Myocastor coypus</i>	62/12
Coypu	<i>Myocastor coypus</i>	22/13
Brown hare	<i>Lepus europaeus</i>	22/7
Stoat	<i>Mustela erminea</i>	22/31
Stoat	<i>Mustela erminea</i>	22/33
Red fox	<i>Vulpes vulpes</i>	21/28
Hedgehog	<i>Erinaceus europaeus</i>	72/32
Sika	<i>Cervus nippon</i>	57/31
Horse	<i>Equus caballus</i>	57/24
Beaver	<i>Castor fiber</i>	63/25
Sheep	<i>Ovis aries</i>	58/31
American mink	<i>Neovison vison</i>	AMX01
American mink	<i>Neovison vison</i>	AMX02
Wildcat	<i>Felis silvestris</i>	Z.2015.118.1
Wildcat	<i>Felis silvestris</i>	Z.2015.118.2

194 **Table S7.** Maximum number of reads subtracted to control for contamination and/or
 195 tag switching for each wild species in each eDNA sampling type (water or sediment)
 196 and the type of blank in which the reads were identified (Field, Extraction and PCR).
 197 Species indicated by * were not identified as eDNA positive records.

198

Common name	Scientific name	Blank	Reads
Red deer	<i>Cervus elaphus</i>	Field	164
Water vole	<i>Arvicola amphibius</i>	Extraction	7479
Field vole	<i>Microtus agrestis</i>	Field	324
Wood mouse	<i>Apodemus sylvaticus</i>	None	0
Brown rat	<i>Rattus norvegicus</i>	None	0
Pygmy shrew	<i>Sorex minutus</i>	Field	1
Water shrew	<i>Neomys fodiens</i>	Extraction	1
Common shrew	<i>Sorex araneus</i>	Field	2
Mountain hare	<i>Lepus timidus</i>	Field	76
European rabbit	<i>Oryctolagus cuniculus</i>	Field	38
Stoat*	<i>Mustela erminea</i>	Field	68
Badger	<i>Meles meles</i>	None	0
Otter	<i>Lutra lutra</i>	Extraction	1
Red fox	<i>Vulpes vulpes</i>	None	0
Pine marten	<i>Martes martes</i>	None	0
Cat	<i>Felis</i> spp.	None	0
American mink*	<i>Neovison vison</i>	Extraction	343
Red squirrel	<i>Sciurus vulgaris</i>	Extraction	1
Grey squirrel	<i>Sciurus carolinensis</i>	None	0
Edible dormouse	<i>Glis glis</i>	None	0
Human 1	<i>Homo sapiens</i>	Field	547
Human 2	<i>Homo sapiens</i>	Field	110107
Human 3	<i>Homo sapiens</i>	Field	1
Cattle	<i>Bos</i> spp.	Extraction	1630
Sheep	<i>Ovis</i> spp.	Field	122
Pig	<i>Sus scrofa domesticus</i>	Field	99
Dog	<i>Canis lupus familiaris</i>	Field	135
Horse	<i>Equus przewalskii</i>	None	0

FIGURES

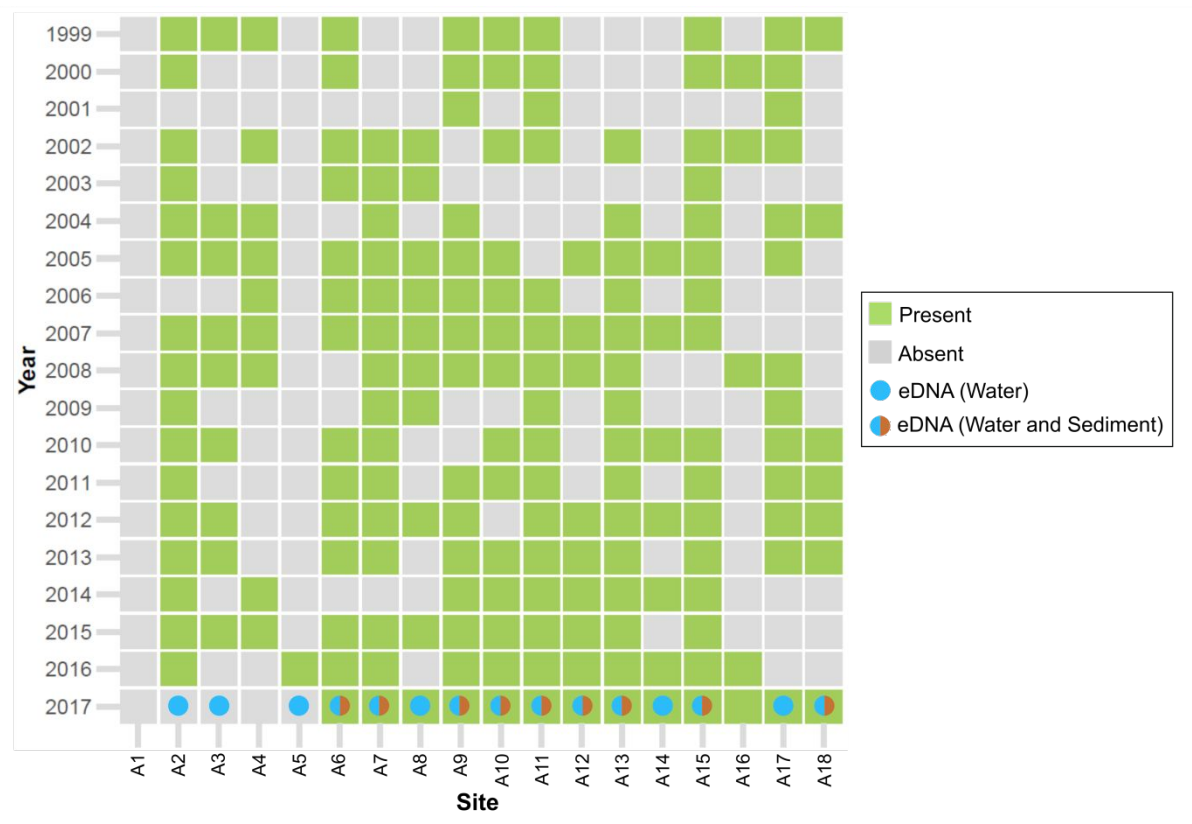


Figure S1. Presence and absence of water voles (*Arvicola amphibius*) from 1999-2017 using latrine surveys (X. Lambin, *unpublished data*) from sites A1-A18. Positive detections using environmental DNA (eDNA; water; and water and sediment) indicated in 2017.

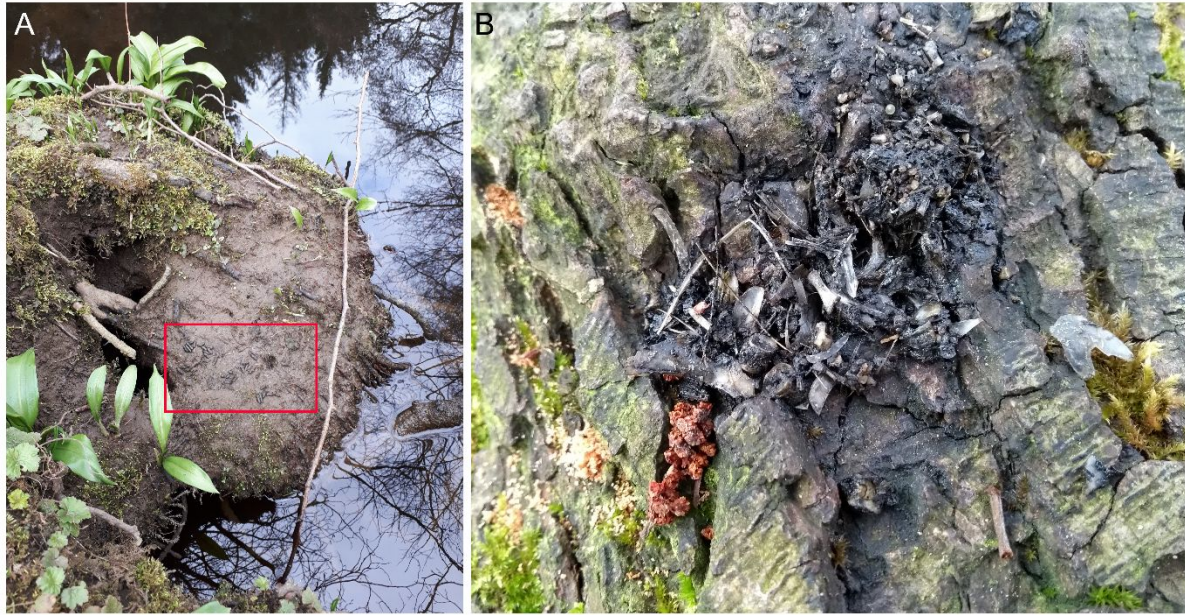


Figure S2. Example of a water vole latrine with faecal pellets, highlighted in the red rectangle in (A), and an otter spraint in (B). Both are from site P1 in the Peak District.



Figure S3. Examples of camera trap photographs for six species. Photographs have been manually adjusted to increase visibility of the species. Red boxes are used to highlight where the smaller mammals are positioned within the photograph. A: weasel (*Mustela nivalis*); B: red deer (*Cervus elaphus*); C: water vole (*Arvicola amphibius*); D: field vole (*Microtus agrestis*); E: Eurasian otter (*Lutra lutra*) and F: water shrew (*Neomys fodiens*).

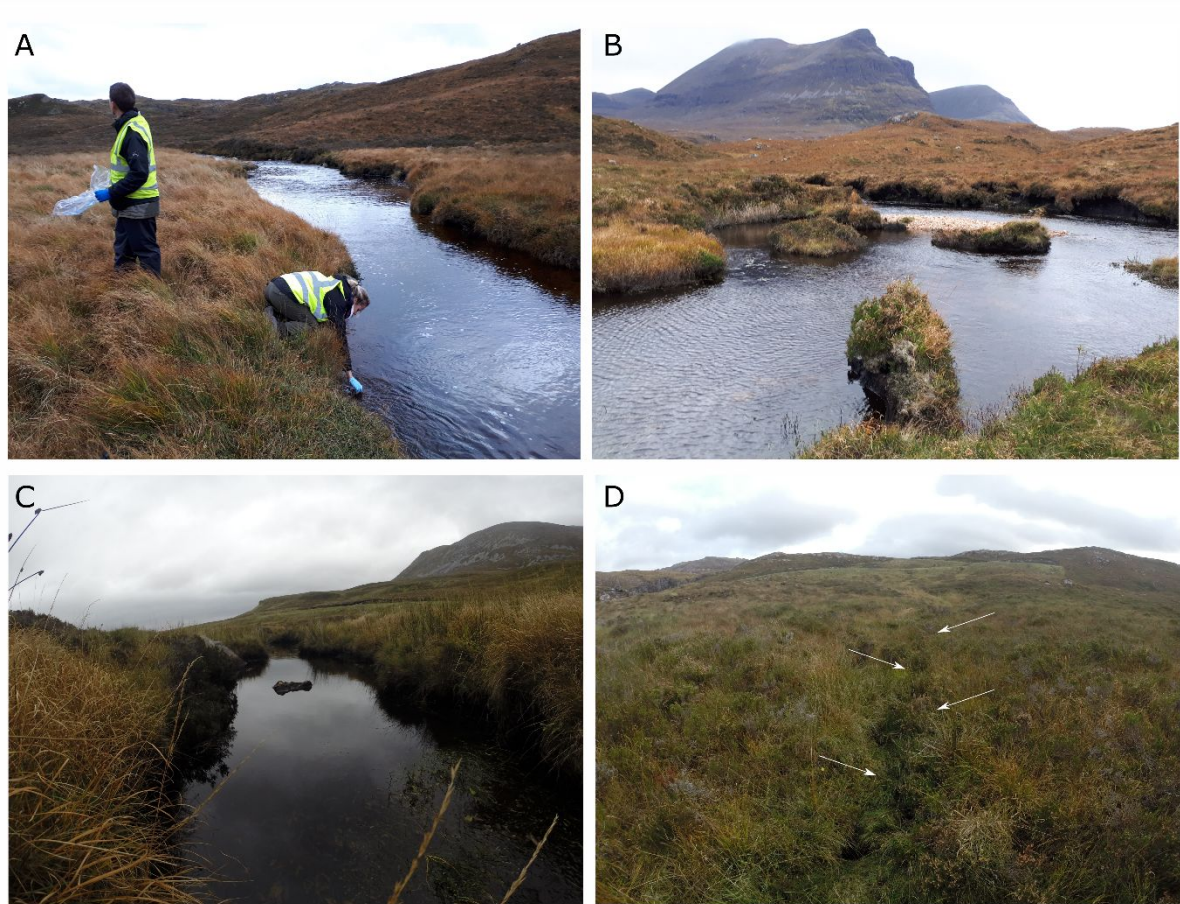


Figure S4. Examples of four sampling areas for environmental DNA (eDNA): A = A8; B = A12; C = A16 and D = A11. Sites A8, A11 and A12 returned positive eDNA records for the water vole, site A16 was negative. Sampling at site A11 was conducted in a narrow stream that is not visible here but is indicated by the white arrows (D). Sampling methodology for eDNA is indicated in (A), where sampling was conducted along the edge of the river/stream for both water and sediment samples.

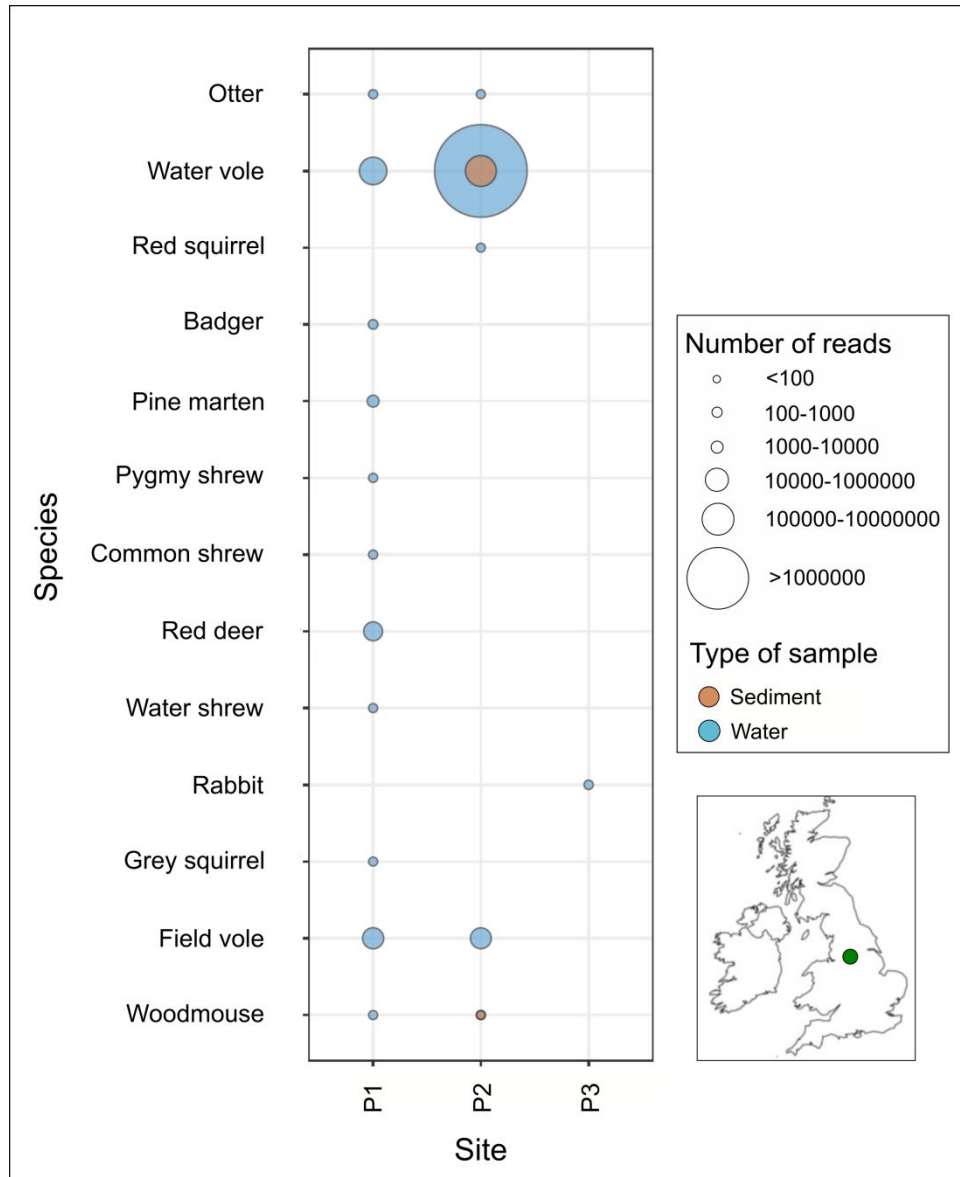


Figure S5: A bubble graph representing presence-absence and categorical values of the number of reads retained (after bioinformatic filtering) for eDNA (water in blue and sediment in orange) from each wild mammal identified in each site (P1-P3) in the Peak District National Park. The location of the Peak District is indicated in the inset map but the actual sampling sites can not be disclosed due to conservation and persecution concerns around certain protected species.

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