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

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Abstract

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

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

3. eDNA metabarcoding detected a large proportion of the expected mammalian community within each area. Common species in the areas were detected at the majority of sites. Several key species of conservation concern in the UK were detected by eDNA in areas where authenticated records do not currently exist, but potential false positives were also identified for several non-native species.

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

5. *Synthesis and Applications.* eDNA metabarcoding represents an extremely promising tool for monitoring mammals, allowing for the detection of multiple species simultaneously, and provides comparable results to widely-used conventional survey

methods. eDNA from freshwater systems delivers a 'terrestrial dividend' by detecting both semi-aquatic and terrestrial mammalian communities, and provides a basis for future monitoring at a landscape level over larger spatial and temporal scales (i.e. long-term monitoring at national levels).

Keywords: biomonitoring, camera trapping, eDNA metabarcoding, latrine surveys, mammals, occupancy modelling, rivers

Introduction

Environmental DNA (eDNA) metabarcoding (the simultaneous identification of multiple taxa using DNA extracted from an environmental sample, e.g. water, soil, based on short amplicon sequences) has revolutionised the way we approach biodiversity monitoring in both marine and freshwater ecosystems (Thomsen & Willerslev, 2015; Valentini et al., 2016; Deiner et al. 2017). Successful applications include tracking biological invasions, detecting rare and endangered species and describing entire communities (Holman et al., 2019). Since water has been shown to be a reliable source of eDNA (Deiner et al., 2017), most eDNA metabarcoding applications to date have been focused on monitoring fishes, amphibians and macroinvertebrates (Fernández et al., 2018; Hänfling et al., 2016; Valentini et al., 2016). What has become apparent from studies in lentic systems (ponds and lakes) is that semi-aquatic and terrestrial mammals can also be detected using universal primer sets for vertebrates, despite not being the focal taxonomic group (Hänfling et al., 2016; Harper et al., 2019). As a result, there has been an increasing focus on the use of both vertebrate (Harper et al., 2019) and mammal-specific primer sets (Ishige et al., 2017; Ushio et al., 2017; Leempoel et al., 2019) for detecting mammalian communities using eDNA metabarcoding.

Mammals include some of the most imperiled taxa, with over one fifth of species considered to be threatened or declining (Visconti et al., 2011), hence monitoring of mammalian biodiversity is essential. Given that any optimal survey approach is likely to be species-specific, very few species can be detected at all times when they are present. This imperfect detection (even greater for elusive and rare species) can lead to biased estimates of occurrence and hinder species conservation (Mackenzie et al., 2003). For mammals, repeated surveys using several monitoring methods are usually

applied, such as indirect observations of latrines, faeces, hair, or tracks, or direct observations such as live-trapping or camera trapping surveys over short time intervals such that closure/invariance can be assumed and detectability estimated (Nichols et al., 2008). Each of these methods has associated efficiency, cost and required expertise trade-offs, which become more challenging as the spatial and temporal scales increase.

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

The aim of this study was to assess the efficiency of eDNA for detecting semi-aquatic and terrestrial mammals in natural lotic systems in the UK. We conducted eDNA sampling in rivers and streams in two areas (Assynt, Scotland and Peak District National Park, England), which together have the majority of UK semi-aquatic and terrestrial mammalian species present (Table S1). Our objectives were two-fold: first, we sought to establish whether eDNA metabarcoding is a viable technique for

106 monitoring semi-aquatic and terrestrial mammals by comparing it to the mammalian
107 communities expected from historical data, a group for which eDNA sampling has
108 rarely been evaluated in a natural setting. Secondly, we evaluate the detection
109 efficiency of water- and sediment-based eDNA sampling in one of these areas
110 (Assynt) for multiple species compared to multiple conventional non-invasive survey
111 methods (latrine surveys and camera trapping).

Material and Methods

Latrine surveys

Assynt, a heather-dominated upland landscape in the far northwest of the Scottish Highlands, UK (Fig. 1A), is the location of an ongoing 20-year metapopulation study of water voles (*Arvicola amphibius*) led by the University of Aberdeen (Fig. S1). Here, we mainly focus only on data collected in 2017. The metapopulation is characterized by 116 discrete linear riparian habitat patches (ranging from 90 m to nearly 2.5 km) distributed sparsely (4% of waterway network) throughout the 140 km^2 study area (Sutherland et al., 2014). Water voles use prominently placed latrines for territory marking (Fig. S2A). Using latrine surveys, a reliable method of detection (Sutherland et al., 2014), water vole occupancy status was determined by the detection of latrines that are used for territory marking (Sutherland et al., 2013). During the breeding season (July and August), latrine surveys were conducted twice at each site. In addition to water vole latrines, field vole (*Microtus agrestis*) pellets are also easily identifiable, and so field vole detections were also recorded along waterways as a formal part of the latrine survey protocol. Live-trapping was then carried out at patches deemed to be occupied by water voles according to latrine surveys to determine their abundances (this was used to determine which sites were sampled for eDNA; Fig. 1A).

Camera Trap Data

Camera traps were deployed at the beginning of July and thus overlapped temporally with the latrine survey in Assynt. As part of an assessment of the value of cameras for monitoring water voles, data were collected from cameras deployed at seven of these patches for the purpose of this study. Within each of these patches, cameras were

deployed at the midpoint of the areas where active signs (latrines, grass clipping, burrows) were detected, and if no signs were detected, at the midpoint of historical water vole activity (J. Drake, C. Sutherland and X. Lambin, *pers. comm*.). These will also capture images of any species present in the area that come within close proximity of the camera (Fig. S3A-F).

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

eDNA sampling

A total of 18 potential water vole patches were selected for eDNA sampling in Assynt from 25-27th October 2017. The time lag between the latrine/live-trapping and eDNA surveys was because of two main reasons: (i) legitimate concerns around cross-site DNA contamination during latrine/live-trapping where researchers moved on a daily basis between sites as well as regularly handled and processed live animals and (ii)

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

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

eDNA Laboratory Methods

DNA was extracted from the sediment samples using the DNeasy PowerMax Soil kit and from the water samples using the DNeasy PowerWater Kit (both QIAGEN Ltd.) in a dedicated eDNA laboratory in the University of Salford. In order to avoid the risk of contamination during this step, DNA extraction was conducted in increasing order of

187 expected abundance in the eDNA samples (all field blanks were extracted first,
188 followed by the sites with supposedly zero water vole abundance, up to the highest
189 densities last). Along with field blanks (Assynt = 8, Peak District = 2), six lab extraction
190 blanks were included (one at the end of each daily block of extractions). A
191 decontamination stage using a Phileas 25 Airborne Disinfection Unit (Devea SAS) was
192 undertaken before processing samples from different locations. eDNA was amplified
193 using the MiMammal 12S primer set (MiMammal-U-F, 5′-
194 GGGTTGGTAAATTTCGTGCCAGC-3′; MiMammal-U-R, 5′-
195 CATAGTGGGGTATCTAATCCCAGTTTG-3′) (Ushio et al., 2017) targeting a ˜170bp
196 amplicon from a variable region of the 12S rRNA mitochondrial gene. A total of 147
197 samples, including field collection blanks (10) and laboratory negative controls (12,
198 including six DNA extractions blanks and six PCR negative controls), were sequenced
199 in two multiplexed Illumina MiSeq runs. Briefly, a set of 96 primers pairs with seven-
200 base sample-specific oligo-tags (Multiplex Identifier [MID] tags) and a variable number
201 (2-4) of fully degenerate positions to increase variability in amplicon sequences were
202 used. PCR amplification was conducted using a single-step protocol and to minimize
203 bias in individual reactions, PCRs were replicated three times for each sample and
204 subsequently pooled. Illumina libraries were built using a NextFlex PCR-free library
205 preparation kit according to the manufacturer's protocols (Bioo Scientific) and pooled
206 in equimolar concentrations along with 1% PhiX (v3, Illumina). The libraries were run
207 at a final molarity of 9pM on an Illumina MiSeq platform using the 2 x 150bp v2
208 chemistry.

209 Bioinformatic analysis were conducted using OBITools metabarcoding package
210 (Boyer et al., 2016) and the taxonomic assignment was conducted using ecotag
211 against a custom reference database (see Appendix 1). To exclude MOTUs/reads

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

A complete description of PCR conditions, library preparation and bioinformatic analyses are provided in Appendix 1 in the Supplementary Material.

Occupancy/Detection Analysis in Assynt

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

(a) Latrine: two latrine surveys at 116 patches.
(b) w-eDNA: three water-based eDNA samples at 18 of the 116 patches surveyed.
(c) s-eDNA: three sediment-based eDNA samples at 18 of the 116 patches surveyed.
(d) Camera: six one-week occasions of camera trapping data at seven of the 18 patches surveyed by both Latrine and eDNA (w-eDNA + s-eDNA) surveys.

We chose to focus on three species that were detected by at least three of the four methods: water voles, field voles and red deer (*Cervus elaphus*). Water voles and field voles were recorded using all four survey methods and had detection histories for 14 surveying events ((Latrine × 2) + (w-eDNA × 3) + (s-eDNA × 3) + (Camera × 6)). Red deer were not recorded during latrine surveys and had detection histories for 12 surveying events ((w-eDNA × 3) + (s-eDNA × 3) + (Camera × 6)). To demonstrate the relative efficacy of the four surveying methods, we restricted the analyses to the 18 sites where both latrine surveys were conducted and eDNA samples were taken, seven of which had associated camera trapping data. Although each surveying method differs in terms of effort and effective area surveyed, each are viable surveying methods that are readily applied in practice. So, while the specific units of effort are not directly comparable, the relative detection efficacy per surveying method-specific unit of effort is of interest and will provide important context for designing future monitoring studies and understanding the relative merits of each surveying method. Analyzing the data using occupancy models allowing for method-specific detectability enables such a comparison in per unit effort efficacy between eDNA metabarcoding and multiple conventional survey methods.

A single season occupancy model (MacKenzie et al., 2002) was applied to the ensemble data where detection histories were constructed using each of the surveying events as sampling occasions (MacKenzie et al., 2017). The core assumption here is that the underlying occupancy state (i.e. occupied or empty) is constant over the sampling period, and therefore, every sampling occasion is a potentially imperfect observation of the true occupancy status. Because occasions represent method-specific surveying events, we used “surveying method” as an occasion-specific covariate on detection (Latrine, w-eDNA, s-eDNA and Camera). Our primary objective

was to quantify and compare method-specific detectability, so we did not consider any other competing models. For comparing the methods, we compute accumulation curves as (MacKenzie & Royle, 2005):

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

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

Results

Mammal Detection via eDNA metabarcoding

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

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

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

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

Occupancy Analysis

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

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

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

347 The number of surveying events, *k*, required to achieve $p^*_{psm} \geq 0.95$ for red deer was
348 3 samples, 30 samples, and 29 weeks, for water eDNA, sediment eDNA, and cameras
349 respectively (see also Fig. 2).

Discussion

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

Detection of mammalian communities using eDNA metabarcoding

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

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

In addition to lifestyle (semi-aquatic or terrestrial), the number of individuals of each species (i.e. group-living) may be important for eDNA detection (Williams et al., 2018). As a counter example to this, otters and weasels were notably absent in the eDNA samples in Assynt despite being picked up by camera traps (Fig. S3 and Table S5). Otters were present in the water eDNA samples at two sites in the Peak District, albeit at a lower number of reads in comparison to most of the other species detected (Fig. S5; Table S2). This mirrors previous studies where eDNA analysis has performed relatively poorly for otter detection in captivity and the wild (Harper et al., 2019; Thomsen et al., 2012). Carnivores were generally detected on fewer occasions (e.g. red foxes, badgers and pine martens; Figs. 1B and S5) or not at all (e.g. stoats and American mink in addition to those discussed above) in comparison to smaller mammals and red deer, and a similar pattern has been shown with North American carnivores in a recent study using eDNA from soil samples (Leempoel et al., 2019). For some of these species, species ecology/behavior such as a relatively large home range and more solitary nature (e.g. red foxes) may go some way towards explaining a lack of, or few, eDNA records. This may mean adopting different eDNA sampling strategies depending on the type of mammal (e.g. carnivore, rodent etc.) being targeted. Those with larger home ranges may require additional sampling at both broad and fine spatial scales to provide reliable detections. Furthermore, as demonstrated by Ushio et al. (2017) poor efficiency for amplifying some mammal species might be associated to suboptimal experimental conditions (e.g. inadequate primer design, concentration, and/or annealing temperatures).

Regarding the sampling medium for eDNA, here we demonstrated that water is a more effective method for detection of mammal eDNA than sediment (Table 1; Figs. 1B and S5). For one of our focal species, the water vole, 75% of sites which were deemed unoccupied by latrine surveys and those with ≤2 individuals (8 sites) in Assynt, returned a non-detection for sediment eDNA as opposed to 37.5% of sites for water (Figs. 1A, 1B and S1). Distinct temporal inferences are provided by eDNA recovered from water and sediment samples. DNA bound to sediments can remain detectable for a longer period (i.e. up to hundreds of years) and provide historical data, whereas, eDNA retrieved from water samples provide more contemporary data due to a faster degradation in the water column (Turner et al., 2015). It is worth investigating further if sediment eDNA could indicate the presence of a more 'established' population, where a certain threshold of individuals and long-term occupation (i.e. historical) is required for detection in sediment (Fig. S1; Turner et al., 2015; Leempoel et al., 2019).

Importantly, sparse or single eDNA records should be carefully verified. The edible dormouse and grey squirrel sequences identified within the Assynt samples (Fig. 1B) and red squirrel within the Peak District (Fig. S5) highlights the caveats associated with this technique. Should management have relied on eDNA evidence alone, as the edible dormouse and grey squirrel are classified as invasive species within Great Britain, false positives for these species could lead to unnecessary resources being allocated for management/eradication programmes. These potentially arose due to sample carryover from a previous sequencing run on the same instrument (a known issue with Illumina sequencing platforms; Nelson et al., 2014) which included those species for the reference database construction (the dormouse reads showed evidence of degradation by not exactly matching the sequence from the

tissue-derived sequence for example; Table S2). Controlling for false positives is certainly a huge challenge in eDNA metabarcoding and the need to standardize and optimize thresholds for doing so is an ongoing debate (Ficetola et al., 2015; Harper et al., 2019).

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

448 *Comparisons between surveying methods*

449 Comparisons of species detection by traditional survey approaches and eDNA
450 analysis are now numerous in the literature, and mainly focus on what is and what is
451 not detected within and across different methods (Hänfling et al., 2016; Leempoel et
452 al., 2019). Yet, there has been growing incorporation of occupancy modelling to
453 estimate the probability of detecting the focal species, in comparison to one other
454 survey method, either for a single species (Lugg et al., 2018; Tingley et al., 2019) or
455 multiple species (Valentini, et al., 2016; Abrams et al., 2019). Simultaneous multi-
456 method comparisons for multiple species have been lacking and this study directly
457 addresses this for the first time.

458 The probability of detecting the water vole and field vole was higher for the
459 latrine surveys than eDNA sampling (both water and sediment) and camera traps
460 (Table 1; Fig. 2). However, when considering confidence intervals, there was
461 considerable overlap between latrine, water-based eDNA and camera traps for both
462 species, with only sediment-based eDNA yielding a low probability of detection (Table
463 1). Detection probabilities for water-based eDNA and camera traps were similar for
464 water voles, with camera traps less likely to detect the field vole than water-based
465 eDNA. For the red deer (for which no latrine survey was undertaken), water-based
466 eDNA had a much higher probability of detection than either sediment-based eDNA
467 or camera traps (which performed similarly; Table 1). Despite the increasing adoption
468 of camera traps in providing non-invasive detections for mammals (Hofmeester et al.,
469 2019), camera traps were outperformed by water-based eDNA metabarcoding for the
470 three focal species in this component of the study. Camera traps are certainly limited
471 by their photographic range and placement (amongst many other factors; Hofmeester
472 et al., 2019). Here, camera traps were deployed so as to sample the habitat of the

water vole (see Fig. S3), which may explain lower detection for other terrestrial species in comparison to eDNA metabarcoding (see above). Studies focusing on a single species often report that eDNA analysis outperforms the conventional survey method in terms of detection probabilities (e.g. Lugg et al., 2018). For metabarcoding, there is clearly a need to carefully consider the potential for 'cross-talk' between samples and how false positives could impact detection probabilities using occupancy modelling. However, we have demonstrated general congruence between surveying methods. Multi-species metabarcoding studies may trade-off a slightly lower (but comparable) detection probability than other survey methods for individual species (Fig. 2) in favour of a better overall "snapshot" of occupancy of the whole mammalian community (Ushio et al., 2017).

The comparison between survey 'effort' for the four methods to reach a probability of detection of ≥0.95 is highly informative and provides a blueprint for future studies on mammal monitoring. For the water vole, three latrine surveys would be required. A total of four water-based and 10 sediment-based eDNA replicates or five weeks of camera trapping would be required to achieve the same result (Fig. 2). This increases for the field vole in the same habitat, with five latrine surveys and six water-based eDNA replicates. Sediment-based eDNA would be impractical for this species and camera trapping would take 14 weeks. The red deer would require three water-based eDNA replicates and 29-30 events for sediment-based eDNA and camera trap detection.

What is important here is the spatial component and the amount of effort involved in the field. Taking 4-6 water-based eDNA replicates from around one location within a patch could provide the same probability of detecting these small mammals with three latrine surveys. In many river catchments, there may be 100s to 1000s of

498 kilometres to survey that would represent suitable habitat, and only a fraction of that
499 may be occupied by any given species. This is particularly relevant in the context of
500 recovery of water vole populations post-translocation or in situations where remnant
501 populations are bouncing back after invasive American mink (*Neovison vison*) control
502 has been instigated. On a local scale, finding signs of water voles through latrine
503 surveys is not necessarily difficult, but monitoring the amount of potential habitat
504 (especially lowland) for a species which has undergone such a massive decline
505 nationally is a huge undertaking (Morgan et al., 2019). The use of eDNA
506 metabarcoding from freshwater systems to generate an initial, coarse and rapid
507 'distribution map' for vertebrate biodiversity (and at a relatively low cost) could
508 transform biomonitoring at a landscape level. Then, on the basis of this, practitioners
509 could zoom in to further investigate specific areas for confirmation of rare or invasive
510 species for example.

511 It is clear that eDNA metabarcoding is a promising tool for monitoring semi-
512 aquatic and terrestrial mammals in both lotic (this study) and lentic systems (Harper
513 et al., 2019; Ushio et al., 2017). We detected a large proportion of the expected
514 mammalian community (Table S1), including the possible presence of priority species.
515 Water-based eDNA is comparable or out-performs other non-invasive survey methods
516 for several species (Fig. 2). However, there remain challenges for the application of
517 this technique over larger spatial and temporal scales. Technical issues of
518 metabarcoding in laboratory and bioinformatic contexts have been dealt with
519 elsewhere (Harper et al., 2019) but understanding the distribution of eDNA transport
520 in the landscape and its entry into natural lotic systems is at an early stage (and
521 incorporating such variables in occupancy modelling approaches). The characteristics
522 of streams and rivers undoubtedly influence eDNA transportation through the

523 environment (Pont et al., 2018). This clearly requires more detailed and systematic
524 eDNA sampling than undertaken here, particularly in an interconnected river/stream
525 network with organisms moving between aquatic and terrestrial environments.
526 Leempoel et al. (2019) recently demonstrated the feasibility for detecting terrestrial
527 mammal eDNA in soil samples but this study has shown that sampling a few key areas
528 in freshwater ecosystems (e.g. larger rivers and lakes) within a catchment area could
529 potentially provide data on a large proportion (if not all) of the mammalian species
530 within it, even when some species are present at low densities (Deiner et al., 2017).
531 In this regard, future studies might also investigate the value of citizen science, where
532 trained volunteers can contribute to data collection at key sites, thus scaling the reach
533 of research whilst raising public awareness and significance of mammalian
534 conservation concerns though public participation in scientific research (Parsons et
535 al., 2018).
536

538 **Data accessibility**

539 Data and scripts will be made available in public repositories 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 and JD's ongoing work
544 in Assynt. JD and EB carried out the latrine surveys and live-trapping. JD analysed the
545 camera trap data. DAD provided information and data on mammals in the Peak
546 District. ADM, NGS, SSB and MBM carried out the eDNA sampling. MBM, NGS, SSB,
547 CB and ADM performed the laboratory work. NGS, OSW, LRH, MBM, CB and ADM
548 carried out the bioinformatic analyses. NGS, ADM, IC and MBM analysed the eDNA
549 data. CS and JD conducted the occupancy modelling. ADM, NGS, CS, JD, MBM and
550 LRH wrote the paper, with all authors contributing to editing and discussions.

551

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701 **Tables**

702

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

Species	Occupancy	Detection probability			
		Latrine	*w-eDNA*	*s-eDNA*	*Camera*
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)

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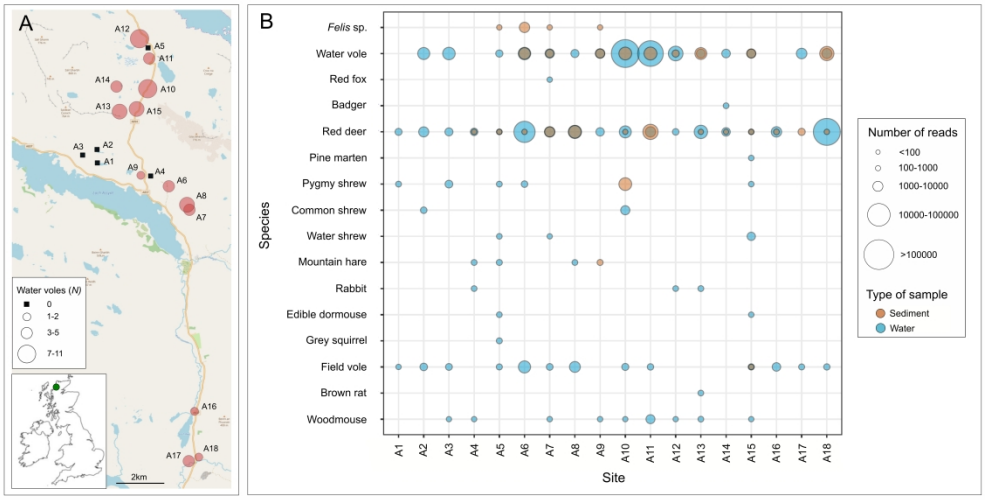
FIGURES

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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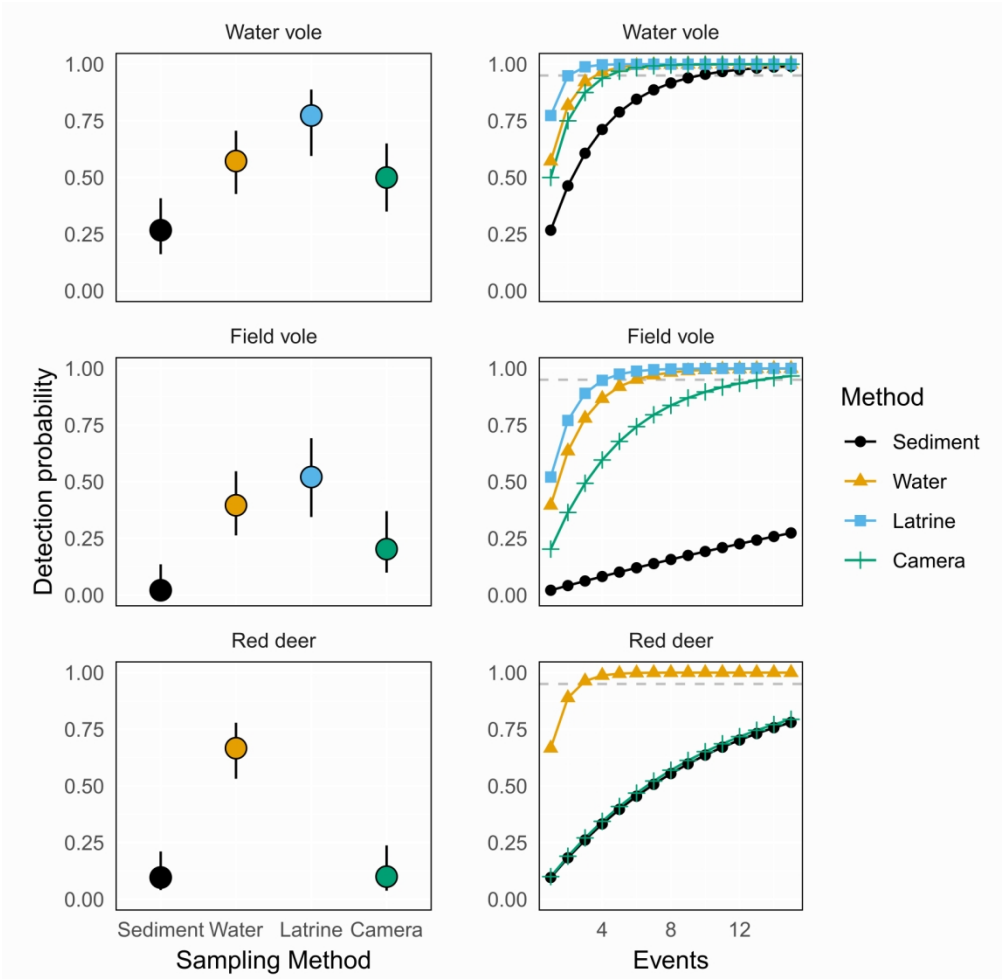

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SUPPLEMENTARY MATERIAL

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

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Appendix 1

eDNA sample collection

Three water sample replicates (two litres each) and three sediment sample replicates (50 ml falcon tube, approximately half-filled) were taken at each site in Assynt, always within a reachable distance from the river's edge and at a depth where sediment samples could be taken (Fig. S3A). Water samples were filtered on site using a Sterivex 0.45 µm filter unit (Merck Millipore) and filters were stored in silica beads in the field (1-3 days; Majaneva et al., 2018) then frozen until DNA extraction. Sediment samples were stored in 100% ethanol. Appropriate decontamination precautions were taken (use of disposable gloves and all equipment and surfaces were decontaminated by using 50% bleach solution) and collection, extraction and PCR negative controls were included. Samples from the Peak District were filtered within 5 hours in the University of Salford laboratory facilities due to its close proximity to the sampling locations. A single filter was used for each replicate in Assynt and the Peak District, and the volume filtered varied between each, ranging from 150 ml to 2 L (see Tables S2 and S3). The amount of sediment collected also varied, with 4 to 10g used in the extractions. A Pearson's correlation was performed to determine if the amount of water/sediment influenced the amount of retained reads for mammals after bioinformatic filtering.

Reference database

Given that this project proposed to use mammal-specific primers (MiMammal-U, Ushio et al., 2017) to target the same region of 12S as the MiFish primers, an *in silico* evaluation was first performed using ecoPCR (Ficetola et al., 2010) against a custom,

phylogenetically curated reference database for mammals distributed in the UK and Ireland. This database was one of several databases constructed for UK vertebrates and used in an eDNA metabarcoding study of pond biodiversity (see Harper et al. 2019 for details). The mammal database was updated in July 2018 for the purposes of the present study. Parameters were set to allow a fragment size of 50-250 bp and different number of mismatches (0, 1, 2, 3) between each primer and each sequence in the reference database. Reference sequence data was available for 103 mammal species (91.96%) in the UK. The nine species that were not represented were either cetaceans or bats. Of those species with reference sequence data (N = 103), 44 (42.72%), 65 (63.11%), 72 (69.90%), and 82 (79.61%) mammals were amplified when 0, 1, 2, and 3 primer-sequence mismatches were allowed respectively. Species that did not amplify under any scenario and of relevance to this study were the European water vole (*Arvicola amphibius*), greater white-toothed shrew (*Crocidura russula*), Millet's shrew (*Sorex coronatus*), Eurasian pygmy shrew (*Sorex minutus*), field vole (*Microtus agrestis*), common vole (*Microtus arvalis*), grey squirrel (*Sciurus carolinensis*), and European polecat (*Mustela furo*).

Targeting a fragment of the 12S gene, a reference database of 32 UK terrestrial mammals was created from ethanol-preserved tissues samples obtained from National Museums Scotland (Table S6). DNA was extracted using the ISOLATE II kit according to the manufacturer's protocol. These DNA samples were then included in a large barcoding project using the MiFish (Miya et al., 2015) primers (O. Wangensteen et al., *unpublished data*) primarily targeting fish species. Although these primers were originally designed to amplify fishes, they are known to amplify mammals also and target the exact same region as the MiMammal primers (Ushio et al., 2017). Of these mammals, only *Sorex araneus* and *Neomys fodiens* failed to generate

reference sequences. PCRs were then carried out on a subset of the tissue-extracted DNA (see Table S6) and Sanger-sequenced (Macrogen Inc.). Amplicons of 172bp from a variable region of the mitochondrial 12S rRNA gene were obtained with the MiMammal-U primers (Ushio et al., 2017).

eDNA Laboratory Methods

A set of 96 primers pairs with seven-base sample-specific MIDs and a variable number (2-4) of fully degenerate positions (leading Ns) to increase variability in amplicon sequences were used. PCR amplification was conducted using a single-step protocol and to minimize bias in individual reactions, PCRs were replicated three times for each sample and subsequently pooled. The PCR reaction consisted of a total volume of 20 µl including 10 µl Amplitaq; 0.16 µl of BSA; 1.0 µl of each of the two primers (5 µM); 5.84 µl of ultra-pure water, and 2 µl of DNA template. The PCR profile included an initial denaturing step of 95°C for 10 min, 40 cycles of 95°C for 30s, 60°C for 45s, and 72°C for 30s and a final extension step of 72°C for 5 min. Amplification were checked through electrophoresis in a 1.5% agarose gel stained with GelRed (Cambridge Bioscience). PCR products were pooled in two different sets and a left-sided size selection was performed using 1.1x Agencourt AMPure XP (Beckman Coulter). Illumina libraries were built from each set, using a NextFlex PCR-free library preparation kit according to the manufacturer's protocols (Bioo Scientific). Libraries were then quantified by qPCR using a NEBNext qPCR quantification kit (New England Biolabs) and pooled in equimolar concentrations along with 1% PhiX (v3, Illumina). The libraries were run at a final molarity of 9pM on an Illumina MiSeq platform using the 2 x 150bp v2 chemistry.

Bioinformatic analysis

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

A stringent approach was applied to our analyses to avoid false positives and exclude MOTUs/reads putatively belonging to sequencing errors or contamination. The final dataset included only MOTUs that could be identified to species level (>0.98), and MOTUs containing less than 10 reads and with a similarity to a sequence in the reference database lower than 98% were discarded (Cilleros et al., 2019). Singleton reads within individual replicates were also discarded. The maximum number of reads detected in the controls for each MOTU in each sequencing run were removed from all samples (Table S7). For water voles, field voles and red deer (the most abundant wild mammals in terms of sequence reads in our dataset), this equated to a sequence frequency threshold of ≤0.17%, within the bounds of previous studies on removing sequences to account for contamination and tag jumping (Cilleros et al., 2018; Schnell, Bohmann, & Gilbert, 2015). The number of retained reads per replicate was not significantly correlated with the volume of water filtered (Pearson's correlation: r =

0.213; $p = 0.094$) or the amount of sediment collected (Pearson's correlation: $r = 0.076$; $p = 0.556$).

TABLES

Table S1. Species (and the Order to which they belong) that are expected to be found within Assynt (based on Matthews et al. 2018) and the Peak District (Alston et al. 2012) and whether or not they were detected by eDNA. A * indicates species where presence is uncertain from Matthews et al. (2018).

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

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

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

Additional file: TableS2_Reads_Water.xlsx

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

Additional file: TableS3_Reads_Sediment.xlsx

Table S4. Number of reads obtained after all filtering steps applied to remove non-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 (*Sus, Bos, Equus, Ovis, Canis*)	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 (*Sus, Bos, Equus, Ovis, Canis*)	500,473
MOTUs with minimum identity of 0.98	465,997

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

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

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

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

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

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

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

FIGURES

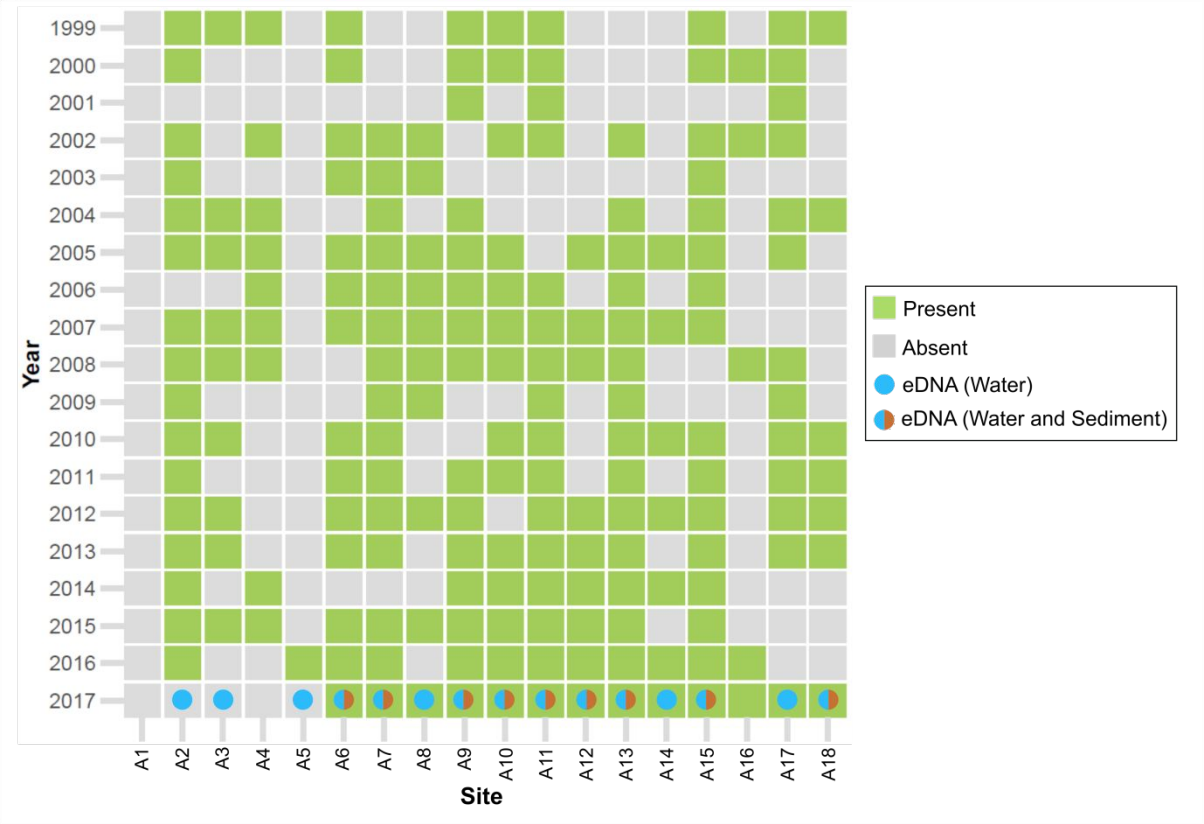

Figure S1. Presence and absence of water voles (*Arvicola amphibius*) from 1999-2017 using latrine surveys (X. Lambin, *unpublished data*) from sites A1-A18. Positive detections using environmental DNA (eDNA; water; and water and sediment) indicated in 2017.

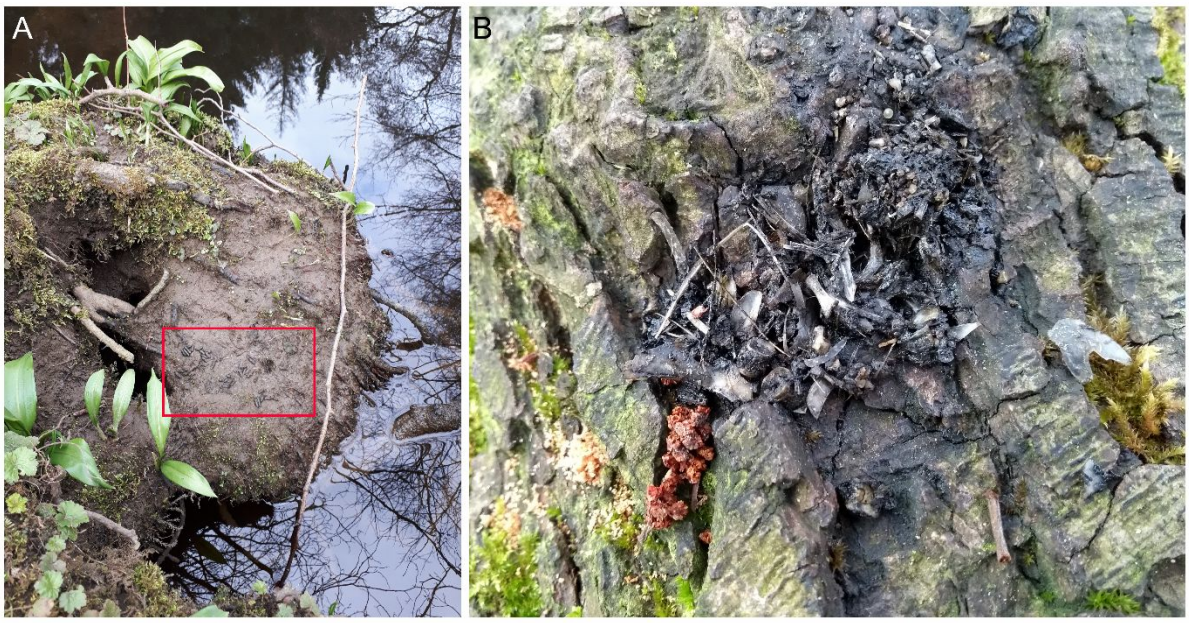

Figure S2. Example of a water vole latrine with faecal pellets, highlighted in the red rectangle in (A), and an otter spraint in (B). Both are from site P1 in the Peak District.

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*).

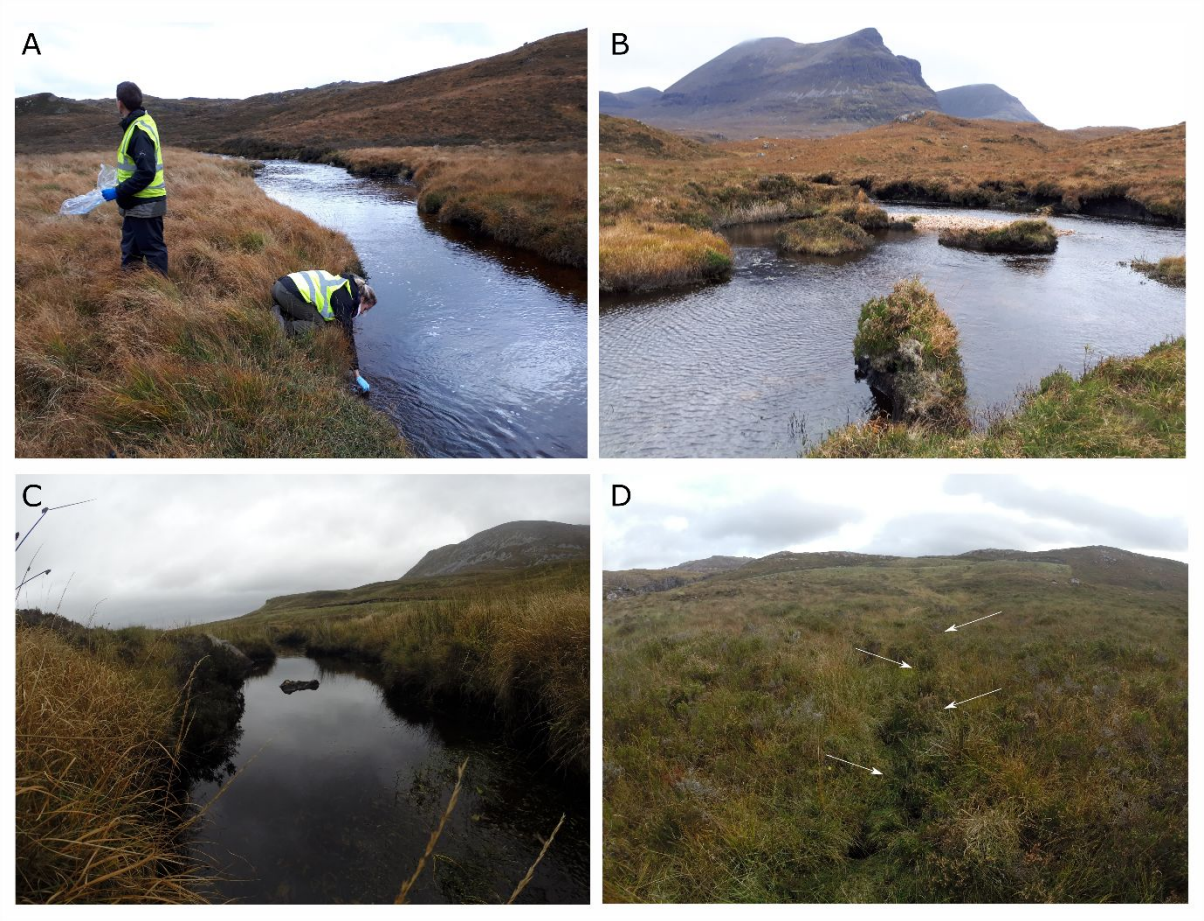

Figure S4. Examples of four sampling areas for environmental DNA (eDNA): A = A8; B = A12; C = A16 and D = A11. Sites A8, A11 and A12 returned positive eDNA records for the water vole, site A16 was negative. Sampling at site A11 was conducted in a narrow stream that is not visible here but is indicated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

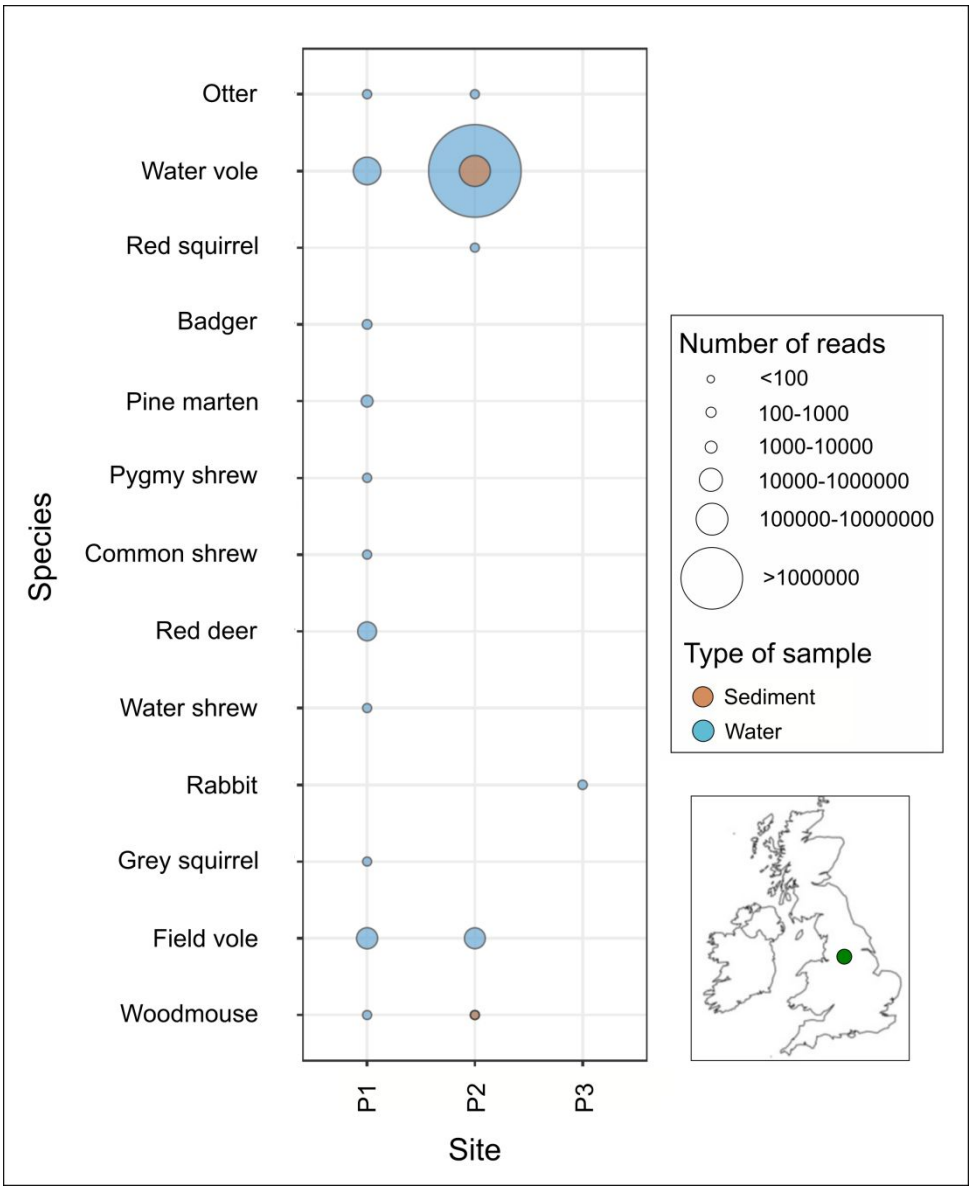

Figure S5: A bubble graph representing presence-absence and categorical values of the number of reads retained (after bioinformatic filtering) for eDNA (water in blue and sediment in orange) from each wild mammal identified in each site (P1-P3) in the Peak District National Park. The location of the 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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Common name	Scientific name	Match	A1_1	A1_2	A1_3	A2_1
Red deer	*Cervus elaphus*	1.000	303	606	375	5451
Wood mouse	*Apodemus sylvaticus*	1.000	0	0	0	0
Pygmy shrew	*Sorex minutus*	1.000	120	0	0	0
Mountain hare	*Lepus timidus*	1.000	0	0	0	0
Grey squirrel	*Sciurus carolinensis*	1.000	0	0	0	0
Badger	*Meles meles*	1.000	0	0	0	0
Brown rat	*Rattus norvegicus*	1.000	0	0	0	0
Otter	*Lutra lutra*	1.000	0	0	0	0
Red squirrel	*Sciurus vulgaris*	1.000	0	0	0	0
Red fox	*Vulpes vulpes*	1.000	0	0	0	0
European rabbit	*Oryctolagus cuniculus*	0.994	0	0	0	0
Water shrew	*Neomys fodiens*	0.994	0	0	0	0
Edible dormouse	*Glis glis*	0.994	0	0	0	0
Water vole	*Arvicola amphibius*	0.994	0	0	0	0
Field vole	*Microtus agrestis*	0.994	0	4	0	0
Pine marten	*Martes martes*	0.994	0	0	0	0
Common shrew	*Sorex araneus*	0.983	0	0	0	657
Human	*Homo sapiens*	1.000	0	0	0	0
Human	*Homo sapiens*	1.000	0	0	0	0
Human	*Homo sapiens*	1.000	0	0	0	0
Cattle	*Bos*	1.000	0	0	0	0
Sheep	*Ovis*	1.000	0	0	0	0
Dog	*Canis lupus familiaris*	1.000	0	0	0	0
Pig	*Sus scrofa*	0.994	0	0	0	0
Horse	*Equus przewalskii*	1.000	0	0	0	0
Total reads			423	610	375	6108
Water filtered (ml)			600	500	500	600

A2_2	A2_3	A3_1	A3_2	A3_3	A4_1	A4_2	A4_3	A5_1
1614	3118	1935	0	1326	1527	395	130	0
0	0	0	0	2	2	0	2	0
0	0	2159	2	485	0	0	0	0
0	0	0	0	0	134	0	0	0
0	0	0	0	0	0	0	0	190
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	71	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
6765	13340	9267	0	13894	0	0	0	0
832	1382	981	0	0	0	0	0	242
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	19	0
0	0	0	0	0	1008	0	0	0
0	0	0	0	0	276	0	0	0
0	0	0	0	0	118	0	0	13
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
9211	17840	14342	2	15707	3065	466	151	445
550	500	500	500	500	1100	1000	950	550

A5_2	A5_3	A6_1	A6_2	A6_3	A7_1	A7_2	A7_3	A8_1
74	0	21332	51170	39896	2034	3038	6253	2314
0	0	0	0	0	9	0	3	0
0	113	671	0	0	0	0	0	0
66	140	0	0	0	0	0	0	98
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	4	0	0
0	0	0	0	0	0	0	0	0
0	46	0	0	0	0	0	2	0
0	61	0	0	0	0	0	0	0
0	1558	0	7954	14618	0	0	6824	0
0	0	982	14968	4961	0	851	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	367	0	2807	0	0	0	0
0	0	5073	0	8143	0	0	0	0
0	0	0	0	20	0	0	0	0
0	1660	0	6934	386	0	0	6125	38
160	154	0	621	0	0	0	0	0
0	0	0	0	0	0	0	52	0
0	0	0	2066	0	0	0	0	0
111	0	0	0	0	0	0	0	0
411	3732	28425	83713	70831	2043	3893	19259	2450
500	300	2000	2000	2000	1600	1800	1700	700

A8_2	A8_3	A9_1	A9_2	A9_3	A10_1	A10_2	A10_3	A11_1
21496	3882	0	1987	2242	4244	0	15686	0
0	0	0	0	10	0	2	0	6113
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
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0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
3361	0	0	2988	4666	25896	109505	91400	86347
12834	1108	0	0	0	0	0	1303	544
0	0	0	0	0	0	0	0	0
0	0	0	0	0	6572	0	0	0
0	0	2760	0	0	0	0	0	0
0	0	33316	0	0	0	0	0	0
0	0	10	0	0	0	0	0	0
0	0	0	0	0	0	0	252	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	235	0	0	0	0	0
0	0	0	0	0	0	0	0	0
37691	4990	36086	5210	6918	36712	109507	108641	93004
700	500	800	800	700	700	900	800	800

A11_2	A11_3	A12_1	A12_2	A12_3	A13_1	A13_2	A13_3	A14_1
11504	0	681	0	0	1146	27780	0	4406
0	0	0	2	0	80	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	95
0	0	0	0	0	78	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	62	0	0	126	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
92040	4974	0	36069	2964	0	5777	0	4768
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	1439	0	0
0	0	0	0	0	0	577	0	0
0	0	0	0	0	0	0	0	0
0	40	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
103544	5014	743	36071	2964	1430	35573	0	9269
650	700	600	650	650	900	1000	900	1400

A14_2	A14_3	A15_1	A15_2	A15_3	A16_1	A16_2	A16_3	A17_1
0	39	0	38	0	0	11459	0	0
0	0	2	0	0	0	0	0	0
0	0	0	29	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	3956	0	0	0	0	0	0
0	0	0	16	0	0	0	0	0
0	0	4505	0	1258	0	0	0	0
0	0	334	0	50	0	0	4631	0
0	0	0	25	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	1545	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	528	552	0	2035	6402	1120	0
0	0	0	140	33	0	0	0	0
0	0	231	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	111	0	0	0	0	0
0	39	11101	911	1341	2035	17861	5751	0
1300	1300	300	150	220	300	280	300	300

A17_2	A17_3	A18_1	A18_2	A18_3	P1_1	P1_2	P1_3	P2_1
0	0	3302	176471	28581	39	19204	200990	0
0	0	0	0	0	2	0	3	4
0	0	0	0	0	0	0	5	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	2	0
0	0	0	0	0	218	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	3	5	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	4	0
0	0	0	0	0	0	0	0	0
1881	10397	0	10303	0	31691	84663	67604	1463657
0	211	424	0	0	0	44876	33400	40892
0	0	0	0	0	0	4293	0	0
0	0	0	0	0	0	0	3	0
0	0	0	0	0	0	0	0	5704
0	0	0	0	0	91848	342666	477325	120447
0	0	0	0	0	0	0	215	0
0	0	0	7912	346	780029	797189	599080	0
0	0	0	0	0	186768	179282	129040	0
0	0	0	0	0	0	30862	0	0
0	0	19	0	0	27313	31285	16706	0
0	0	0	0	0	0	0	2	0
1881	10608	3745	194686	28927	1117911	1534325	1524379	1630704
280	270	500	500	500	700	700	700	1500

P2_2	P2_3	P3_1	P3_2	P3_3
0	0	0	0	0
4	6	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
0	2	0	0	0
0	2	0	0	0
0	0	0	0	0
0	0	63	0	0
0	0	0	0	0
0	0	0	0	0
1170897	1085809	0	0	0
20597	14167	0	0	0
0	0	0	0	0
0	0	0	0	0
0	9876	0	10408	0
0	202101	0	44526	329119
0	0	0	0	0
0	0	0	2950	50971
0	0	271	67697	43330
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0
1191498	1311963	334	125581	423420
1500	1500	1200	1000	1000

Common name	Scientific name	Match	A1_1	A1_2	A1_3	A2_1
Red deer	*Cervus elaphus*	1.000	0	0	0	0
Pymgy shrew	*Sorex minutus*	1.000	0	0	0	0
Mountain hare	*Lepus timidus*	1.000	0	0	0	0
Water vole	*Arvicola amphibius*	0.994	0	0	0	0
Field vole	*Microtus agrestis*	0.994	0	0	0	0
Wood mouse	*Apodemus sylvaticus*	1.000	0	0	0	0
Cat	*Felis*	0.982	0	0	0	0
Human	*Homo sapiens*	1.000	0	0	4422	5
Human	*Homo sapiens*	1.000	0	0	0	0
Cattle	*Bos*	1.000	0	0	0	0
Sheep	*Ovis*	1.000	0	0	0	0
Dog	*Canis lupus*	1.000	25	0	0	0
Total reads			25	0	4422	5
Sediment (g)			4	7	10	10

A2_2	A2_3	A3_1	A3_2	A3_3	A4_2	A4_3	A5_1	A5_2
0	0	0	0	0	4	4	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	2
0	0	31830	0	36352	2	115018	0	7392
0	0	0	0	0	0	3488	0	0
0	0	0	0	2	0	4	0	0
0	0	0	0	4	0	0	0	0
0	0	0	0	0	5	0	0	0
0	0	31830	0	36358	11	118514	0	7394
7	7	10	10	10	9	8	10	10

A5_3	A6_1	A6_2	A6_3	A7_1	A7_2	A7_3	A8_1	A8_2
4	6	0	4	2	10560	2	23797	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	17198	0	0	3742	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	11203	2	0	0	0	0
19205	36842	0	47593	115249	0	4	81615	0
0	0	0	0	390	0	0	0	2
0	3	0	4	0	0	0	0	0
0	0	0	0	0	2	0	2	0
25021	0	0	0	3	2	0	0	0
44230	54049	0	58804	119388	10564	6	105414	2
6	10	10	10	9	9	10	10	10

A8_3	A9_1	A9_2	A9_3	A10_1	A10_2	A10_3	A11_1	A11_2
0	0	0	0	0	0	2	0	0
0	0	0	0	0	0	24860	0	0
0	0	0	220	0	0	0	0	0
0	6832	0	0	25025	0	0	0	224
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	2	0	0	0	0	0	0
0	7	21449	20446	0	0	2	0	0
0	0	0	2695	0	0	0	0	0
0	0	0	2	5	0	0	0	94
0	0	0	0	0	0	0	0	0
0	0	0	0	2	0	0	0	2
0	6839	21451	23363	25032	0	24864	0	320
10	9	10	10	10	10	5	10	10

A11_3	A12_1	A12_2	A12_3	A13_1	A13_2	A13_3	A14_1	A14_2
43744	0	0	0	0	0	4	2	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
25645	1627	0	0	0	8234	11003	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
26432	2595	0	76100	11040	0	2	2	18909
0	0	0	2	0	0	0	0	0
10	0	0	5	0	0	11531	0	0
11421	0	0	0	0	0	65	0	0
2	0	0	0	0	0	0	0	0
107254	4222	0	76107	11040	8234	22605	4	18909
10	7	10	10	10	10	10	10	10

A14_3	A15_1	A15_2	A15_3	A16_1	A16_2	A16_3	A17_1	A17_2
0	3	3	3	0	139	0	1492	2
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	6545	0	0	0	0	0
0	0	0	2	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	16411	42796	10183	14355	26196	0	35298	0
0	2	0	0	0	9991	0	0	0
0	0	0	8	2	0	0	6	19719
0	0	2	2	0	0	0	4	0
0	0	0	0	0	0	0	0	0
0	16416	42801	16743	14357	36326	0	36800	19721
10	10	10	9	10	10	10	10	10

A17_3	A18_1	A18_2	A18_3	P1_1	P1_2	P1_3	P2_1	P2_2
0	7	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	18814	2701	13683	0	0	0	257874	0
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	3	0
0	0	0	0	0	0	0	0	0
41244	377	1901	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
0	12243	3	0	32427	9760	416	0	0
0	4	409	0	19732	0	30549	0	0
0	2	0	0	0	0	0	0	0
41244	31447	5014	13683	52159	9760	30965	257877	0
10	10	9	9	10	10	9	10	10

P2_3	P3_1	P3_2	P3_3
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
0	0	0	0
10	8	9	10