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ORIGINAL ARTICLE

Holistic monitoring of freshwater and terrestrial vertebrates by camera trapping and environmental DNA

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Abstract

The anthropogenic impact on the world's ecosystems is severe and the need for non-invasive, cost-effective tools for monitoring and understanding those impacts are therefore urgent. Here, we combine two such methods in a comprehensive multi-year study; camera trapping (CT) and analysis of environmental DNA (eDNA), in river marginal zones of a temperate, wetland Nature Park in Denmark. CT was performed from 2015 to 2019 for a total of 8778 camera trap days and yielded 24,376 animal observations. The CT observations covered 87 taxa, of which 78 were identified to species level, and 73 were wild native species. For eDNA metabarcoding, a total of 114 freshwater samples were collected from eight sites in all four seasons from 2017 to 2018. The eDNA results yielded a total detection of 80 taxa, of which 74 were identified to species level, and 65 were wild native species. While the number of taxa detected with the two methods were comparable, the species overlap was only 20%. In combination, CT and eDNA monitoring thus yielded a total of 115 wild species (20 fishes, 4 amphibians, one snake, 23 mammals, and 67 birds), representing half of the species found via conventional surveys over the last ca. 20 years (83% of fishes, 68% of mammals, 67% of amphibians, 41% of birds, and 20% of reptiles). Our study demonstrates that a holistic approach combining two non-invasive methods, CT, and eDNA metabarcoding, has great potential as a cost-effective biomonitoring tool for vertebrates.

KEYWORDS
biodiversity, camera traps, Denmark, eDNA, metabarcoding, monitoring

1 | INTRODUCTION

Freshwater ecosystems and their bordering terrestrial habitats cover a small fraction of the Earth's surface yet support about a third of all known vertebrate species (Strayer & Dudgeon, 2010). These habitats are highly vulnerable to human activities, such as urban development, agriculture, nutrient and waste-water runoff, aquaculture, fisheries, and damming (Arthington et al., 2006;

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Dudgeon et al., 2006; Naiman et al., 2002), necessitating efficient methods for monitoring their biodiversity. Conventional methods for such monitoring include direct visual or acoustic observations, or indirect detections via, e.g., tracks, scat or sloughed feathers or fur. In the past decades, camera trapping (CT) has proven to be a minimally invasive and highly efficient method for detection and long-term monitoring of vertebrate biodiversity (e.g., Ahumada et al., 2013; Mugerwa et al., 2013; Silveira et al., 2003). The method allows detection of elusive (Trolle & Kéry, 2005), rare (Azlan & Lading, 2006), and novel species (Rovero et al., 2008), and while CTs are often used to study mammals in tropical areas (Burton et al., 2015; Havmøller et al., 2019), they have also proven effective in temperate forests and open areas (Parsons et al., 2018; Rovero et al., 2014). More recently, environmental DNA (eDNA) analysis has emerged as another cost-effective and non-invasive method for biodiversity monitoring (Ficetola et al., 2008; Taberlet et al., 2012; Thomsen & Willerslev, 2015). This method has been used for species inventories across a wide range of habitat types, although most applications to date are in aquatic systems (e.g., Goldberg et al., 2016; Pedersen et al., 2015; Thomsen et al., 2012). The use of eDNA in terrestrial ecosystems has grown a lot in the past few years with several interesting studies published across the globe (e.g., Johnson et al., 2019, 2023; Mena et al., 2021; Ryan et al., 2022).

All the biomonitoring methods have their strengths and weaknesses in terms of taxonomic coverage, ease of use, survey effort and requirements of taxonomic expertise, and not one method can capture the entire vertebrate diversity of an ecosystem. For instance, combining eDNA metabarcoding and CTs for monitoring of marine fishes has resulted in detection of a larger richness than any of these approaches alone (Boussarie et al., 2018; Stat et al., 2018). Similarly, metabarcoding analysis of eDNA from stream water (Lyet et al., 2021) and terrestrial sediments (Leempoel et al., 2020) combined with CTs has been found to be efficient for monitoring terrestrial mammals. The number of such vertebrate studies combining water eDNA and CTs is growing rapidly, in covering all sorts of habitats from reefs (Boussarie et al., 2018; Stat et al., 2018) to ponds (Harper et al., 2019; Mas-Carrió et al., 2022).

Here, we combine 1 year of aquatic eDNA sampling and 4 years of CT data collection to investigate the vertebrate fauna in a Danish wetland and Nature Park in temperate Northern Europe. We provide an updated inventory of the diversity of species in the park, their commonness and conservation status, and evaluate the complementarity, strengths, and weaknesses of monitoring aquatic eDNA versus monitoring with CTs and compare our results with baseline data for the same locality collected by conventional biodiversity monitoring methods over the past two decades. We expect that the CT method will be effective for mammals and birds and that the eDNA method will be effective for fish and mammals, since the primers used were designed for those groups.

2 | MATERIALS AND METHODS

2.1 | Study site

Field work was performed in a wetland area within Nature Park Åmosen (hereafter referred to as Åmosen), West Zealand, Denmark (N 55.618860, W 11.329161). Åmosen comprises a stream system of approximately 45 km from Undløse in the east to the Great Belt in the west (Figure 1). It consists of a mixed set of habitats including streams, wetlands, forests, fens, meadows, bogs, and thickets, as well as agriculture and some urban development. Åmosen holds a unique flora and fauna including several red-listed species and about 80% of the park is designated as a Natura 2000 area (area no. 156, H137 and area no. 157, H138, F100). Natura 2000 is a network of nature protection areas in the European Union. The areas preserve and protect habitat types, wild animals and plants which are rare, endangered, or characteristic for EU countries (Naturstyrelsen, 2016a, 2016b; Schmidt, 2017). The Appendix S1 lists which species are red-listed and endangered (Table S11).

2.2 | Camera trapping

We monitored the vertebrate fauna of Åmosen by deploying up to 18 camera traps (CTs) at six locations over a period of 4 years from the May 20, 2015 to August 12, 2019 (Table 1). The number of CTs varied by location and season, as some sites were more suitable for deployment than others, and as cameras were occasionally lost due to theft and flooding. We used a water-resistant CT model (IR PLUS BF HD) equipped with a passive infrared sensor and a 940 nm light-emitting diode flash source. All the CTs were placed facing the catchment area and angled to cover both the stream and the opposite stream bank, as suggested by Matsubayashi et al. (2006). The CTs were programmed to record photos and/or 10 s videos with normal sensitivity and no trigger interval, and no bait or lures were used. Batteries and memory cards were replaced at regular intervals.

Photos and videos from CTs were manually examined and identified to the lowest possible taxonomic level based on morphological traits, movement patterns and sounds with help from taxonomic experts at the Natural History Museum of Denmark. To avoid artificial inflation of observations, a camera event (CE) was defined as all detections of a certain species within 30 min at the same location (O'Brien et al., 2003; Zimmermann & Rovero, 2016). To assess the commonness of each taxon, we estimated the relative abundance index (RAI) as the number of CEs of a given taxon per 100 camera trap days (O'Brien, 2011; Rovero et al., 2014), and the naïve occupancy (NO) as the proportion of sites that recorded at least one CE of the target species (e.g., Hedwig et al., 2017; Jenks et al., 2011; Rovero et al., 2014).

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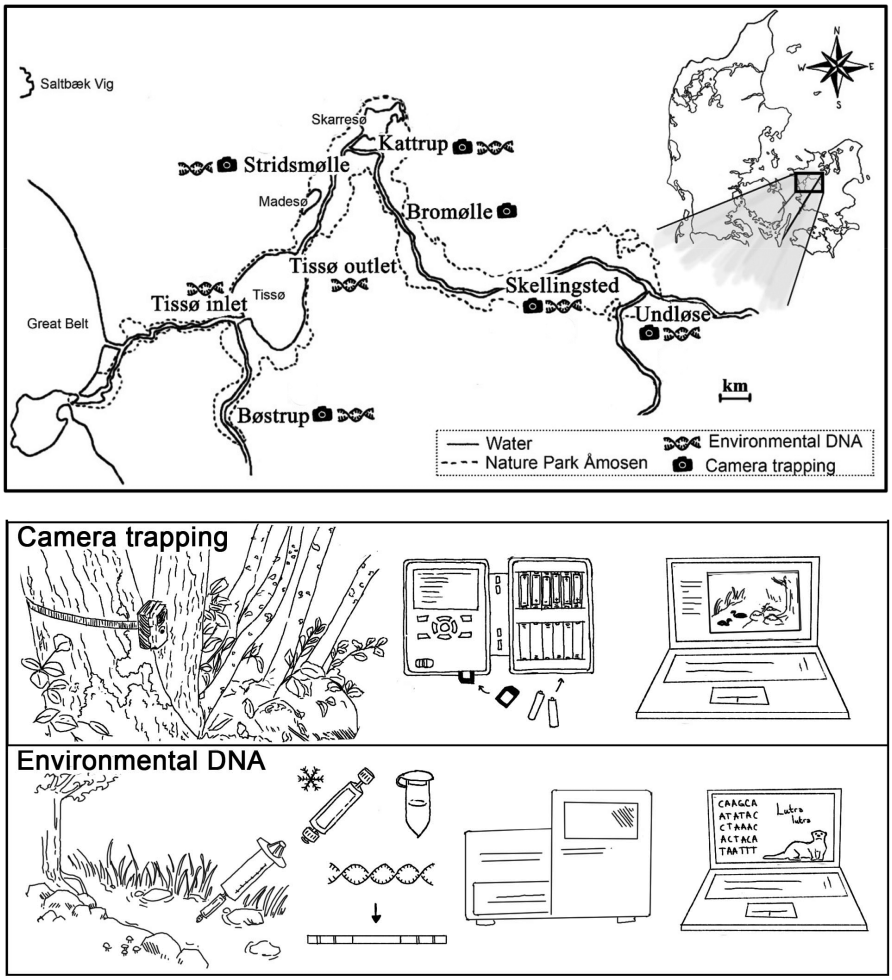

FIGURE 1 The Åmosen Nature Park sampling sites as well as schematic illustration of the camera trapping and environmental DNA methods used to monitor vertebrate diversity. The distance from the eastern part of the park (Undløse) to the sea in west is ca. 35 km. Illustrations by AMRH.

2.3 | Environmental DNA

In addition to monitoring by CTs, we performed eDNA-based monitoring of vertebrates by collection of water samples from September 2017 to December 2018. A total of eight sampling days at all seven locations with flowing waters were done (Table 1, Figure 1). At each sampling event, two to three sample replicates were collected within a few meters from one another Each sample replicate consisted of up to 500 mL of water taken with a 60 mL syringe (Soft-Ject, HSW, Tuttlingen, Germany) and filtered through a Sterivex filter unit of 0.22 µm pore size (polyethersulfone, Merck Millipore, Germany). To avoid cross contamination between sampling locations and dates, a clean 60 mL syringe was used for each location. All the filters were only used one time. Syringes were reused, but were rinsed thoroughly in 0.5% bleach and 70% ethanol, and left to dry out, before being reused. The samples were transported in a cooler and stored at −18°C until DNA extraction. At the end of each sampling day, a negative control sample was taken by filtering mineral water in the Åmosen area, before returning to the laboratory, resulting in a total of ten field blanks.

All laboratory work was performed in separate laboratories designated for DNA extraction and eDNA metabarcoding precedures, respectively. Across ten rounds the environmental DNA was extracted from the filters using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilsen, Germany) with a modified protocol (Sigsgaard et al., 2020; Spens et al., 2017) (Supporting text A and Figure S1). Each round of extraction from filters included a negative extraction control that each serves to identify any eventual cross contamination in the laboratory. Polymerase chain reaction (PCR) amplification was performed using the primer set Mamm01 (mamm01_F: 5′-CCGCCCGTCACCCTCCT-3′, mamm01_R: 5′-GTAYR CTTACCWTGTTACGAC-3′) (Taberlet et al., 2018), and the primer set MiFish-U (MiFish-U_F: 5′-GTCGGTAAAACTCGTGCCAGC-3′, MiFish-U_R: 5′-CATAGTGGGGTATCTAATCCCAGTTTG-3′) (Miya et al., 2015). These primer sets target regions of approximately 59 bp and 170 bp (excluding primers), respectively, around 390–400 bp apart in the 12S mitochondrial gene. Primer sets and extractions were tested out in initial quantitative PCR (qPCR) to infer optimal concentrations of template from extractions. Reagents, volumes, concetrations, and thermocycler conditions for the subsequent metabarcoding PCR setup are provided in the Supporting text B. The samples were divided across three unique PCR setups (Supporting text C, and Tables S1–S3). Each PCR setup included one PCR replicate of each extraction of eDNA from water samples, together with negative PCR controls and positive mock samples. For each primerset both forward and reverse primer were

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TABLE 1 Sampling details for each study site (see Figure 1) and overall.

	Bromølle	Bøstrup	Kattrup	Skellingsted	Stridsmølle	Tissø inlet	Tissø outlet	Undløse	All sites
Site characteristics	Stream, forest and settlements	Stream, reed beds and agriculture	Stream in forested landscape	Regulated stream in agricultural landscape	Stream in forested landscape	Lake in agricultural landscape	Lake in agricultural landscape	Regulated stream in agricultural landscape	
Camera									
Period	6/4-2017-12/8-2019	6/4-2017-12/8-2019	20/5-2015-12/8-2019	6/4-2017-12/8-2019	20/5-2015-12/8-2019	–	–	6/4-2017-12/8-2019	
N	4	1	8	2	1	–	–	2	18
CDs	670	97	6487	514	1007	–	–	106	8881
CEs	770	107	6907	546	578	–	–	181	9089
Taxa	34	20	71	37	34	–	–	34	87
eDNA									
Period	–	27/2-13/12-2018	27/9-2017-13/12-2018	27/2-13/12-2018	17/9-2017-13/12-2018	27/2-13/12-2018	27/2-13/12-2018	27/2-13/12-2018	
N	–	15	19	15	22	14	14	15	114
Taxa	–	40	50	46	49	45	46	42	80
Taxa									
Aves	20	22	53	23	26	21	23	26	74
Mammalia	14	9	24	20	17	7	5	15	29
Actinopterygii	0	14	19	18	17	16	17	12	20
Amphibia	0	2	1	1	3	1	1	3	4
Squamata	0	0	0	1	0	0	0	0	1
Domestic	2	4	7	4	4	4	3	4	9
Total	34	47	97	63	63	45	46	56	137

Note: Number (*N*) of camera traps (CTs) at each study site, camera days (CDs) (collected from May 20, 2015 to of August 12, 2019), and number of camera events (CE's) (observations of animal with at least a 30-min interval). eDNA samples were collected all through 2018 (January*, February, March, May, September, and December), a few samples were taken in the end of 2017 (September** and October**). *only for Stridsmølle. **only for Kattrup and Stridsmølle.

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ordered in many pairs equipped with matching tags. Matching tags could then be used for each single replicate sample, and later on be sorted by their tags (Table S5). Because of this, this setup did not allow for reusing the same unique tagged forard and reverse primer pair more than once per library. Only paired matching tags were used to help lower the risk of getting tag jumps (Schnell et al., 2015). The mock comprised genomic DNA from exotic species unlikely to be found in Denmark, including mammals, fish, and a frog (Olds et al., 2016; Thomsen et al., 2016). One mock was prepared for the first and second setup (Table S7) and another mock was prepared for the third setup (Table S8). Positive controls and negative non-target PCR controls were included in all three setups. The negative extraction controls and field blanks were only included in the third setup with the MiFish-U primerset (Tables S1–S3). Each PCR setup was run twice, giving a total of six PCR replicates of each sample pool for each setup, resulting in a total of 18 libraries (Supporting text C and D, and Tables S1–S4).

All the PCR products were verified on a 2% agarose gel stained with GelRed (Biotium). If the negative controls returned amplified products, we performed the metabarcoding PCR with a lower number of cyles, to avoid amplifying contamination in the negative controls, before continuing with the preparation of libraries. Once we only had positive amplification in the extractions from water samples and the positive control we continued with the library preparation. From each of the 18 libraries (Tables S1–S4) we pooled 10 μL to a total of 120 μL. The 120 μL was then purified using the MinElute (Qiagen) PCR purification kit (cat. no. 28006), following the supplied protocol with modifications (Appendix S1, Supporting text D). Twelve 150 bp paired-end libraries (six for the Mamm01 primer set in the first setup with three technical replicates, six for the MiFish-U primer set in the second setup with three technical replicates, and six for the third setup for the MiFish-U primer set with two technical replicates) were prepared with an Illumina TruSeq DNA PCR-free LT Sample Prep kit (Illumina, San Diego, California), spiked with 8% phiX, and sequenced on two Illumina MiSeq3 flow cells (six libraries on each, the Mamm01 libraries from the first setup on one flow cell, six from the MiFish-Uin the second setup on another flow cell, and six from the MiFish-U in the third setup on a third flow cell) at the GeoGenetics Sequencing Core, University of Copenhagen, Denmark.

Sequence reads were demultiplexed using the software package Cutadapt (Martin, 2011) and a custom python script (available at https://github.com/tobiasgf/Bioinformatic-tools/tree/master/Eva_Sigsgaard_2018) (Sigsgaard et al., 2020). Reads shorter than 10 bp or including ambiguities or with >2 expected errors were removed (Sigsgaard et al., 2020). We then used DADA2 (Callahan et al., 2016) to correct PCR and sequencing errors in the raw sequencing output, and forward and reverse reads with a minimum of 5 bp overlap and no mismatches were then merged. Sequences were blasted against the National Center for Biotechnology Information (NCBI) GenBank database using BLASTn (Altschul et al., 1990) on the March 20, 2020. BLASTn settings were set to a maximum of 3000 hits per query (−max_target_seqs 3000), minimum thresholds of 90% query coverage per high-scoring segment pair (−qcov_hsp_perc 90), and 80% sequence similarity (−perc_identity 80). The output format was set to: -outfmt "6 std qlen qcovs sgi sseq ssciname staxid". BLAST hits displaying incomplete final query coverage were removed. After initial attempts with different settings of coverage and similarity, we opted for a coverage of 90% and a similarity score of 80%, as using BLAST with other settings appeared to return either a diversity that was too broad and implausible for the habitat, or too narrow a diversity that appeared unable to cover the relatively common species known from the habitat. We then classified hits taxonomically in R v.3.6 (R Core team, 2020), using the package 'taxize' (Chamberlain & Szocs, 2013). To reduce data processing time, BLAST hits were then compared against a list of regional vertebrate species and hits to species that are exotic to northern Europe were removed. Comparison of obtained BLAST hits was done with an R code, that compared the BLAST hits with a list of the plausible vertebrate species that potentially can occur in the habitat (Table S10). We removed exotic species with >95% match with the mock species (Table S10). The naïve occupancy (NO) is defined as the proportion of sites a given species is present. It was calculated across all eDNA samples and for each study site, respectively, as the number of eDNA sites/samples where a given taxon was detected divided by the total number of eDNA sites/samples (Table 1).

2.4 | Method comparison

In the overall comparison of CT and eDNA data, it is important to note that this comparison is somewhat biased by our sampling of CT and eDNA data not overlapping completely in collection period and number of sample sites. Still, we decided to compare CT and eDNA data, as the aim was to see if there might be congruence in the diversity obtained.

Species accumulation curves were made for for both CT and eDNA efforts, using the function 'specaccum' from the 'vegan' package (Oksanen et al., 2019) in RStudio v1.2.1335, R v. 3.6.0, here a randomized accumulation curve is found together with the 95% confidence intervals of the mean and the actual cumulation curve. To compare our CT and eDNA-based species detections with previous biodiversity monitoring efforts, we summarized data from traditional/conventional vertebrate surveys performed in Åmosen over the last two decades (2000–2020). Species presence data was compiled from BirdLife Denmark (DOF) (Grell, 1998 and recent data from Michael Fink), Baagøe and Jensen (2007), Carl and Møller (2012), and the Danish species portal Arter.dk, as well as from additional direct visual observations, trapping, excrements, tracks, roadkill done during the CT and eDNA field work and museum collections. The purpose of compiling such background data was to evaluate if the two methods used to generate new data were able to generate a reasonable coverage of the vertebrate fauna known from the area.

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3 | RESULTS

3.1 | Camera trapping

The CT yielded a total of 8778 camera days with 24,376 animal sightings across 8674 CEs. These sightings represented 87 vertebrate taxa, of which 78 (90%) were identified to species level (Table 1). While birds (57 taxa) and mammals (29 taxa) dominated, a grass snake (*Natrix natrix*), and a northern pike (*Esox lucius*) caught by a gray heron (*Ardea cinerea*) were also observed (Figures 2 and 3, Table S9). Most observed taxa were wild species, but domestic animals such as cat (*Felis catus*), dog (*Canis lupus*), cattle (*Bos taurus*), chicken (*Gallus gallus*), and Muscovy duck (*Cairina moscata*) were also detected. The taxa differed markedly in detection frequency with 53% of the taxa being detected in less than 10 CEs and only 18% of the taxa being observed at more than 100 CEs (Figure 2a, Table S9). The most observed bird was the mallard (*Anas platyrhynchos*) with a total of 1422 CEs, amounting to an RAI of 16.2 (detection at 16.2% of all CEs on average) and an NO index of 1.0 (detection at all monitoring sites) (Figure 2b,c). Other frequently and/or widely detected birds included common wood pigeon (*Columba palumbus*), gray heron and Eurasian blackbird (*Turdus merula*). The mammal accounting for the most CEs was the roe deer (*Capreolus capreolus*), although this species was only observed at half of the sites (CEs = 1172; RAI = 13.4; NO = 0.50), whereas the brown rat (*Rattus norvegicus*) was both frequently and widely observed (CEs = 719; RAI = 8.2; NO = 1.0). Other frequently and/or widely encountered taxa included pine marten (*Martes martes*) and other mustelids, red squirrel (*Sciurus vulgaris*) and red fox (*Vulpes vulpes*). A rare surprise was the Eurasian otter (*Lutra lutra*), thought to be locally extinct at the time of the study but

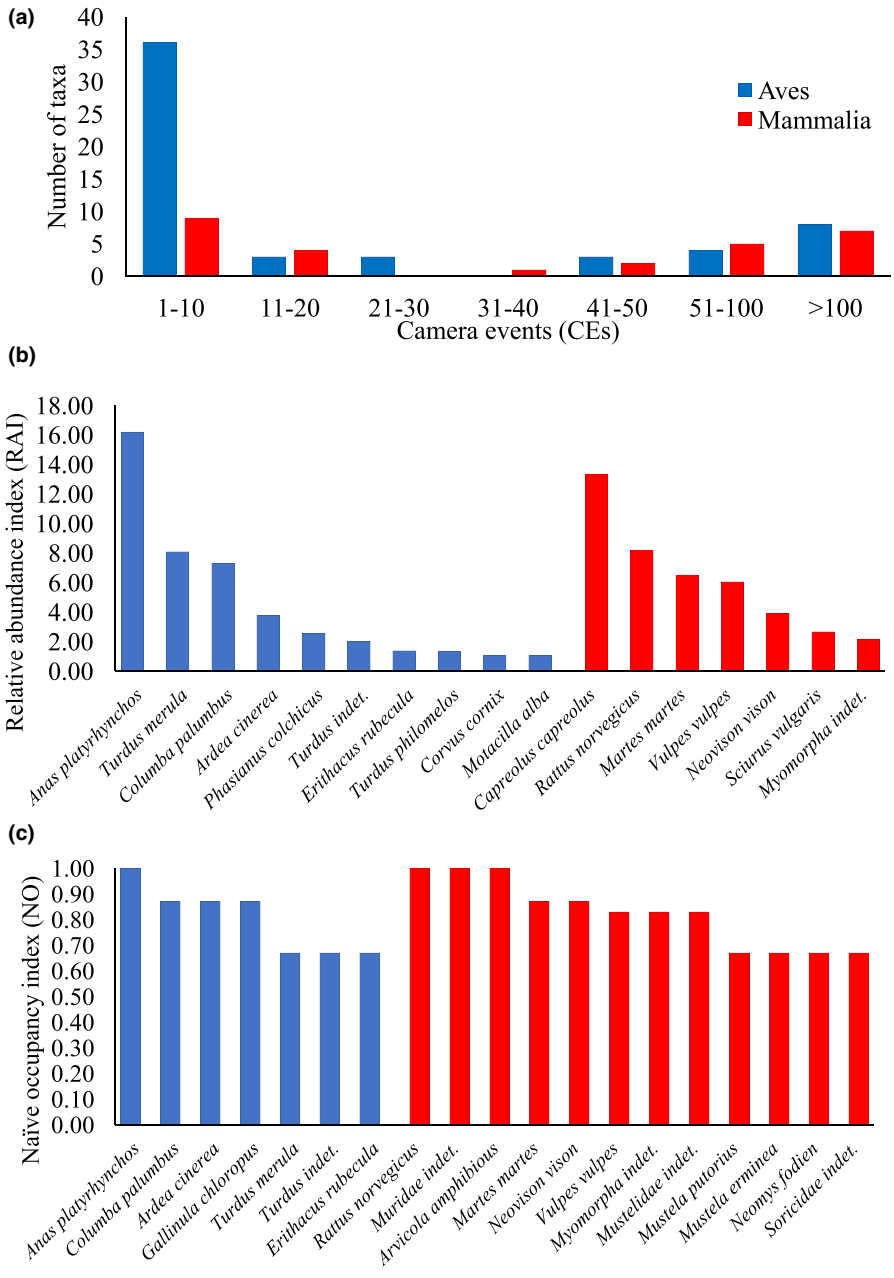

FIGURE 2 The bird and mammal taxa detected by camera traps differed greatly in their abundance and occupancy. (a) The number of taxa in different CE categories with a few very common taxa and many rare. (b) The most abundant bird and mammal taxa defined by a relative abundance index RAI >1.0. (c) The most common taxa defined by naïve occupancy index. A full species list is provided in Table S9.

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found here in 35 CEs, and first time May 28, 2016 (Figure 3). Human activity was recorded at all six study sites, in a total of 472 sightings (472/24,376 = 2%) and 111 individual CEs (111/8674 = 1%).

3.2 | Environmental DNA

The Illumina MiSeq platform produced a total of 25,408,796 raw paired-end reads. After removing mock sample species, non-target species (e.g., prokaryotes and fungi) and human reads, a total of 12,154,093 reads from target vertebrates remained. Totally, 48% of the reads being retained. Across the two primer sets, in the proportion of reads retained, matching vertebrates, 4% were identified as non-target vertebrates: The proportion of non-vertebrate sequence reads was much higher for the Mamm01 primer set (50%–65%) than for the MiFish-U primer set (10%–20%). The retained sequence reads represented 80 taxa, of which 74 were identified to species level (Table 1, Table S9). Both primer sets amplified mitochondrial DNA (mtDNA) from amphibians, fish and mammals, but while 49 taxa were identified by both primer sets, nine taxa were solely identified by the Mamm01 primer set and 22 taxa solely by the MiFish-U primer set. As expected, the MiFish-U primer set yielded more fish species than the Mamm01 primer set, but the Mamm01 primer set did not yield more mammal species (Figure S1).

Humans were detected at all study sites, and nine of the detected species were domestic, including cat, cattle, chicken, dog, horse (*Equus caballus*), Muscovy duck, pig (*Sus scrofa*), sheep (*Ovis aries*), and turkey (*Meleagris gallopavo*). The common roach (*Rutilus rutilus*), and two other taxa dominated the eDNA data with more than one million sequence reads per taxa, while 20 of the taxa were detected in less than 1000 reads and five taxa in less than 100 reads (Figure 4a). The NO analysis also revealed large differences in species occupancy with a few bird (domestic), mammals and fish taxa being detected at all eDNA study sites, including undetermined ducks, mallard (*Anser platyrhynchos*), Eurasian coot (*Fulica atra*), cow, pig, dog, undetermined arvicolines (voles and muskrats), common roach, Eurasian perch (*Perca fluviatilis*), ide (*Leuciscus idus*), northern pike, European eel (*Anguilla anguilla*), and rudd (*Scardinius erythrophthalmus*) (Figure 4b, Table S9).

3.3 | Method comparison

Our review of conventional monitoring data from the Åmosen region yielded 263 wild vertebrate species. Of these, 29 species were deemed outliers as they were, e.g., presumed locally extinct or were extremely rare visitors (Table S11), resulting in a total of 234 final species for comparison with our CT and eDNA data (Figure 5a, Table S11, Supporting text E). We detected 137 taxonomic units (Table 1) including 115 identified as wild species with eDNA and CT combined, including 20 fish, four amphibians, one snake, 23 mammals, and 67 birds (Figure 5, Table 1, Table S9). Thus, the total number of wild species we detected during roughly 15 months of eDNA and 4 years of CT vertebrate monitoring comprise about half (115/234 = 49%) of the species observed in the region through decades of more conventional biomonitoring.

The accumulation curve for CT shows that new taxa are discovered throughout the first 7200 CD with no additional taxa found thereafter. The majority of detected taxa have been found (81 taxa, or 90%) around 6300 CD (Figure 5f). The accumulation curve for eDNA shows that new taxa are added after each collection event, with a major increase in species at sampling event no. 4, but only little increase hereafter (Figure 5g).

Only 30 species were detected with both eDNA and CTs, including one fish, seven mammals, and 15 birds (Figures 5 and 6). The aquatic eDNA detections were biased toward fish and amphibians, whereas CT detections were limited to mammals and birds, except for a single fish detection, which was a result of a gray heron (*Ardea cinerea*) catching a northern pike (*Esox lucius*) close to the camera (Figure 3b). The 115 species detected by CT and aquatic eDNA represented a large diversity in terms of body size, biomass, behavior, life-history, habitat requirements and conservation status, including 19 species (16.5%) categorized as vulnerable, endangered, or critically endangered on the Danish Red List and seven species on the Natura 2000 list (EU Habitat Directive and/or Bird Directive) (Moeslund et al., 2019; Figure S8, Table S9).

4 | DISCUSSION

Our study demonstrates that CT and eDNA sampling can serve as complementary methods for a more holistic monitoring of the vertebrate fauna in a temperate European wetland, nature park Åmosen. We were able to verify the presence of 115 vertebrate species, which is nearly half of the total reported species from Åmosen ($n = 263$) over the last 20 years (see Tables S9 and S11). The taxa found with both CT and eDNA monitoring represent nearly 50% of the eDNA taxa and around 46% of the CT taxa, confirming the benefit of using the two methods in combination. These ratios are not much different from a terrestrial study of vertebrates in southwestern Australia combining soil eDNA and CT, with around half and one third of the total taxa occurring in eDNA and CTs, respectively (Ryan et al., 2022). It should, however, be considered that the CTs in the present study spanned across nearly 4 years and the eDNA monitoring only 1 year, making the comparison somewhat unbalanced.

With CTs, contrary to eDNA monitoring studies, the life stage of detected species can sometimes be determined, e.g., juveniles of American mink (*Neovison vison*), mallard, pine marten, and stoat (*Mustela erminea*) detected by CTs in the present study. Foraging behavior was observed in several species including American mink, common wood pigeon, red fox, and white wagtail (*Motacilla alba*). On the other hand, some species can be hard to detect by CT due to their behavior, life stage, or seasonal changes, potentially leading to biased results (Gotelli & Colwell, 2001). For such elusive species, parallel monitoring of eDNA is especially relevant for complementing

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CT and traditional monitoring methods. Amphibians can be hard to detect as they differ greatly in behavior and appearance between life stages. Valentini et al. (2016) found that eDNA had a much higher detection rate of amphibians than traditional survey methods, provided that the sampling of eDNA is carried out while the amphibians are in their aquatic stage.

Monitoring of semi-aquatic animals, like Eurasian otter, can be problematic with CT (Lerone et al., 2015), and proved challenging when monitoring eDNA. Even though the primer set Mamm01 (Taberlet et al., 2018) was found to have no mismatches with otter DNA sequences obtained from NCBI GenBank database, we did not detect any eDNA from otter. Neither did we detect eDNA from any of the other seven species of mustelids (Figure 6, Table S9) detected by CTs even though comparison of the primers and the mtDNA target region in mustelid species did not show mismatches. Our CT data show that almost all mammal species were in contact with the freshwater stream at some point, and previous studies have shown that when terrestrial mammals drink from, or are otherwise in contact with, a water body, their DNA is often detectable in water samples (Matsubayashi et al., 2006; Rodgers & Mock, 2015; Ushio et al., 2017). Williams et al. (2017) found that even when only the snout of a pig was in contact with water, pig DNA could be detected in the water afterwards. Past studies have also shown difficulties in detecting eDNA from otter even when using a species-specific primer set (Andersen et al., 2018; Harper et al., 2019; Thomsen et al., 2012), but the mammal primers used here have detected mustelids in previous metabarcoding studies (Mena et al., 2021). Of all the seven Danish

FIGURE 3 Examples of animals observed by CTs in Åmosen. (a) birds and fish 1. *Tachybaptus ruficollis* and *Anas platyrhynchos*, 2. *Erithacus rubecula*, 3. *Phalacrocorax carbo*, 4. *Rallus aquaticus*, 5. *Buteo buteo*, 6. *Ardea cinerea* and *Esox lucius*, and (b) mammals (1. *Martes martes*, 2. *Rattus rattus*, 3. *Lutra lutra*, 4. *Cervus elaphus*, 5. *Vulpes vulpes* and 6. *Meles meles*.

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FIGURE 3 (Continued)

mustelid species (Table S9), otters spend the most time in water (Baagøe & Jensen, 2007), but eDNA detection is likely challenging due to low-populations sizes and/or nocturnal behavior. When sampling in stream obviously the obtained data to some degree is a result of the up-stream faunal activity, and habitat features may also confound results in flowing water (Hinlo et al., 2018), but for a holistic overview of the fauna the method is still very effective. Also, the sampling time in eDNA studies can be very important across a day (Jensen et al., 2022) and across the year (Sigsgaard et al., 2017), so the lack of detections might be caused by the mismatch of sampling at daytime, when the otter is active only at night. In 2019 only 8% of eDNA monitoring studies had targeted mammals (Tsuji et al., 2019), but since then the field have expanded rapidly with many studies monitoring eDNA from semi-aquatic and fully terrestrial mammals (e.g., Coutant et al., 2021; Lyet et al., 2021; Sales et al., 2020).

4.1 | Future perspectives

Efficient nature conservation and restoration increasingly requires non-invasive, cost-effective methods for monitoring biodiversity. The two methods used in the current study are already very useful for this task and they are still improving. CT is widely used for monitoring mammals but and standardized protocols have been developed to estimate, e.g., densities of large carnivores as well as factors affecting them (Havmøller et al., 2019). There is, however, no single camera trap protocol that enables full insight into a vertebrate community, and CT will unavoidably have taxon-specific biases (Burton et al., 2015). One of the most time-consuming factors with camera traps is data annotation, which is still largely done manually, although there are advances with annotation through machine learning (Whytock et al., 2021). In our study, 30% of all CT records contained an animal, while the rest were recordings

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triggered by moving water, vegetation or heat spots from the sun. Camera traps are becoming cheaper and more efficient as the technology is developing. It is still considered a somewhat costly method, as equipment costs can be high but the approach is comparatively cheap in the long-term. CT monitoring does not require experts in the field but can instead rely on locals and volunteers, which has also been shown to broaden environmental awareness in local communities (Hönigsfeld-Adamič & Smole, 2011; Parsons et al., 2018).

Our study confirmed that the monitoring of eDNA is effective for monitoring the distribution and occurrence of both aquatic and semi-aquatic vertebrates as shown in other studies (Taberlet et al., 2018; Thomsen et al., 2012). Monitoring aquatic eDNA allowed for detection of all species of fish known from the area with the exception of a few rare species. Of the undetected species, grass carp (*Ctenopharyngodon idella*) and the Wels catfish (*Silurus glanis*) are known only from private ponds near the stream; the flounder (*Plectichtys flesus*) is mainly a marine species that occasionally migrates upstream to Tissø (Carl & Møller, 2012) and the burbot (*Lota lota*) went locally extinct in 1927 (Carl & Møller, 2012). The only common species not detected was the Crucian carp (*Carsassius carassius*), a species mostly found in lentic waters, which might explain its absence in the river water samples. DNA metabarcoding is continuously being refined for more detailed multispecies detection (Creer et al., 2016), but we consider the aquatic eDNA metabarcoding method ready for large-scale monitoring of fish in European freshwater habitats. More terrestrial mammals might have been detected if eDNA from soil, dung, or air samples had been included as well (Leempoel et al., 2020; Lynggaard et al., 2022; Sales et al., 2020; van der Heyde et al., 2021).

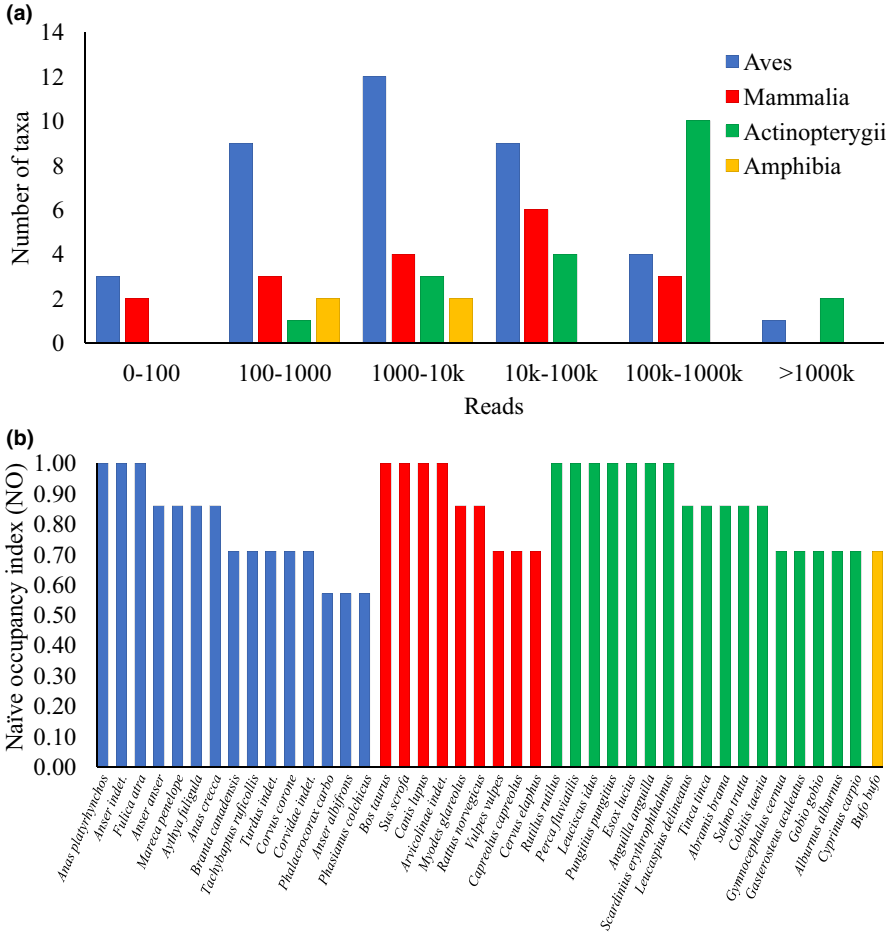

FIGURE 4 The bird, mammal, fish, and amphibian taxa detected by eDNA water sampling differed greatly in their frequency. (a) The number of taxa in different DNA sequence read categories. (b) The most common taxa defined by a naïve occupancy index defined as the proportion of sites where the species was detected. Notice that the three most detected mammals were domestic animals (cow, pig and dog). A full species list is provided in Table S9.

FIGURE 5 Evaluation of monitoring approach. (a) The number of taxa detected in Nature Park Åmosen for each vertebrate class as detected by camera trapping (73 species), (b) eDNA (65 species), (c) both methods (115 species), and (d) previous traditional surveys (234 species). (e) camera trapping and eDNA data compiled by each five vertebrate classes. (f, g) Species accumulation curves for vertebrate taxa detected in Åmosen by camera trapping and eDNA sampling. The black line are the detected taxa, stippled line is randomized accumulation curve estimated in specaccum (vegan package in R), and light gray shading is the 95% confidence intervals. (h) Birds (blue) and mammals (red) with large difference between camera trap naïve occupancy (NO_{CT}) and eDNA naïve occupancy (NO_{eDNA}). Species above or below the horizontal line are overrepresented in camera traps or eDNA, respectively. The figure illustrates the selectivity of the methods, CT being more effective for some species than eDNA and vise versa. Illustrations by AMRH. A full species list is provided in Table S9.

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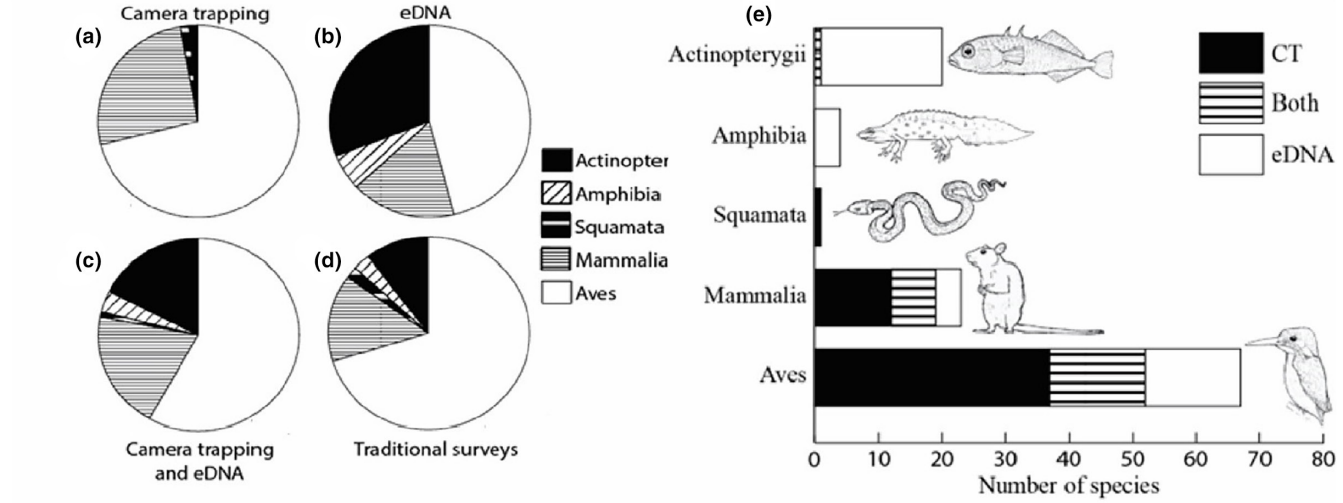

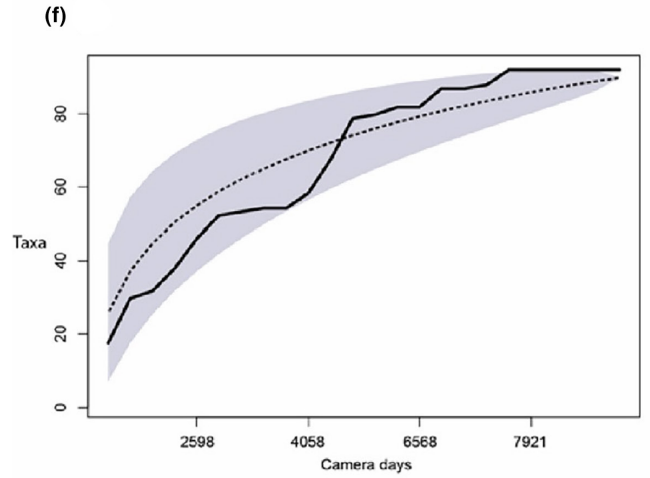

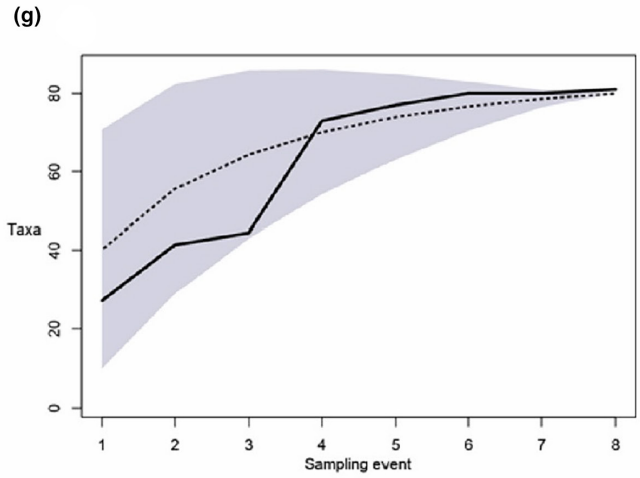

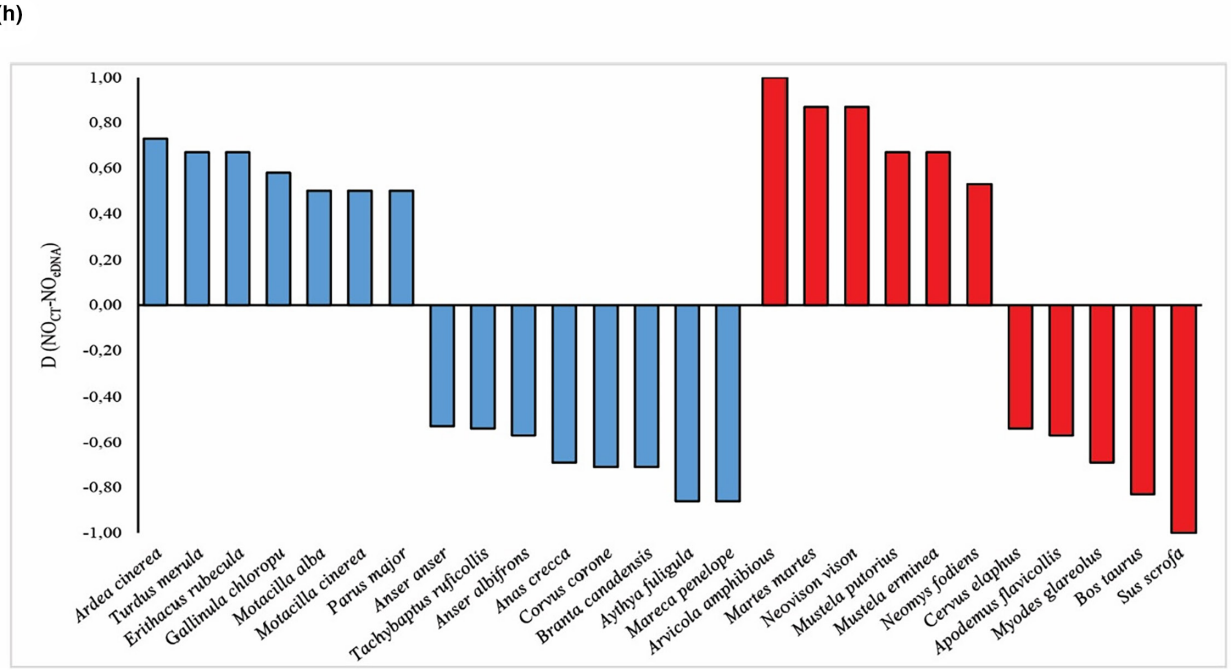

Like many other Danish nature parks and national parks, Nature Park Åmosen is a mosaic of cultural landscapes and more natural habitats mixed with human installations, roads, cities, and agriculture. As demonstrated in the present study these parks can host a variety of wildlife, especially in small pockets of old forest and around near-natural rivers. Such a biodiversity hot-spot is our sampling site

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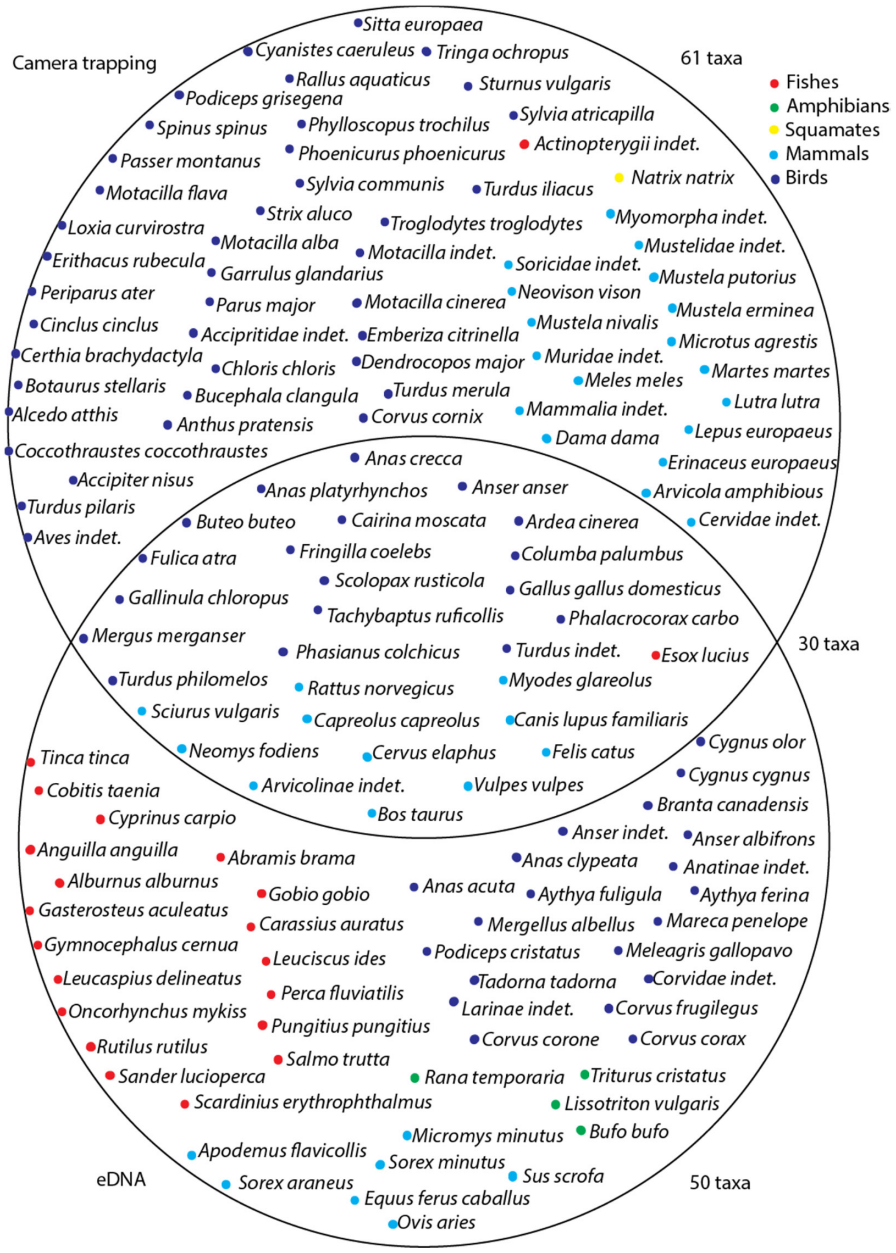

FIGURE 6 Overview of taxa found from camera trapping an eDNA. *Venn diagram* showing the overlap between the qualitative results obtained from camera trapping and eDNA metabarcoding of freshwater in Åmosen. Both methods detected 30 taxa, while 50 taxa only were detected by eDNA and 61 taxa only were detected by camera trapping.

Kattrup, with almost twice as many species as the other sites. This is also where we first found the otter, which is extremely rare on the island of Zealand. Combining CTs and eDNA metabarcoding could be an efficient future means for vertebrate biodiversity monitoring in wetlands and other wildlife habitats.

AUTHOR CONTRIBUTIONS

Anne Marie Rubæk Holm: Writing – Original draft preparation, Conceptualization, Data curation, Methodology, Visualization, Drawings and Illustrations. Steen Wilhelm Knudsen: Methodology, Supervision, Writing – Reviewing and Editing. Malene Månsson: Conceptualization, Investigation. Ditte Elmgreen Pedersen: Investigation, Data curation. Pauli Holm Nordfoss: Investigation. Daniel Klingberg Johansson: Investigation. Rasmus Worsøe Havmøller: Methodology, Writing – Reviewing and Editing. Eva Egelyng Sigsgaard: Validation, Methodology, Reviewing and Editing. Philip Francis Thomsen: Validation, Methodology, Reviewing and Editing. Marthe Gramsbergen: Conceptualization, Investigation. Morten Tange Olsen: Conceptualization, Investigation, Visualization, Supervision, Writing – Reviewing and Editing. Peter Rask Møller: Project administration, Conceptualization, Investigation, Supervision, Writing – Reviewing and Editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

DATA AVAILABILITY STATEMENT

eDNA data are available on the following github repository: https://github.com/monis4567/aamosen_edna_metabarcoding, and from this DRYAD data repository: https://doi.org/10.5061/dryad.00000008k. Camera trap photos of all detected species are up-loaded to iNaturalist.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Not relevant.

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