

# airDNA Can Estimate Avian Species Richness in Woodlands

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## Abstract

DNA filtered directly from air samples (airDNA) is showing great promise as a tool for surveying biological assemblages. This study provides evidence that air samples, taken in a natural, open environment, can provide estimates of species richness for a targeted taxonomic group that are analogous to estimates produced using standard survey techniques. airDNA and audio-visual surveys were carried out across sixteen small broadleaf woodlands in central Scotland. While the total species richness recorded by airDNA was lower than that of the standard survey, rarefaction analysis provided comparable estimates. Our study demonstrates that airDNA survey may represent a viable complement to existing biodiversity monitoring methods and, with refinement and the development of a best practice approach, could become a rapid, reliable and non-invasive standalone surveying technique.

## Introduction

Anthropogenic pressures are driving a global decline in biodiversity, with climate change and land usage identified as major ongoing threats (Sala *et al.*, 2000; Brondizio *et al.*, 2019). Unprecedented population losses have been recorded in terrestrial species, with hundreds of species extinctions every year (Ceballos *et al.*, 2017). Bird populations, as the most diverse group of terrestrial vertebrates, have shown widespread declines in both abundance and diversity (Burns *et al.*, 2021; Rigal *et al.*, 2023). Understanding and monitoring population trends is vital for developing conservation strategies, and although bird populations have been monitored closely in many countries (Foppen, 2020; Tobias *et al.*, 2022), conventional monitoring techniques such as point counts (Fontúrbel *et al.*, 2020), acoustic recordings (Pérez-Granados and Traba, 2021), ringing and geolocators (Heim *et al.*, 2020) are often time consuming, cost prohibitive, and require a high level of expertise, as well as involving potential disturbance to the birds.

Environmental DNA (eDNA) analyses have been repeatedly demonstrated to have powerful biomonitoring capabilities. eDNA techniques target the DNA discarded in biological matter i.e. urine, faeces, skin or tissue cells, or deceased bodies (Rees *et al.*, 2014) which can persist within an

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environment to be collected from areas such as soil, sediment, or water, regardless of current organism presence. Depending on conditions, eDNA can persist for the span of a few days in an environment such as temperate water (Thomsen *et al.*, 2012), and up to hundreds of thousands of years in permafrost (Willerslev *et al.*, 2003). This is a well-established monitoring technique in aquatic settings, successful in surveying biodiversity in both marine and freshwater settings (Lim *et al.*, 2016; Stat *et al.*, 2017), detecting invasive species (Rees, Maddison, *et al.*, 2014; Thomas *et al.*, 2020), and monitoring single species that are under threat (Rees, Bishop, *et al.*, 2014), but eDNA survey techniques for terrestrial species is less established.

Characterising terrestrial communities using eDNA often relies on samples taken from secondary sources such as flowers (Walker *et al.*, 2022; Newton *et al.*, 2023), spiderwebs (Newton *et al.*, 2024) or insects such as carrion flies (Calvignac-Spencer *et al.*, 2013), where DNA has accumulated. These samples are limited in their representation of species, detecting only those that interacted with that particular vector. Samples taken from water sources such as small streams (Mena *et al.*, 2021), stagnant water (Williams, Huyvaert and Piaggio, 2017) or small, transient water sources such as inside tyre tracks in a forest (Marshall *et al.*, 2022) have shown success in characterizing vertebrate communities but face the same limitations: detection is limited to the DNA collected from water runoff, or through interactions with the water source such as bathing or drinking.

More recently, however, metabarcoding and qPCR studies using airborne DNA (airDNA) have demonstrated its potential as a survey tool to detect and characterize terrestrial communities. In enclosed environments such as animal housing rooms, bat roosts and classrooms, airDNA sampling has been successfully utilized to detect naked mole rats (Clare *et al.*, 2021), big brown bats (Serrao *et al.*, 2021) and characterize tropical bat communities (Garrett, Watkins, Francis, *et al.*, 2023; Garrett, Watkins, Simmons, *et al.*, 2023). In zoological parks, Clare *et al.* (2022) and Lynggaard *et al.* (2022) independently showed the successful detection of birds and mammal species from the surrounding area, both captive within the zoos and native to the sample area. Although highlighting the capability for airDNA samples to detect terrestrial vertebrates, these studies sampled from enclosed or semi-enclosed areas that are likely to contain elevated levels of airborne DNA when compared to more natural 'wild' settings. In their papers, both Clare *et al.* (2022) and Lynggaard *et al.* (2022) highlighted the importance of expanding airborne DNA research into wider, less concentrated natural systems where the airborne DNA may be more dilute.

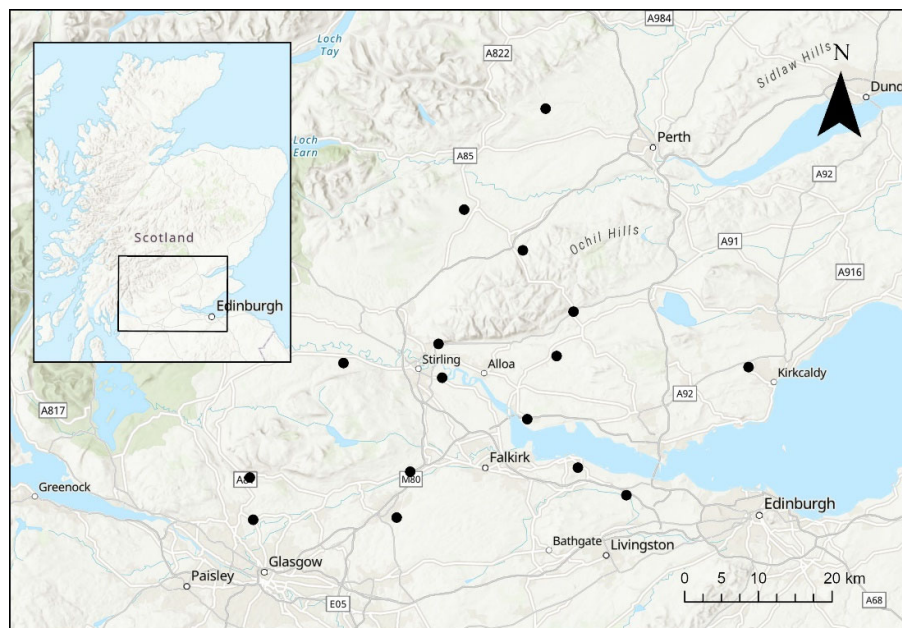
In a study utilising airDNA to document insect species, Roger *et al.*, (2022) collected airborne DNA from open, forest-dominated habitats in southern Sweden using liquid cyclone samplers. This method detected nine vertebrate species, notably including three bird species. Similarly, passive water-based airDNA collectors successfully detected bird, fish and mammal airDNA, accumulated over 24 hours at both coastal and grassland sites (Klepke *et al.*, 2022). Despite not targeting bird species specifically, these studies suggest that not only is airborne DNA a viable means to detect bird populations, but that this can be done using even without the aid of eDNA accumulation caused by enclosed areas and monitored populations such as those at zoological parks, although many uncertainties remain surrounding the persistence, movement, and degradation of airborne DNA in these 'wilder' settings.

This study utilised active sampling techniques to collect air samples from wooded areas in Scotland, alongside standard bird surveys of the sample area in order to assess the ability of airDNA metabarcoding to detect avian species compared to audio-visual surveys. AirDNA samples detected 11 target taxa, seven of which were identified to species level while the remaining four were identified to species clusters. Despite being limited by a lack of robust, avian specific primers suitable for eDNA analysis, the results indicate that airDNA sampling is capable of providing accurate estimates of the total number of species within an ecosystem compared to audio-visual surveys, emphasising its potential as a biodiversity monitoring tool to be used alongside traditional techniques.

## Methods

### *Study Area*

The study was carried out across central Scotland at sixteen woodland sites which are part of the Woodland Creation and Ecological Networks (WrEN) project (Watts *et al.*, 2016). Existing WrEN data were used to select small, secondary mixed broadleaf woodlands (size: 0.5 - 2.2 ha; age: 20 - 160 years) which represented a range of avian species richness values when surveyed previously in 2015 (number of species: 8-20; see Whytock *et al.*, 2018). The sample sites were embedded within agricultural landscapes and provided a good representation of small Scottish woodlands in their early growth (Figure 1).



**Figure 1. Survey locations in central Scotland. Reprinted from ArcGIS under a CC BY license, with permission from Esri, original Copyright 2024 Esri (Basemaps supported by Esri, Esri UK, Tom Tom, Garmin, FourSquare, USGS).**

### *Field Sampling*

Standard woodland bird survey methods (hereafter 2021 AV surveys) were conducted in parallel with airDNA sampling from 19<sup>th</sup> – 30<sup>th</sup> May 2021. Two woodland sites were surveyed per day by an experienced ornithologist in the early morning. Survey effort was standardised to forty minutes at each site with an additional 10 minutes per hectare for sites > 1.5 ha (five sites with none > 2.5 ha). Bird surveys followed a modified version of the Common Bird Census Method by Marchant *et al.* (1990), whereby species presence was recorded within the survey area of the woodland site based on positive visual or auditory identification. To improve chances of detecting territorial species, and reduce the effect of variation in woodland shapes, all areas of the woodland were approached on foot by the observers to within a minimum distance of fifty metres, depending on accessibility. These surveys replicated methods previously used by Whytock and colleagues in 2015, except that each

woodland was surveyed just once rather than three times (Whytock *et al.* 2018). We also use the data from the Whytock, 2015 surveys in our comparative analyses (hereafter 2015 AV surveys).

A single airDNA sample was collected immediately after each conventional bird survey was finished. To collect the sample an Apex 2 vacuum pump (Casella, Bedford, UK) was used to draw air through a closed 49 mm diameter acrylic filter holder (Sartorius Biotech, Germany) containing a 0.45  $\mu\text{m}$  pore size, cellulose nitrate filter (Whatman, Cytiva, UK). The pump was run continuously at a flow rate of 4  $\text{L min}^{-1}$  for two hours per site with the filter inlet held in position at 160 cm above the ground, facing away from the researcher, and moved throughout the woodland. Once sampling was complete, the filter was carefully folded using DNA free forceps and placed into 1 ml Longmire's buffer (Williams *et al.* 2016) and stored at room temperature until processing (max. 6 weeks). To prevent cross contamination during field sampling all sampling equipment; the filter housing, associated tubing and forceps were soaked in 10% bleach for twenty minutes, rinsed with water and allowed to dry between each survey. At a sub-sample of five woodlands, two airDNA samples were collected simultaneously to assess the level of reproducibility between results.

### *DNA extraction*

All benches and pipettes were decontaminated using CHEMGENE HLD4L wipes (STARLAB), before and after each step. In addition, a one-way workflow was used to spatially separate each step of the process, each with its own designated space, equipment, reagents, and consumables. The air filters and any captured DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. The filters were extracted in two sets of X samples and each set included an extraction control of (200  $\mu\text{l}$  sterile distilled water) to indicate whether cross contamination occurred during the DNA extraction. At the final step DNA was eluted into 100  $\mu\text{l}$  TE buffer and quantified using a Qubit dsDNA HS Assay Kit and a Qubit 4.0 fluorometer (both Thermo Fisher Scientific, UK) following the manufacturer's instructions.

### *PCR amplification*

For the 12S ribosomal RNA vertebrate assay (Riaz *et al.* 2011; Kelly *et al.* 2014), amplifications were performed via a two-step PCR process. In total 12 PCR replicates were performed on each air derived DNA sample. The vertebrate eDNA amplification mixture contained 1X DreamTaq Green PCR Master Mix (Thermo Scientific), 0.4  $\mu\text{M}$  of each primer, 0.25  $\mu\text{M}$  of the (F and R) human blocking primer 12S\_V5\_blkhum (Calvignac-Spencer *et al.*, 2013), 0.8 mg/ml Bovine Serum Albumin (BSA, Thermo Scientific), 3% Dimethyl Sulfoxide (DMSO, Thermo Scientific), 0.9  $\mu\text{l}$  of template DNA, and PCR grade water (Thermo Scientific), to a total reaction volume of 8  $\mu\text{l}$ . PCR amplification profile for the vertebrate primers consisted of: initial denaturation at 95°C for 2 min; 10 cycles at 95°C for 20 s, a 30 s touchdown annealing step (-0.5°C per cycle) starting at 60°C, extension step of 72°C for 40 s; 35 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 40 s; and a final elongation step at 72°C for 5 min.

Three no template controls (NTC) using PCR grade water (PCR negative controls) were included in each PCR run to assess for cross-contamination during the set up and analysis. Amplification success was confirmed via gel electrophoresis. If no amplification was observed in the NTC on the agarose gel those samples did not proceed to be sequenced.

## *Sequencing*

All PCR replicates per sample per marker were pooled and purified using Mag-Bind® TotalPure NGS (Omega Bio-tek) magnetic beads. A sequencing library was prepared from the purified amplicons using a combinational dual index approach, following Illumina's 16S Metagenomic Sequencing Library Preparation protocol but using 1X DreamTaq PCR Master Mix (Thermo Scientific). Indexed PCR products were again purified using Mag-Bind® TotalPure NGS (Omega Bio-tek) magnetic beads. The purified products were quantified using a Qubit dsDNA BR Assay Kit, normalized and pooled. The pooled purified index PCRs were sized using a TapeStation D1000 ScreenTape System (Agilent). The libraries were sequenced on an Illumina MiSeq with a V3 MiSeq Reagent kit, the final library was loaded at 10 pM with a 20% PhiX control spike.

## *Bioinformatics*

Sequences were demultiplexed with `bcl2fastq` and subsequently processed via a single analysis pathway with parameters optimised for each assay where appropriate. Paired-end FASTQ reads for each sample were merged with USEARCH (Edgar 2010). Forward and reverse primers were trimmed from the merged sequences using `cutadapt` (Martin 2011) and a length filter applied as appropriate for the assay. These sequences were quality filtered with USEARCH and dereplicated by sample, retaining singletons. Unique sequences from all samples were denoised in a single analysis with UNOISE2 (Edgar 2016), to yield ASVs or zero-radius OTUs (ZOTUs), which allow studying intraspecific diversity.

Taxonomic assignments were made via sequence similarity searches of the ZOTU sequences against reference databases appropriate for the assay. The NCBI nucleotide (NCBI nt) database was queried using BLASTn (Altschul et al. 1990; Camacho et al. 2009) and required hits to have a minimum e-score of  $1e^{-20}$  and cover at least 90% of the query sequence. The taxonomic identification associated with all hits was converted to match the GBIF taxonomic backbone to enable results from different databases to be combined.

Assignments were made to the lowest possible taxonomic level where there was consensus, with minimum similarity thresholds for species, 99% genus, 97% and higher-level assignments of 95% for vertebrates. ZOTUs were then clustered at 97% similarity with USEARCH to obtain OTUs. An OTU-by-sample table was generated by mapping all dereplicated reads for each sample to the OTU representative sequences with USEARCH at an identity threshold of 97%. Finally, low abundance detections were omitted, with filter thresholds set at a percentage of the total reads per sample (vertebrate eDNA: 0.02%). Results are presented for OTUs identified to the avian taxonomic group only.

## *Statistical Analysis*

Results from the airDNA surveys were compared with the rapid audio-visual survey results (2021 AV) and with the full audio-visual surveys undertaken at the same locations in 2015 (2015 AV). Species rarefaction curves, sample coverage and Chao2 estimates of expected species richness were calculated based on the pooled incidence of bird species across all woodlands using the R package `iNEXT` (Hsieh *et al.* 2006). The five replicate airDNA surveys collected to assess reproducibility were excluded from these analyses. Two analyses of the AV survey data are presented, the first includes all data to species level, the second clustered species into taxonomically identifiable groups based on the

12S genetic region (see table 1). airDNA results were analysed both with and without the species which are not typical residents of small woodlands (all the anseriformes) to take account of DNA being present from species which may not be directly utilising the wooded areas, but flying over or being kept locally for agricultural purposes. All analyses were carried out in R version 4.0.5 (R Core Team, 2021).

## Results

The results from the two audio-visual surveys (2015 and 2021) yielded similar results. A total of 33 species were recorded during the 2021 surveys carried out in this study with an average species richness of 10.9 per site (95% CI = 1.7; range 5 to 18). This compares with a total of 26 species in the 2015 AV surveys with an average species richness of 11.5 per site (95% CI = 1.4; range 6 to 16).

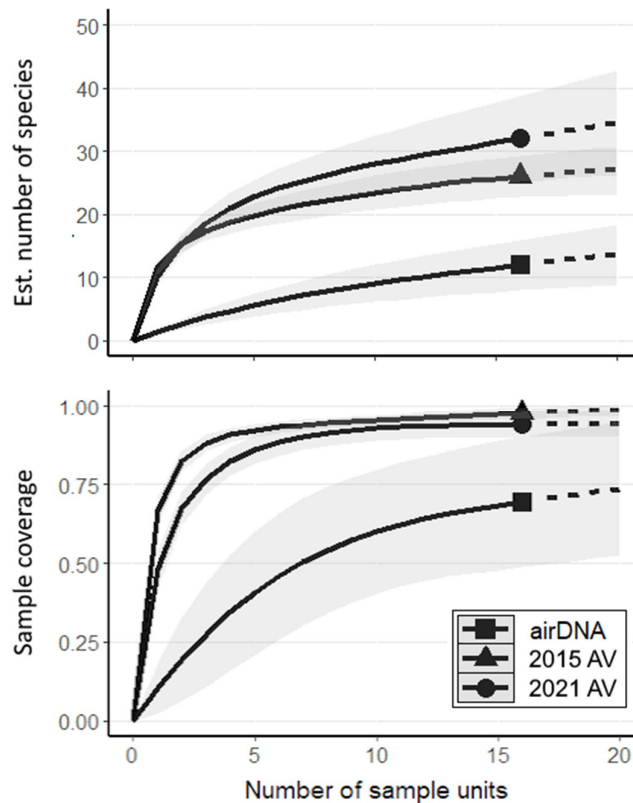
A total of 7680 litres of air were sampled from across all woodlands (480 litres per woodland). The metabarcoding analysis generated high-quality avian sequence data from all 16 sites representing a minimum total of 13 bird species. Of the 13 avian species identified in the samples two were excluded from analyses: domestic chicken and turkey (*Gallus gallus* and *Meleagris gallopavo*) are commonly considered a contaminant species and unlikely to be part of the woodland avifauna. Of the remaining 11 target taxa detected, seven were identified to species level, with the remaining four identified to species clusters (Table 1). One recovered sequence showed extremely high (99.1%) similarity to the continental European species *Certhia brachydactyla*, a rare vagrant to the UK. The closely related species *Certhia familiaris*, is more commonly found in UK woodlands and was reported in the standard survey, however currently there are no publicly available 12S sequences. We therefore cautiously assigned this sequence to *C. familiaris*. There was an average species richness of 1.0 (95% CI = 0.3) per site and ranged from zero to three species detected. Of the 11 taxa found the most commonly detected were *Anas platyrhynchos* and a Columbidae species cluster, each of which were reported from a total of five samples (table 1).

A minimum of twenty-one species were recorded solely in the standard AV surveys, accounting for those which can't be distinguished using the 12S gene, while one species, *Sitta europaea*, was recorded only via airDNA. Reproducibility of the airDNA results was low, with no species being recorded in both replicates at any of the five sites where simultaneous samples were collected.

Rarefaction analysis indicated that sample coverage was 69% for the airDNA and 94-98% for the standard surveys (figure 2). While the estimated species richness for the airDNA results was lower than the standard surveys, there was considerable overlap between the upper limit of the airDNA-derived species richness estimates and lower limits of the standard surveys (figure 3). These comparisons are based on visual inspection of the data as there is currently no validated statistical model for directly comparing Chao estimators; observations of statistical significance are therefore made conservatively.

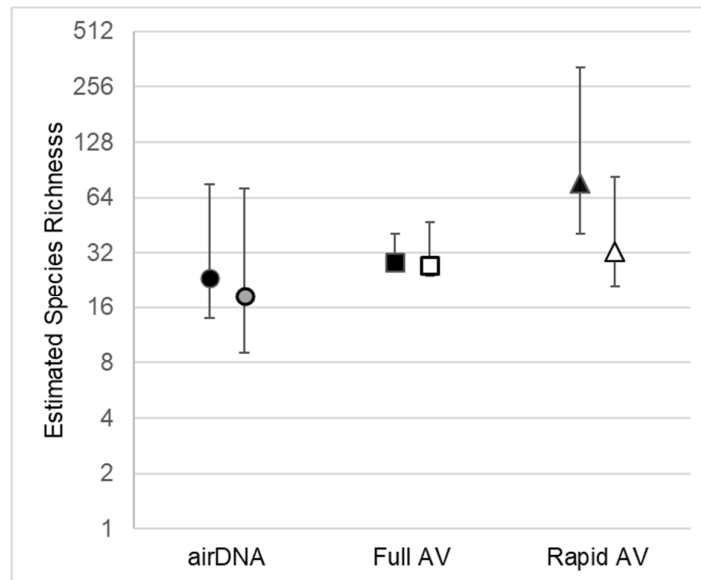
**Table 1: Avian taxa identified from airDNA samples and number of sites at which each species group was recorded.**

Order	Family	Species / Group	No. Sites
Anseriformes	Anatidae	<i>Anas platyrhynchos</i>	5
	Anatidae	<i>Anser sp., Cygnus sp. or Branta sp.</i>	2
Columbiformes	Columbidae	<i>Columba livia, C. oenas, C. palumbus, Streptopelia decaocto</i>	5
Galliformes	Phasianidae	<i>Phasianus colchicus</i>	1
	Certhiidae	<i>Certhia ?familiaris</i>	1
	Corvidae	<i>Corvus corax, C. corone, C. frugilegus, C. monedula, Pica pica, Garrulus glandarius</i>	4
Passeriformes	Fringillidae	<i>Carduelis carduelis</i>	1
	Fringillidae	<i>Motacilla alba, M. cinerea, Pyrrhula</i>	1
	Motacillidae / pyrrhula		
	Muscicapidae	<i>Erithacus rubecula</i>	2
	Paridae	<i>Parus major, Cyanistes caeruleus</i>	1
	Sittidae	<i>Sitta europaea</i>	2



**Figure 2. Species rarefaction curves and sample coverage for airDNA and audio-visual bird surveys. Dotted lines are extrapolated values.**

The results were relatively unaffected by the manipulations designed to make airDNA and standard survey results more comparable. Neither clustering species recorded using AV survey by identity in the 12S region nor removing anseriformes from the airDNA results had a strong effect on the estimated species richness (figure 3).



**Figure 3.** Mean and 95% confidence intervals for the estimated species richness for  $n = 16$  woodlands based on the Chao2 estimators. Black symbols represent rarefaction results from all data; the grey symbol illustrates the results from the airDNA when non-woodland species were removed from the dataset; open symbols represent the results from the audio-visual surveys (2015 and 2021) with species merged by identity at the 12S gene region. Note the  $\log_2$  scale of the y-axis.

## Discussion

In this study we have shown that DNA extracted from the air in woodlands within a ‘wild’ landscape context can be used to detect avian species and that the results, although sparse, provide an accurate estimate of the total number of species within an ecosystem when compared to the results of standard audio-visual surveys. As such, we believe that active airDNA sampling shows promise as a method for providing quantitative measures of biodiversity. The sampling of airDNA has been shown to be feasible in areas that are likely to be high in airborne DNA, due to high densities of animals (i.e. in zoos) and regular disturbance (e.g. Clare *et al.* (2022) and Lynggaard *et al.* (2022)) as well as via large scale sampling of airborne particulate matter (Littlefair *et al.* 2023). This study provides further evidence that airDNA may provide another tool in the emerging panoply of eDNA techniques for use in biodiversity survey.

Refining the sampling methodology is now an important step in the development of airDNA techniques. In total, we filtered 7,680 litres of air across sixteen woodlands using a small portable suction device. Whilst this volume is approximately fifty times greater than that filtered by Clare *et al.* (2022) which identified 25 species of bird and mammal from air taken from a zoo, it is still too low for open habitats where airborne DNA will be present at much lower concentrations. However, if the amount of air sampled were to be significantly increased, it is probable that the quality of the data returned would increase in terms of both the number of species recorded and the reproducibility of

the results. Extrapolation of the rarefaction analysis indicated that the sample coverage would continue to increase with greater sample volume. Assessing the efficacy of sampling different quantities of air on the results would be a useful future research step. However, caution must be applied to ensure that pump volumes do not cause unwarranted disturbance in natural environments. Our pumps measured 58 dB which was deemed unlikely to cause disturbance, particularly given the nature of the woodlands which embedded within anthropogenic landscapes where anthropogenic noises are common.

Due to the lack of robust bird-specific primers that are suitable for eDNA analyses, this study used vertebrate specific primers targeting the 12S gene. This worked well as a proof of principle, but future airDNA studies would benefit from using primer sets which have greater taxonomic resolution and by revising the sampling techniques to avoid swamping the samples with human DNA. Despite in-field precautions and the use of human blocking primers in the metabarcoding process (see Valentini *et al.* 2016), the results were dominated by the presence of human DNA that may have masked the presence of other avian species. Avoiding sampling of non-target DNA may also be assisted by developing automated sampling regimes that do not rely on humans being present during sample collection. The development and use of a greater variety of reliable taxon-specific primers sets.

While these demonstrations are far from a perfected technique, we believe that airDNA could be a useful ornithological tool for two main reasons: it may provide consistency of detection throughout the year and allow the detection of cryptic and/or elusive species. The majority of avian surveys are undertaken audio-visually. As birds are generally large, mobile and vocal, as well as having the benefit of having excellent identification materials and, usually, access to expert surveyors, audio-visual techniques can often provide a good representation of the species within a habitat. However, the detectability of many species via audio-visual techniques changes throughout the year for example, during periods of moulting, many species become much harder to detect as they prioritise predator avoidance behaviours due to decreased evasion capacity (Lind *et al.* 2010). This means that audio-visual bird surveys are generally limited to periods when birds are most detectable. We contend that airDNA detectability may be more consistent across different behavioural seasons, assuming that enough air can be sampled to allow for between-sample consistency, since DNA will continually be shed into the atmosphere. An alternative hypothesis would be that detectability may even increase for airDNA at some periods of the avian lifecycle when audio-visual detectability decreases e.g. during the moult or, in species where both parents provision young, brood rearing.

We also expect that airDNA may provide benefits for the detectability of cryptic and/or elusive species. It is notable that airDNA reported *Sitta europaea*, the Eurasian Nuthatch, from two sites while it was not recorded via any of the standard AV surveys. While not a scarce species, *S. europaea* is a woodland species which is hard to spot and generally most vocal in late winter and early spring. By May, when our surveys were taking place, nuthatch vocalisations are very infrequent and therefore it is fairly unsurprising that it was not detected in AV surveys at this time of year. However, while elusive, it is an active species, foraging throughout the woodland strata and therefore may shed DNA widely throughout its woodland range. We suggest that other avian species which are hard to survey using standard AV methods should be assessed for their suitability of airDNA survey and, where appropriate, the development of highly sensitive single-species, qPCR-based assays be developed.

While we are confident that airDNA can be a useful technique in many cases, there is a clear need for further testing on the source and fate of airborne DNA to be studied. As demonstrated by the identification of chicken and turkey DNA in the samples, it is probable that airborne DNA from outside a specific study area will be captured. Contamination control will be vital to ensuring that airDNA results are reliable – this is likely to be a combination of carefully planning sampling techniques to, for

example, minimise the transfer of DNA between sites by surveyors and developing clear guidance on the bioinformatic analysis frameworks to provide clear probability levels of species presence.

We conclude that airDNA is an interesting and potentially useful addition to the eDNA toolkit with multiple possible applications (see Clare *et al.* 2021). Of particular interest should be its potential to provide a robust and reproducible metric of the biodiversity that can complement other techniques.

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## References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J., 1990. 3.1 Sequence searches-challenges. *Molecular Biology*, 215, pp.403-410.
- Banerjee, P., Dey, G., Antognazza, C.M., Sharma, R.K., Maity, J.P., Chan, M.W., Huang, Y.H., Lin, P.Y., Chao, H.C., Lu, C.M. and Chen, C.Y., 2021. Reinforcement of Environmental DNA Based Methods (*Sensu Stricto*) in Biodiversity Monitoring and Conservation: A Review. *Biology*, 10(12), p.1223.
- Bohmann, K., Evans, A., Gilbert, M.T.P., Carvalho, G.R., Creer, S., Knapp, M., et al. (2014) Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology & Evolution*, 29, 358–367.
- Calvignac-Spencer, S., Merkel, K., Kutzner, N., Kühn, H., Boesch, C., Kappeler, P.M., Metzger, S., Schubert, G. and Leendertz, F.H., 2013. Carrion fly-derived DNA as a tool for comprehensive and cost-effective assessment of mammalian biodiversity. *Molecular Ecology*, 22(4), pp.915-924.
- Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. and Madden, T.L., 2009. BLAST+: architecture and applications. *BMC Bioinformatics*, 10(1), pp.1-9.
- Clare, E.L., Economou, C.K., Faulkes, C.G., Gilbert, J.D., Bennett, F., Drinkwater, R. & Littlefair, J.E. (2021a) eDNAir: proof of concept that animal DNA can be collected from air sampling. *PeerJ*, 9, e11030.
- Clare, E.L., Economou, C.K., Bennett, F.J., Dyer, C.E., Adams, K., McRobie, B., Drinkwater, R. and Littlefair, J.E., (2021b) Measuring biodiversity from DNA in the air. *Current Biology*. 32 (3) 693-700.e5
- Edgar, R., 2010. *USEARCH*. Lawrence Berkeley National Lab (LBNL), Berkeley, CA (United States).
- Edgar, R.C., 2016. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *BioRxiv*, p.081257.
- Fediajevaite, J., Priestley, V., Arnold, R. and Savolainen, V., 2021. Meta-analysis shows that environmental DNA outperforms traditional surveys, but warrants better reporting standards. *Ecology and Evolution*, 11(9), pp.4803-4815.

Gogarten, J.F., Hoffmann, C., Arandjelovic, M., Sachse, A., Merkel, K., Dieguez, P., Agbor, A., Angedakin, S., Brazzola, G., Jones, S. and Langergraber, K.E., 2020. Fly-derived DNA and camera traps are complementary tools for assessing mammalian biodiversity. *Environmental DNA*, 2(1), pp.63-76.

Hsieh, T.C., Ma, K.H. & Chao, A. (2016) iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). *Methods in Ecology and Evolution*, 7, 1451–1456.

Kelly RP, Port JA, Yamahara KM, Crowder LB (2014) Using Environmental DNA to Census Marine Fishes in a Large Mesocosm. *PLOS ONE* 9(1): e86175.

Lind, J., Jakobsson, S. and Kullberg, C., (2010) Impaired predator evasion in the life history of birds: behavioral and physiological adaptations to reduced flight ability. *Current Ornithology* 17, pp.1-30.

Lynggaard, C., Bertelsen, M.F., Jensen, C.V., Johnson, M.S., Guldberg Frøslev, T., Olsen, M.T. & Bohmann, K. (in press) Airborne environmental DNA for terrestrial vertebrate community monitoring. *Current Biology*. 32 (3): 701-707.e5

Marchant, J.H., Hudson, R., Carter, S.P. & Whittington, P. (eds) (1990) Population trends in British breeding birds. British Trust for Ornithology, Tring.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet*, 17(1), pp.10-12.

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.

Rees, H., Maddison, B., Middleditch, D., Patmore, J. & Gough, K. (2014) The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*, 51, 1450-1459

Riaz, T., Shehzad, W., Viari, A., Pompanon, F., Taberlet, P. and Coissac, E. (2011). ecoPrimers: inference of new DNA barcode markers from whole genome sequence analysis, *Nucleic Acids Research*, 39 (21), 1.

Ruppert, K.M., Kline, R.J. & Rahman, M.S. (2019) Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*, 17, e00547

Thomsen, P.F., Kielgast, J., Iversen, L.L., Wiuf, C., Rasmussen, M., Gilbert, M.T.P., et al. (2012) Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, 21, 2565–2573.

Thomsen, P.F. & Willerslev, E. (2015) Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, 183, 4–18.

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P.F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F. and Gaboriaud, C., 2016. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*, 25(4), pp.929-942.

Watts K, Fuentes-Montemayor E, Macgregor NA, Peredo-Alvarez V, Ferryman M, Brown N, Bellamy C & Park KJ (2016). Using historic woodland creation to construct a long-term, large-scale natural experiment: the WrEN project. *Ecology & Evolution* 6: 3012-3025.

Weldon, L., O'Leary, C., Steer, M., Newton, L., Macdonald, H. and Sargeant, S.L., 2020. A comparison of European eel *Anguilla anguilla* eDNA concentrations to fyke net catches in five Irish lakes. *Environmental DNA*, 2(4), pp.587-600.

Whytock, R.C., Fuentes-Montemayor, E., Watts, K., Barbosa De Andrade, P., Whytock, R.T., French, P., et al. (2018) Bird-community responses to habitat creation in a long-term, large-scale natural experiment: Birds and Habitat Creation. *Conservation Biology*, 32, 345–354.

Willerslev, E., Hansen, A.J., Binladen, J., Brand, T.B., Gilbert, M.T.P., Shapiro, B., et al. (2003) Diverse Plant and Animal Genetic Records from Holocene and Pleistocene Sediments. *Science*, 300, 791–795.

Williams, K.E., Huyvaert, K.P. and Piaggio, A.J., 2016. No filters, no fridges: a method for preservation of water samples for eDNA analysis. *BMC Research Notes*, 9(1), pp.1-5.