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From Species to Signals: Exploring the Potential of Airborne eDNA and eRNA Analysis for Pathogen-Agnostic Monitoring at One Health Crossover Points

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Abstract

Land-use change is altering patterns of disease spillover between wild and farmed animals, demanding more agile surveillance at One Health interfaces. Indirect surveillance, which detects host presence rather than pathogens directly, could help inform about disease spillover potential. We collected airborne environmental nucleic acids (eNA), in and around a livestock farm to assess its suitability as a surveillance tool to analyze animal, microbial and viral communities. Host animal presence was compared to three complementary methods; camera trapping, bioacoustics monitoring, and collection of insects for invertebrate derived DNA (iDNA). Air sampling methods were benchmarked in 2024 and finally collected (May 2025) at a farm and two nearby nature estates. The eNA was sequenced using metabarcoding and shotgun metagenomics which identified 53 vertebrate, 324 fungi, 2,739 virus, and 2,753 bacterial taxa across three sites, with the greatest diversity at the farm for all groups except fungi. eNA missed two vertebrate species detected by camera traps but captured six additional species. Bioacoustics outperformed eDNA for bird detection (27 vs 10 families), and iDNA yielded eight vertebrate families. Airborne eNA analysis provides a comprehensive picture of animal, microbial, and viral communities at each crossing-over site, establishing its potential for agnostic host and pathogen monitoring.

Introduction

Spillover of pathogens between wild and farmed animals has always occurred, but increased human population density along with a changing climate, land use changes, and subsequent changes to biodiversity are drastically altering known patterns of interaction [1,2]. This can be attributed to many interconnected factors, including new or increased contact between domesticated and wild animal populations caused by habitat fragmentation and loss, as well as changes to the geographic range of vector species, leading to higher risks of spillover [1].

Existing surveillance systems are not well equipped to capture the changes in disease spillover risk brought by these new patterns of interaction. A majority of the systems currently in place rely on targeted surveillance for known pathogens [3]. While these systems have advantages such as optimized diagnostic

workflows, the previously mentioned ecological changes mean that new pathogens might be emerging, while others are expanding their ranges, making it difficult to know which samples and specific pathogens monitoring programs should target. Instead, an indirect disease surveillance approach which tracks fluctuations in the drivers of disease emergence, such as the presence of certain host species to help to better understand disease risks could be used[2]. This strategy could monitor communities of host animals particularly in areas where wildlife and livestock may (indirectly) interact, such as on farms. Livestock farms are complex systems where humans, wildlife and domestic animals are in close direct and indirect contact through their shared environment, which increases chances for pathogen spillover between the different populations. This makes farms ideal targets for innovative disease surveillance. Changes to the ecosystem surrounding a farm, such as the introduction of a new species or a decrease in biodiversity could be a precursor to disease emergence. Many commonly used methods for observing wildlife rely on experts to identify species and are generally optimized to target specific taxa rather than entire vertebrate communities [4,5]. The addition of total environmental nucleic acids (eNA) based monitoring could add an additional layer to assess crossing-over risks.

eNA consists of any DNA or RNA recovered from environmental samples such as water or soil, which has been shed by any organism in the form of skin cells, feathers, pollen etc. [6]. Through amplicon or shotgun metagenomic sequencing techniques, these fragments can be mapped to species, giving a complete picture of the sampled environment. Studies have found that the air contains genetic traces of vertebrates [7–10], plants [11], and invertebrates [12] as well as being a rich source of information on microbial and viral biodiversity: bacteria (including antimicrobial resistance genes) [13,14], viruses [15], and fungi have been detected [16]. The method has also been shown to be sensitive enough to catch early introductions of invasive or rare plant or animal species without the need to directly observe them [5,11]. In the last few years the collection of airborne eNA has been demonstrated to be a powerful tool for monitoring terrestrial animal populations, both in semi-controlled settings such as zoos [8–10] and in open natural settings [9,36] but there has been limited work on the potential application of the method at livestock farms.

The current study aimed to assess the suitability of airborne eNA analysis as a pathogen-agnostic surveillance tool in and around a livestock farm. The air samples provided information on the vertebrate, microbial, fungal and viral communities present at these sites, demonstrating the method's capability for comprehensive sampling. To help identify strengths and gaps of the method, the vertebrate species detection was compared to three complementary techniques (bioacoustics, camera trapping and invertebrate derived DNA) while microbial detection was assessed against common microbial diversities to be expected at the various sampling sites. Bioacoustics was included to target bird species as they are an important part of the farm landscape and can be missed by other monitoring methods [17,18]. Similarly, farm-dwelling insects interact with diverse wild and domestic hosts and can acquire host DNA on their body surface (iDNA), acting as vehicles for indirect contact between the two groups [19,20].

Methods

Study design

Air samples were collected at three locations, a livestock farm and two nature areas, after which eNA was extracted and analyzed for vertebrate, microbial, fungal and viral presence. A pilot air sampling campaign conducted in 2024 guided the development of the laboratory and bioinformatic methods, including the barcoding gene selection. This was followed by a dedicated sampling campaign in 2025 including three complementary methods of surveying biodiversity (camera trapping, acoustic monitoring for birds, and collection of insects for the evaluation of invertebrate derived DNA (iDNA)). In 2024, air sampling took

place from the 25th of July to the first of August. In 2025 sampling was conducted over two-week periods in May. The study sites are described in detail in Table 1 and visualized in Figures 1A (map of the sampling locations) and 1B (Map of sublocations). Different sampling sublocations were chosen each year due to site accessibility and equipment availability (detailed below in Table 1 and Figure 1B).

Table 1: Sampling location descriptions

Site Name	Description
Tolakker Farm	Farm housing approximately 120 sows, 70 dairy cattle and 200 sheep. The farm is part of the Faculty of Veterinary Medicine of Utrecht University and is surrounded by fields used for grazing the animals.
Oostbroek Estate	Nature reserve managed by the 'Utrechts Landschap'. Includes managed garden areas alongside deciduous forest, meadow and wetland areas. This area has many walking paths that are open to the public.
Vollenhoven Estate	Private estate with managed gardens, as well as deciduous forests, ponds and agricultural fields left unmown to serve as meadowland. The estate is private but frequently hosts events.

Sampling Locations

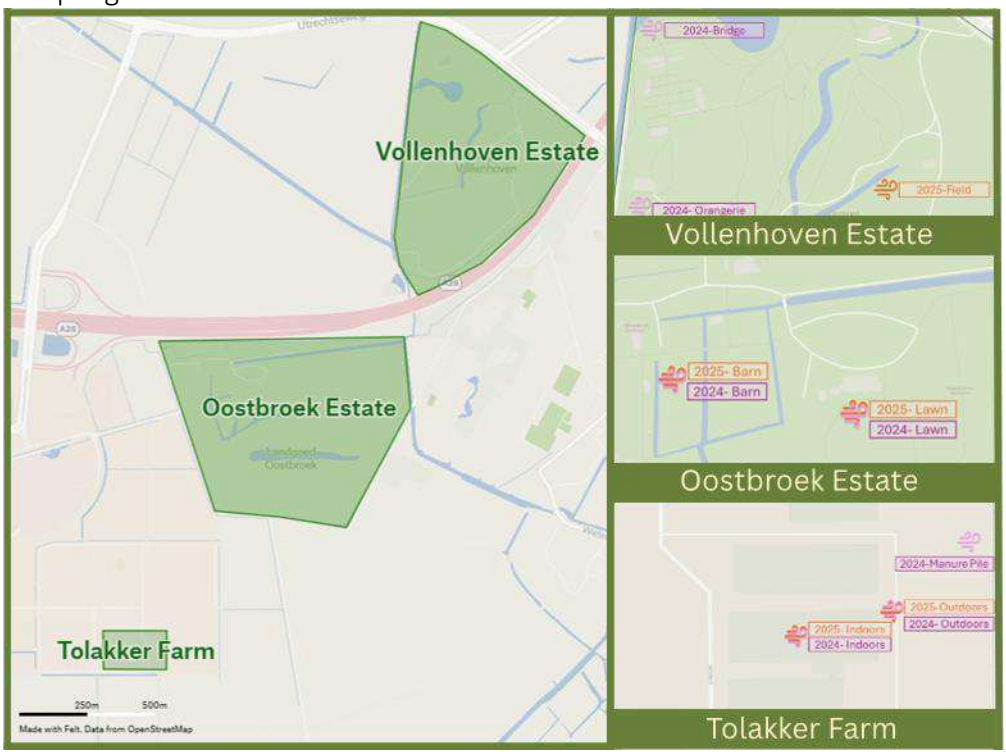


Figure 1A (left): Map of sampling sites. Figure 1B (right): Air sampling locations, 2024 locations are marked in purple, and locations from the 2025 campaign are marked in orange. Map created in Felt (felt.com), basemap © OpenStreetMap contributors.

Sampling locations 2024

At the farm location (the Tolakker) three sublocations around the pig stable were sampled; inside the area of the roof where clean outdoor air is pulled in to ventilate the stable, outdoors directly next to the outdoor

open air section of the pig stable, and outdoors further from the stable. At the Oostbroek estate, air samples were collected at two sites. The first in a section of the garden next to a public walking path and a barn which houses two goats. The second Oostbroek site was in a meadow in a quieter area of the park with fewer visitors. At Vollenhoven samples were again collected at two sites, one near a pond at the edge of a wooded area across the Orangerie building on the estate, and the other was placed next to a small bridge over a stream.

Sampling locations 2025

At the farm location, air samples were taken again in the clean air intake for the pig stables (TI), and outdoors (TO) directly next to the open air portion of the stable. At Oostbroek, the same barn (OB) and lawn (OL)sites were used as in the first year. In Vollenhoven, one air sample was collected at a new site in a meadow area behind a residence on the estate (VF).

Air Sampling

Airborne dust (total dust fraction) was collected using Teflon filters (PTFE 2.0µm 37mm: Pall Corporation) which were placed with a support filter (PE Drain Disc, Whatman) in a Harvard impactor head without size selective impactor (Air Diagnostics and Engineering Inc, Harrison, USA) and connected to a Derenda LSV pump (Comde-Derenda GmbH, Stahnsdorf, Germany). The Harvard impactors were clamped onto metal rods to raise them to 1.5m. The pumps were calibrated to sample a total of 100.8 m^3 of air per week at a rate of 10L per minute. The flow rate was tested with a rotameter, during the initial set up and at the end of each sampling period. At the end of the sampling period the filters were removed from the impactors and stored at -80^oC until NA extraction. Field blanks were also collected at each location and each sampling period to control for contamination of the filters during preparation or transport. For this Harvard impactor heads containing filters but with plastic covering the inlets to stop air from entering the impactor were clamped on the poles with the experimental impactors for the duration of sampling.

Camera Trapping

Between May and June 2025 Browning Dark Ops (BTC-6HDPX) camera traps were installed around the Utrecht Science Park (n=19) and the neighboring Oostbroek Estate (n=7), as part of an annual monitoring program. Of these, two cameras within 200 meters of air samplers were active during the sample period and were analyzed for this study. Camera Trap 13 (CT13) was located ~44m from the Oostbroek Lawn pump, and ~194m from the Oostbroek Barn (OB) pump. Camera Trap 3 (CT3) was ~97m from the Oostbroek Lawn (OL) pump, and ~280m from the OB pump.

The camera traps were attached to a tree at 0.3-0.5 m height and oriented towards places with a relatively unobstructed line of sight. Small vegetation obstructing the field of view was removed, and the devices configured to take bursts of three photographs with night exposure set to ‘long range’ and capture delay to 10 seconds. All photographs were annotated in TimeLapse2 V2.3 [21] and identified to the lowest taxonomic level possible. Only camera observations collected during the same time period as the eDNA sampling are included in the analysis.

Bioacoustic Sampling

Recordings of bird sounds were collected alongside two of the air samplers (outside the stable at the Tolakker, and at Vollenhoven). A recorder was also placed alongside the sampler in Oostbroek, but the data was lost, due to a damaged SD card, so is not included. Bioacoustic data were collected using BirdWeather’s Portable Universe Codec (PUC) recording units, a device equipped with dual microphones able to record soundscapes. Once sounds were detected and recorded, the recording is processed and assigned a species by the BirdNET neural network, developed collaboratively by the K. Lisa Yang Center for Conservation

Bioacoustics at the Cornell Lab of Ornithology and the Chemnitz University of Technology. The species list was then checked by an expert (J.N. Wiegers), and species whose identification could not be confirmed were excluded.

Insect Sampling

At the Tolakker and Vollenhoven sites, insects were actively collected using a sweep net and passively collected using sticky traps (Bug-Scan, Biobest). Insect sweeping was done for six separate days throughout May 2025, and the sticky traps were deployed on the same day as sweeping net sampling and collected on the next scheduled sampling day, with an average sampling duration of three days. Insects were collected from three areas around the outside of the Tolakker, and one area near the air sampler at Vollenhoven.

Active sampling, using the sweep nets, was conducted in a standardized way by walking through a random transect and moving the net sideways consistently for five minutes per day per sampling area. After netting, the net contents were immediately transferred into a plastic collection bag and once returned to the laboratory the bags were stored at -20°C till NA extraction. To check for potential background contamination of the nets, a field negative control was collected by wiping a clean net using a sterile cotton swab. Passive sampling was conducted by deployment of the sticky traps on the pole of the air sampler. As field negative controls, sticky traps were exposed to the open air for 2 minutes (the time needed to deploy the traps at the poles) and put into the sealed collection bag.

Insects collected with the sweep nets were processed by first transferring all insects from each collection bag to a separate 5 mL tube. Larger batches of insects were divided into two pools to prevent damage to the insect bodies from overfilling the tubes, as we aimed to only obtain any potential vertebrate DNA from the outer body of the insects and reduce the amount of DNA coming from the insects themselves or their gut contents. Insect processing from the sticky traps was performed based on protocols adapted from Butterwort et al. (2022) and Maxwell et al. (2011) . Each trap was soaked in 5 mL of HistoClear II (National Diagnostics, Atlanta, GA) and gently shaken by hand to remove the specimens from the glue. Next, a 2 mL mixture of lysis buffer, dPBS, and Tween 20 solutions were added to each pool, and the tubes were rotated one hour to ensure the insects were washed entirely. Ini total, 350 µL of solution was taken from each tube and stored at -80 °C prior to NA extraction. For both methods, the species of individual insects in the samples were not determined, but a photograph of each insect pool was taken for later visual observation.

Nucleic Acid Extraction

Total nucleic acids (DNA and RNA) were extracted from the air filters and insect pools using the modified LGC mag Forensic Nucleic Acid Purification kit, following a protocol adapted to low biomass [24]. Extraction blanks were included for each batch of extractions. The purified total nucleic acid was aliquoted and stored at -80°C.

Vertebrate, bacterial, and fungal metabarcoding

The samples were evaluated for their vertebrate, bacterial and fungal DNA contents. Prior to sequencing, DNA isolates were tested for quality and quantity using quantitative Real Time PCRs (qPCR) following protocols published by Fakunle et al., 2023 [25]. Details on qPCR primers and reaction details can be found in Supplementary tables S1 and S2 respectively. To determine vertebrate species in the samples, the eukaryote 18S ribosomal RNA gene (variable regions v8v9) was initially selected for barcoding based on past success to identify various wild bird species [7]. However, after analyzing the 2024 samples it became evident that 18S-barcoding does not provide enough resolution to differentiate between most mammalian species, therefore in 2025, the detection of vertebrates was executed by the inclusion of mitochondrial 12S

rRNA gene (variable region V5) in combination with mammalian mitochondrial 16S (mt16S) rRNA gene amplicon sequencing as well as increasing the read depth. The median reads as well as number of unique ASVs found using each primer can be found in Supplementary Table S3. Similarly, for the detection of bacterial species, primers targeting the hypervariable v3v4 region of the bacterial 16S rRNA gene were used in 2024, while in 2025 primers with demonstrated ability to detect a wide variety of airborne pathogenic bacterial species (v5v6 region) were used [25,26]. Additionally in 2025, fungal species were assessed based on the inter-transcribed sequence ITS1 region between 18S and 5.8S rRNA genes.

Barcoding targets were amplified in duplicate and subsequently pooled and cleaned from primers and short-artefacts using Ampure XP beads (Beckman Coulter) purification (ratio 1:0.8). Amplicon concentrations were quantified using a Qubit, and amplicons per sample were equimolar mixed after which sequencing indexing adapters were added compatible with Illumina (Nextera XT Index Kit). In 2024, sequencing was done using an Illumina MiSeq Sequencer (Illumina Inc., San Diego, USA)and in the following year the NextSeq 2000 system was used (Illumina Inc., San Diego, USA).). A standardized known 8 bacterial species community positive control was included in all runs (Zymo biomics microbial community DNA standard) as well as field blanks and extraction blanks for each batch.

Shotgun metagenomic sequencing

Aliquots from selected samples from the 2024 campaign and all samples from the 2025 campaign were analyzed for their DNA and RNA virome content. Sequencing libraries were generated from total nucleic acids using the KAPA HyperPlus kit (Roche) and sequenced on using paired-end 300bp sequencing on a NextSeq 2000 (Illumina). Reads were trimmed using fastp v1.1.0 (https://github.com/OpenGene/fastp). *De novo* assembly was performed with metaSPAdes v4.2 (https://github.com/ablab/spades). Virus annotations were provided using DIAMOND blastx against the NCBI non-redundant (nr) reference database (accessed 23/11/2024) [27]. Read-based taxonomic classification was performed using Kraken2 v2.17.1 with the PlusPF database (10/15/2025) (https://github.com/DerrickWood/kraken2). Diversity dynamics were visualized in R 4.3 using the ggplot2 package. Vertebrate associated viral contigs and reads were confirmed with blastn searches. Alpha diversity (shannon index) was calculated using Bracken v1.2.1 (https://github.com/jenniferlu717/KrakenTools). The acquired ARG repertoire was determined by mapping the QCed paired-end reads to the acquired ARG ResFinder database (v4.7.2, https://bitbucket.org/genomicepidemiology/resfinder) using bbmap as a global aligner as described [28].

Meta-taxonomic Bioinformatics

Amplicons per sample were separated based on their unique primer sequences where adaptor primer sequences were simultaneously trimmed using cutadapt (Version 4.4) [29] in paired-end mode such that both primers need to be present. The raw cleaned sequencing data was denoised and converted into pseudoreads (amplicon sequence variants; ASVs) using the dada2 R package (1.30.0). Additional dada2 settings are shown in supplementary Table S4. ASVs were annotated using the naive RDP classifier of dada2 and the SILVA 132.1 nr99) database for bacterial 16S rRNA v5v6 amplicons and the UNITE (V9 Fungi) database for fungal ITS1 ASVs.

BLAST (2.16.0) and the NT database (downloaded 13-sept-2024) were used for both the 12S and mt16S mammalian barcoding data. Since BLAST is not Common Last Ancestor in taxonomic ranks aware, the mt16S and 12S ASV's of all sequences that were scored as 100% identical over 100% ASV length but having a different taxonomic annotation, the last common ancestor (LCA) was determined. This was done by screening all taxonomic levels for 90% or more abundance of one taxon. If the 90% threshold was not met,

this taxonomic level was listed as NA. If for an ASV no 100% identical hit was present, all hits were taken with a 98% or higher identical score over 100% length. If no 98% hit was present, all taxonomic levels were filled with NA.

ASVs that were found in all the samples including the field blanks were considered contaminants and removed from the data. This resulted in the removal of two ASV each from the mt16S and 12S detections, 23 bacterial ASVs from 16S, and no fungal ASVS were removed.

Statistical Analysis

All statistical analyses were performed in R v4.4.2 [30] assisted with the functions from *phyloseq* v1.50.0 [31], *vegan* v2.7-1 [32], *and alxeleratoR* v0.8.2.0 [33]. The Shannon diversity index for the bacterial, fungal and viral communities was calculated using *phyloseq* v1.50.0, using data rarefied to the sample with the lowest number of reads. The similarity of species composition among vertebrates, bacteria, fungi, and viruses across sampling sites and sampling periods was compared using Principal Coordinates Analysis (PCoA) of a Jaccard dissimilarity matrix in *phyloseq* v1.50.0, based on the same rarefied data. PERMANOVAs (999 permutations) were performed to determine the effect of sampling site and time using the adonis2 function from *vegan* v2.7-1. Co-structuring of the bacterial and fungal communities was further assessed using a Procrustes test (protest, 999 permutations) on Bray-Curtis distances ordinated by NMDS [32]. Statistical significance was set at $\alpha = 0.05$ throughout. The heatmap figure was generated using the *pheatmap* v1.0.13 package [34].

Results

An overview of all of the aerosol samples collected during the pilot study in 2024 and the main study in 2025 can be found in Table 2. All of the following analyses were completed on the samples collected during the main study in 2025.

Samples and species diversity

Table 2: Sample and data collection in 2024 (pilot study) and 2025 (main study)

Location	Sampling site	Air samples (n)	Complementary samples
Pilot, 2024			
Tolakker Farm	Indoors – clean air vent	1	None
Tolakker Farm	Outside by stable	1	None
Tolakker Farm	Outside by manure pile	1 (+1 field blank)	
Oostbroek Estate	Lawn	1	None
Oostbroek Estate	Barn	1	
Vollenhoven Estate	Bridge	1	None
Vollenhoven Estate	Orangerie Lawn	1 (+1 field blank)	
2024 subtotal		**7 samples** **2 field blanks**	
Main study, 2025			
Tolakker Farm	Indoors – clean air vent (TI)	2 (1 per week)	None
Tolakker Farm	Outside by stable (TO)	2 (1 per week) + 2 field blanks	Bird recordings Insects collected
Oostbroek Estate	Lawn (OL)	2 (1 per week)	Two camera traps
Oostbroek Estate	Barn (OB)	2 (1 per week) + 2 field blanks	None
Vollenhoven Estate	Field (VF)	2 (1 per week) + 2 field blanks	Bird recordings Insects collected
2025 subtotal		**10 samples** **6 field blanks**	

Vertebrate Results

The merged MT-12S (95 ASVs) and MT-16S (99 ASVs) ASVs were agglomerated on species level leaving 71 unique ASV clusters covering 53 unique taxa. The Tolakker farm was the most diverse site, with 25 vertebrate taxa identified inside the stable, and 35 taxa found in samples from directly outside the stable. Table 3 reports the complete list of vertebrate families detected at all sites, comparing airborne eDNA with the three complementary methods.

DNA belonging to humans (*Homo sapiens*), chickens (*Gallus gallus*), pigs (*Sus scrofa*), and European mole (*Talpa europaea*), as well as DNA from the genera cattle (*Bos*), pigeons (*Columba*), and geese (*Anser*) were found at all five sampling locations. Unique species were also found at each of the sites. The only reptile species detected was the grass snake (*Natrix natrix*) was found at the Oostbroek Barn site. One amphibian species, the common toad *(Bufo bufo),*was found at the Vollenhoven Field (VF), Oostbroek Barn (OB), and Tolakker Inside (TI) sites. Finally, all three of the fish species that could be identified to the species level (*Esox lucius, Tinca tinca*, and *Abramis brama*) were found at the Tolakker Outside (TO) site.

Despite the farm samples containing the highest vertebrate diversity, they also contained the lowest percentage of ASVs which could be assigned taxonomy based on available databases. In the first week of sampling, 20.7% of the total ASVs (vertebrate or otherwise) from the farm samples collected inside the stable could be assigned a taxonomy at the order level or lower, decreasing to 17.1% in the second week. From the TO samples, 21.5% (week 1) and 18.4% (week 2) of ASVs could be assigned taxonomy to the order level or lower. However, in each of these instances, the ASVs which could be assigned any taxonomic level accounted for a majority (>55%) of the sequence reads in the sample. In comparison, in all of the samples collected in the two nature areas, over 52% of reads could be assigned to at least order level.

In addition to the wildlife species, all of the expected domestic livestock species (pigs, sheep and cattle) were detected around the Tolakker farmhouses. DNA from pigs and cattle was found also in the two nature estates while sheep DNA was only recovered at the farm. Two goats are kept at the barn in Oostbroek, and goat DNA was recovered from the samples collected both directly next to the barn, and at the Oostbroek Lawn (OL) site approximately 192 meters away.

As a validation measure, all the observed birds, mammals, reptiles and amphibians were compared against a large database of nature observations by citizens, Waarneming.nl, as well as being checked by an ecologist from one of the sampling sites and confirmed to be present in the area. In the mammal list, one ASV from the TO site was aberrantly labelled as a common warthog (*Phacochoerus africanus*), was excluded as upon inspection, the sequence corresponded to an adenosine receptor with a high degree of identity between domestic pigs and the common warthog. The five detected fish taxa were compared against the iNaturalist database of observations, four of which were confirmed to be native to the Netherlands. The fifth fish taxon, the saltwater genus *Sebastes*, was detected in four samples but is not known to be native to the area but is commercially traded, thus could have originated from products such as from animal feed.

Table 3: Overview of the vertebrate families detected via airborne eDNA, camera traps, bioacoustics and recovered from insects as iDNA. Family names are followed by common names in brackets. Greyed out cells indicate taxa unable to be detected by the methods.

Classification		Tolakker Farm			Oostbroek Estate		Vollenhoven Estate		
Vertebrate - Class	Vertebrate - Family	Airbourne eDNA	Bird Recordings	Insect derived DNA (iDNA)	Airbourne eDNA	Camera Traps	Airbourne eDNA	Bird Recordings	Insect derived DNA (iDNA)
Actinopterygii	Esocidae (Pike)	*							
	Leuciscidae (Carp)	†							
	Sebastidae (Redfish)	*			*†				
	Tincidae (Tench)	†							
Amphibia	Bufonidae (Toads)	*			*		*		
Aves	Accipitridae (Hawks)				*		†		
	Aegithalidae (Long-tailed tits)								
	Alcedinidae (Kingfishers)								
	Anatidae (Ducks, Geese and Swans)	†			†		*†		†
	Apodidae (Swifts)								
	Ardeidae (Herons)								
	Certhiidae (Treecreepers)		Expert ID		*				
	Charadriidae (Plovers)								
	Columbidae (Doves and Pigeons)	*†	Expert ID		†		*†		
	Corvidae (Corvids)	†			†				
	Falconidae (Falcons)		Expert ID						
	Fringillidae (Finches)	*†							
	Haematopodidae (Oystercatchers)								
	Hirundinidae (Swallows)						*		
	Motacillidae (Pipits and Wagtails)								
	Muscicapidae (Old world flycatchers)							Expert ID	
	Oriolidae (Old world orioles)								
	Paridae (Tits)								
	Passeridae (Old world sparrows)	*†	Expert ID						
	Phalacrocoracidae (Cormorants)				†				
	Phasianidae (Pheasants and other ground living birds)	*†			*†		*†		*†
	Phylloscopidae (Leaf warblers)				†				
	Picidae (Woodpeckers)								
	Psittaculidae (Old world parrots)								
	Sittidae (Nuthatches)								
	Sturnidae (Starlings)								
	Sylviidae (Warblers)						†		
	Troglodytidae (Wrens)				*				
	Turdidae (Thrushes)	†					†		

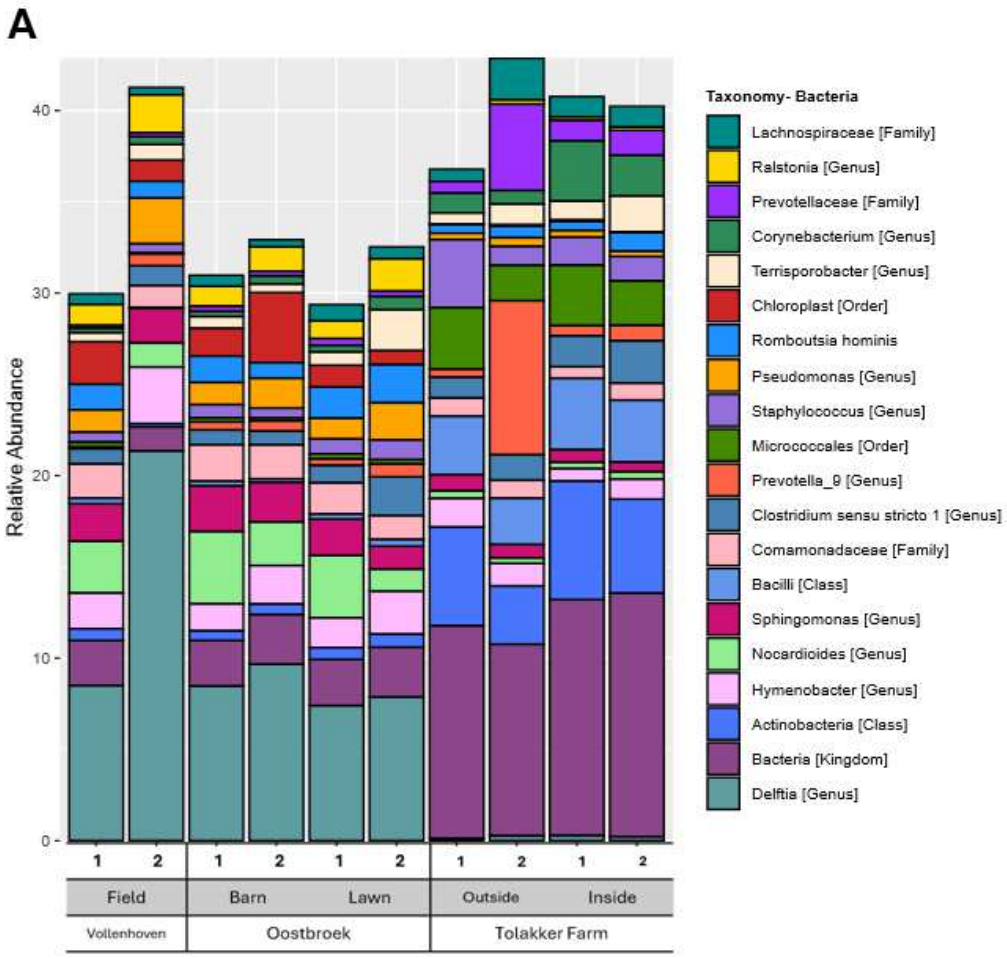
Mammalia	Bovidae (Cattle)	*†		*†	*†		*†		
	Canidae (Canines)	*†			*				
	Cervidae (Deer)				*				
	Cricetidae (New world rats and mice, voles)	*			*				
	Equidae (Horses)	*†			*				
	Felidae (Cats)								*
	Hominidae (Great Apes)	*†		*†	*†		*		*†
	Leporidae (Rabbits and Hares)	*†			*				
	Muridae (Rats and mice)	*†			*†		*†		*
	Mustelidae (Mustelids)	†			*†		†		
	Sciuridae (Squirrels)				*†				
	Soricidae (Shrews)	*							
	Suidae (Swine)	*†		*†	*†		*†		*†
	Talpidae (Moles)	*			*†		*		
Reptilia	Colubridae (Colubrid snakes)				*				

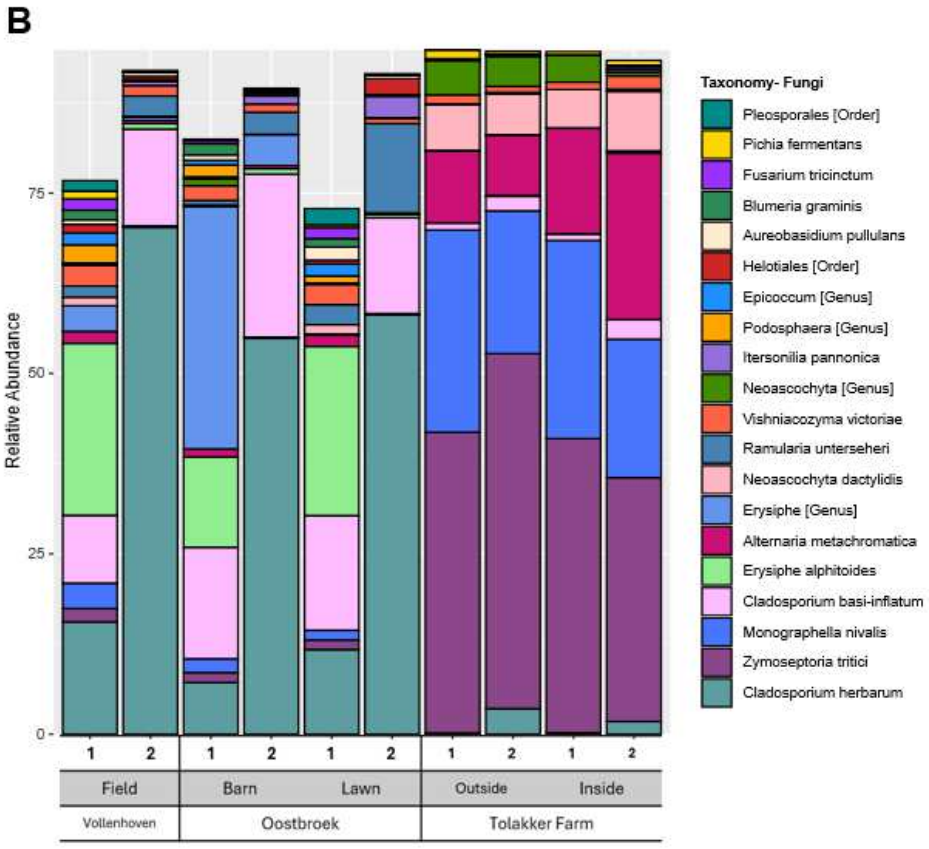
Footnote: Families found from the airborne eDNA and iDNA using the mt16s primer set are marked with "*", and those found using the 12s primer set are marked with "†". Cells marked "expert id " indicate avian families which were identified by the expert on recordings that were misclassified by the algorithm

Bacterial Results

From the bacterial 16S rRNA barcoding, 87,488 ASVs were identified, corresponding to 2,755 unique bacterial taxa. The greatest diversity and evenness was found inside the stable at the Tolakker (TI) during both sampling periods (Shannon Diversity H' = 8.81 and H' = 8.80 for the first and second sampling weeks). The least diverse bacterial community was detected in Vollenhoven in week two (H' = 6.81). The relative abundances of the top 20 most common bacterial taxa from the air samples are shown in Figure 2A. In the farm samples, over 10% of the bacteria could not be classified beyond the kingdom level. However, farm associated taxa including Staphylococcus and Corynebacterium are present in these samples at higher relative abundance than observed in the nature area samples [35].

In the nature area air samples, bacteria from the genus Delfia, which are associated with soil and freshwater, stand out as a dominant genera [36]. This genus is joined by other outdoor air associated taxa such as Bacillus and Pseudomonas, as well as Chloroplast reads from plant 16S sources [14].





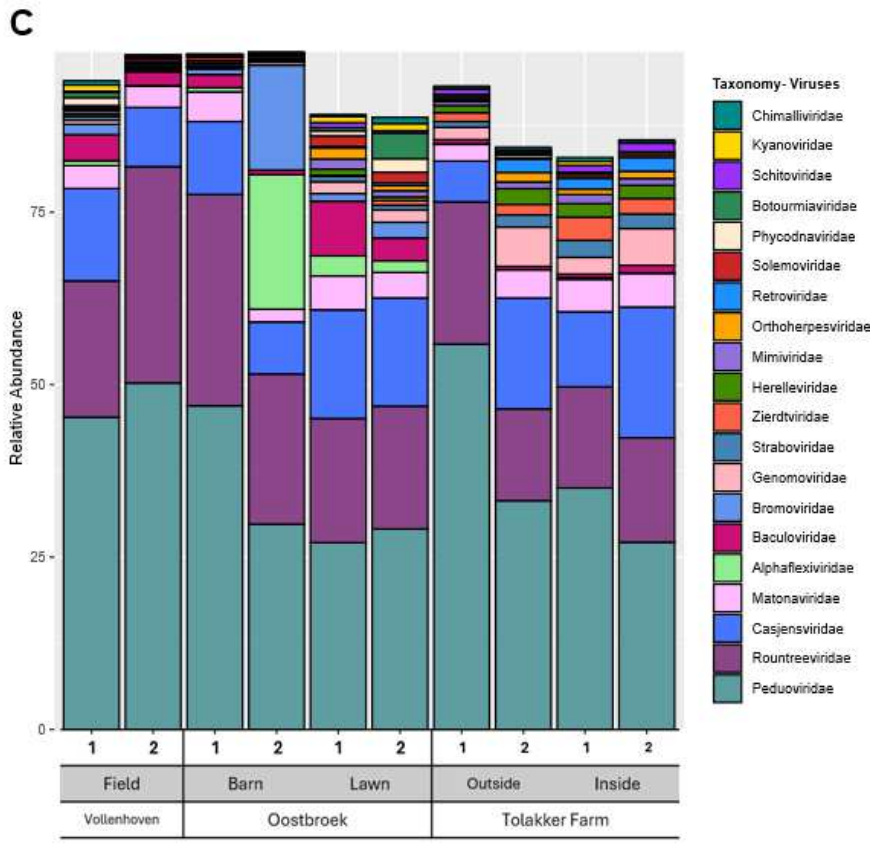


Figure 2: Relative abundances of the top 20 taxa observed in the air samples, showing A (top): bacterial taxa, B (middle) fungal taxa and C (bottom) viral taxa

Fungal Results

ITS1 barcoding revealed 324 fungal taxa from 924 unique ASVs found in the air samples. The Shannon diversity index indicated that the most complex fungal community sampled was collected at the OL site during the first sampling period (H' = 4.15). In contrast, the least complex community was from the Tolakker TI site in the first sampling week (H' = 2.69). Figure 2B depicts the relative abundances of the top 20 most common fungal genera from the air samples. The farm samples are dominated the fungal genera *Zymoseptoria, Monographella, Alternaria* and *Neoascochyta*, which are associated with many grasses including wheat, so these could be associated with the bedding material used in the stables [37]. In both sampling periods, the Oostbroek and Vollenhoven samples contained a high relative abundance of *Cladosporium*, a common plant pathogen. In the first week also a high relative abundance of *Erysiphe* fungi was found in OB. Plant-associated fungi are well represented in the data; however fewer yeast species common to farms such as *Saccharomycotina*, were detected than expected, possibly due to the lack of mechanical lysis step in our extraction protocols [38,39].

Virome Results

Using shotgun metagenomic sequencing 2,739 viral taxa were identified from the air samples. The three most common viral families were all bacteriophages, but some typical farm animal viruses were identified. The top 20 most common viral taxa are shown in Figure 2C. Between the two farm sites, seven different viruses associated with pigs were found, including two porcine bocaviruses and a porcine astrovirus, all of which can cause enteric disease in farmed pigs [40]. The samples from the nature areas notably include families of viruses associated with arthropod hosts (Baculoviridae), and plant mosaic viruses (Bromoviridae) [41,42]. Calculating the Shannon diversity index showed that the four farm samples held the most viral diversity (H'= >3.23), while the lowest viral complexity was found at OB in week 2 (H' = 0.57).

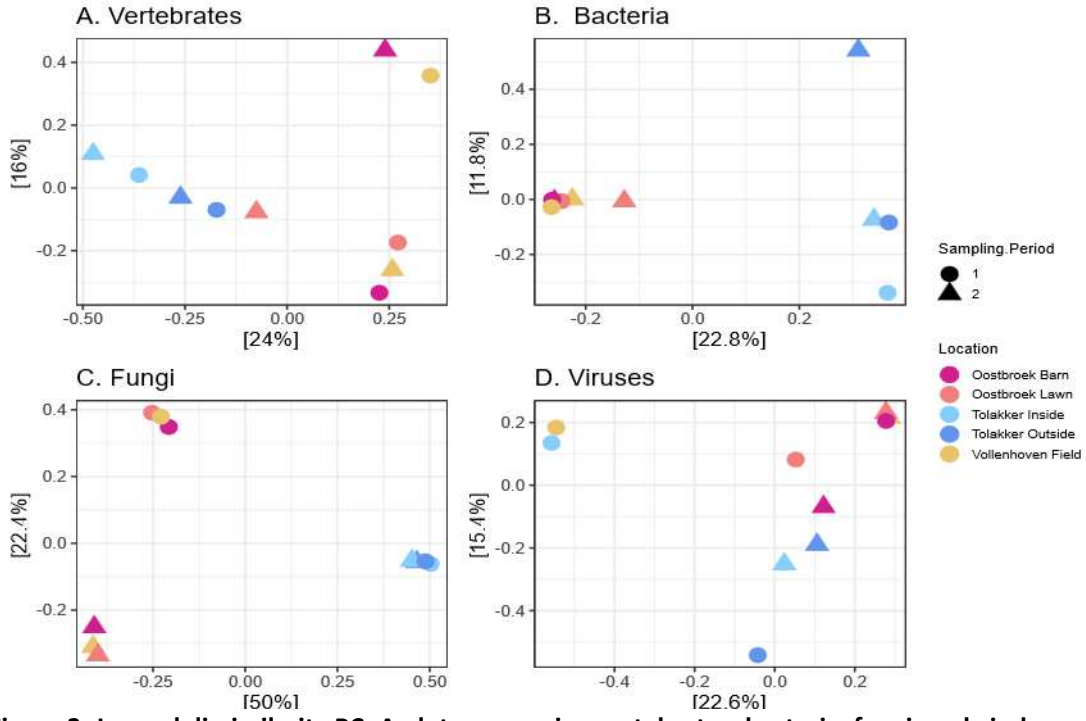


Figure 3: Jaccard dissimilarity PCoA plots comparing vertebrates, bacteria, fungi, and viral species composition between sites. Sites are indicated by color while sampling week is indicated by shape

Species composition of vertebrates, bacteria, fungi, and viruses was compared between sites using Principal Coordinates Analyses (PCoA) (Figure 3). For all but the viruses, the samples collected from the two Tolakker farm sites (TI (light blue) and TO (dark blue)) are generally closer to each other, while being most distinct from those collected in the nature estate locations of Vollenhoven or Oostbroek. The plots indicate that the fungi collected at the farm sites remained stable while in the nature areas, the fungal communities collected from different sites in the same week are more similar than those collected from the same sites a few days later. This is supported by the PERMANOVA analysis indicating significant effects of sampling period and sample location of the composition of the fungal communities (R^2=0.78, p=0.02). The bacterial communities displayed significant variation due to sampling period and time (bacteria, R^2=0.62, p=0.01; 999 permutations. Further Procrustes analysis showed significant co-structuring between the bacterial and fungal communities across the five sampling locations (r=0.85, p=0.001) indicating that their variation may be driven by the same environmental factors. The PERMANOVA analyses on the vertebrate and viral communities did not reveal any significant variation by time or location (p>0.05). While no clear differences can be seen in the configuration of vertebrate species between sampling periods, there were 26% fewer species detected in the second week than in the first.

The Pathobiome

We compared the bacterial and fungal genera found in the samples against lists of potentially pathogenic or allergenic genera [16]. The relative abundance of these genera is illustrated by a heatmap (Figure 4). The samples from the two nature areas cluster by sampling week, and all but one (TI, week 2) of the farm samples are grouped. This ability of eNA to detect changes over time or by sampling location of potentially pathogenic or allergenic genera demonstrates the potential of airborne eNA collection as a complete screening tool.

Acquired AMR genes (ARGs) were found in the shotgun metagenomic sequencing data in each of the air samples. The most common classes across all sites were Tetracycline, Aminoglycoside, and Beta-lactam ARGs. The TI and TO samples contained the most different ARG cluster90 genes (groups of genes which share 90% sequency identity), including common typical farming associated genes tet(w), and cfxA [28].

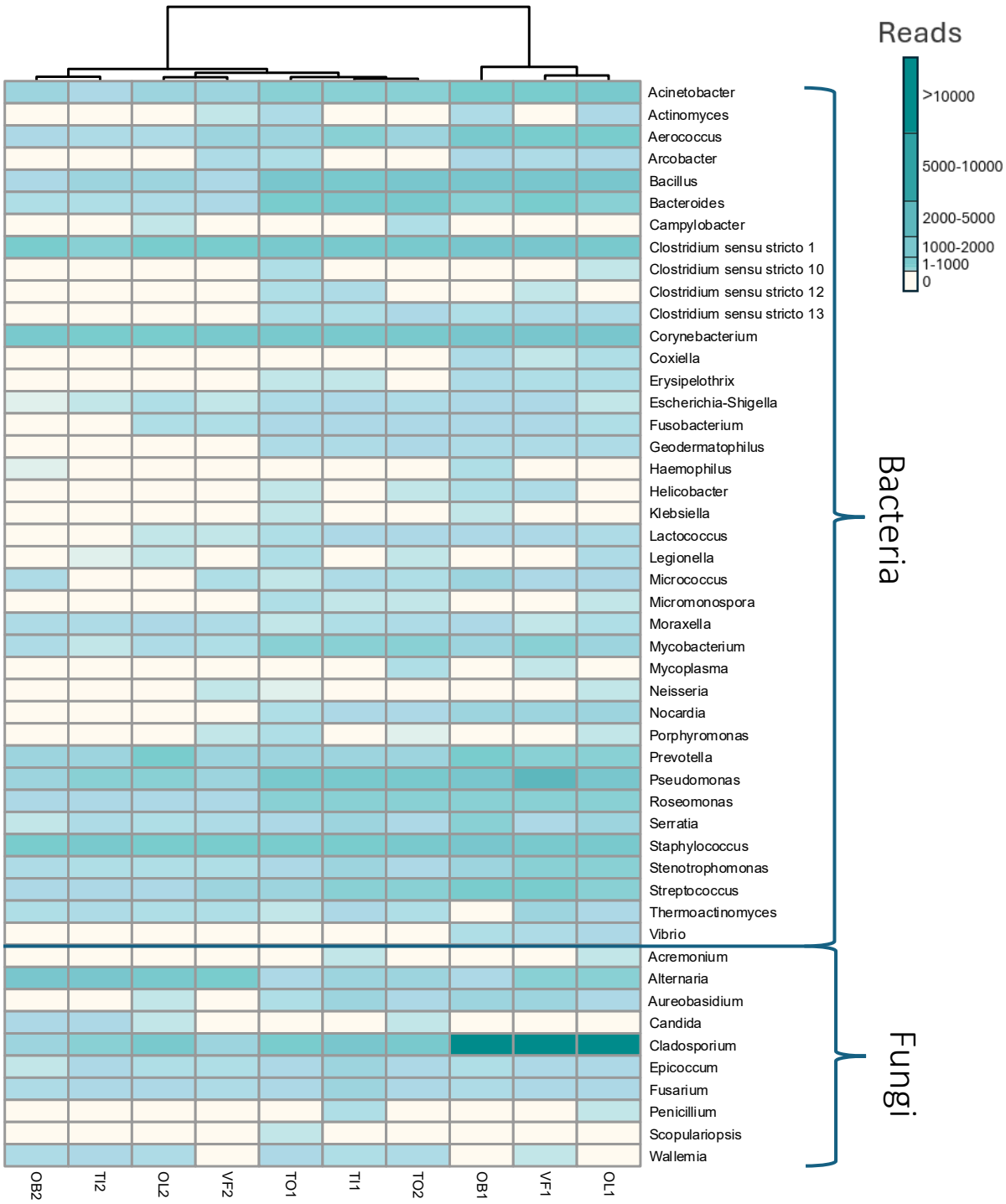


Figure 4: Heatmap indicating the read counts of potentially pathogenic or allergenic genera of fungi and bacteria found in the air samples. The darker shades indicate higher abundance of the genera while the dendritic plot at the top of the heatmap indicates the similarity of different samples.

Comparison of vertebrates found using eDNA and complementary methods

Camera Traps

Overall, more species were detected by eDNA (n=19) than by the camera traps (n=13), though unique species were detected by each method. All the vertebrate families found by the camera traps are reported in Table 3. The cross section of wildlife species indicated by the two airborne eDNA samplers and those

known to be present from the images from the nearby camera's images is illustrated in Figure 5. More small mammal species were detected using eDNA, while the cameras alone detected the presence of roe deer (*Capreolus capreolus*) and various bird species. In addition to the wildlife species, DNA from humans as well as cattle, goats, chickens, pigs, horses and dogs was found in air samples. These domestic animals were absent from the camera detections, however both cameras photographed multiple instances of domestic cats (*Felis catus*), which was not indicated by the eDNA.

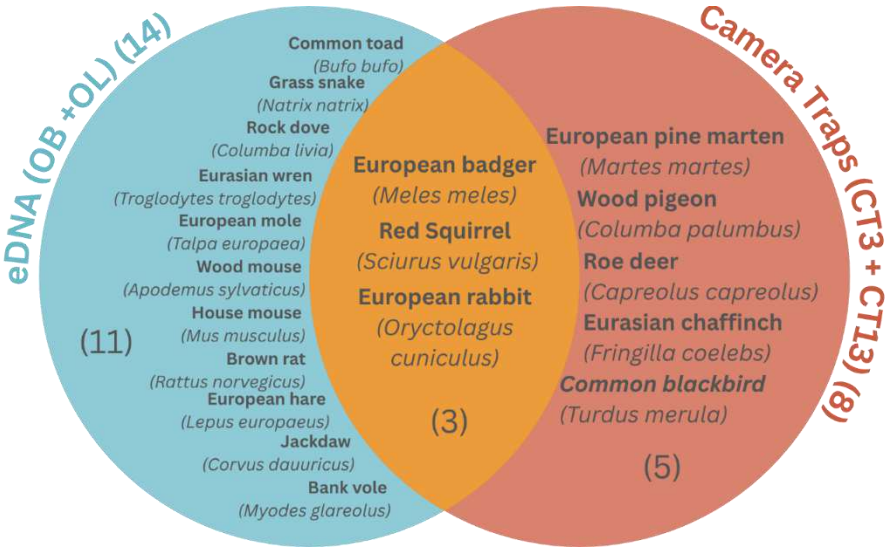


Figure 5. Overlapping wildlife species detected by the two camera traps and two eDNA samplers, located in Oostbroek. Only wildlife identified to the species level and observed in the same sampling period are included in this comparison.

Bioacoustic detection

Bioacoustic results were obtained at the Tolakker farm and Vollenhoven Estate. The PUC placed at the Tolakker detected 24 confirmed bird species. The most commonly acoustically detected species at the Tolakker were the house sparrow (*Passer domesticus*) and the European starling (*Sturnus vulgaris*). In the same location, none of the same species were found by eDNA, but the genus *Passer* which contains the house sparrow was detected. At Vollenhoven, 32 confirmed species were identified by the PUC, four of which were also found using eDNA. Here, the common chaffinch (*Fringilla coelebs*) and the short-toed treecreeper (*Certhia brachydactyla*) were the most commonly reported species by the PUC, neither of which were found in the air samples. All the avian families found in the bioacoustics data that were confirmed by an expert are listed in Table 3. At both sites chickens (*Gallus gallus*) were detected via eDNA but not by the PUCs.

Insect Sampling

From four sampling points from the Tolakker farm and Vollenhoven estate, a total of 641 insects were collected. Of these, 486 individual insects obtained using the sweeping net and 155 insects from sticky traps were analyzed as potential carriers of vertebrate DNA. A total of 21 insect pools consisting of 14 pools from sweep nets (SNs) and 7 pools from sticky traps (STs) were chosen for sequencing based on qPCR results.

DNA from at least one of eight different vertebrate families was recovered from each pool. Vertebrate families detected across locations by both the SN and ST methods combined are presented in Table 3. Of

the eight vertebrate families found, three were detected only in pools collected by sweep netting, four were found both using both methods, and only one family, Anatidae, was recovered only from insects caught with a ST. The iDNA detected the presence of three species, the roe deer (*Capreolus capreolus*), domestic cat (*Felis catus*), and Muscovy duck (*Cairina moschata*) not found in the air samples. No vertebrate viruses were detected in the insect pools using shotgun metagenomic sequencing.

Discussion

In this study we demonstrate that metagenomic sequencing of air samples (eNA) can provide information on vertebrate, bacteria and bacterial AMR genes, viral and fungal communities circulating at sampling sites. Distinct patterns were observed depending on location of sampling. Moreover, we showed that wild vertebrate species could be detected, despite the high livestock DNA load found inside of a livestock farm. Taken together, these findings demonstrate the powerful addition that airborne eDNA monitoring could make to disease surveillance at One Health crossing over points such as livestock farms.

We previously showed that wild waterbird DNA could be detected in air in and around a poultry farm which did not contain any animals after it had been emptied and cleaned following an outbreak of highly pathogenic avian influenza[17]. In our current study we show that genetic material from free living animals can also be detected in the air inside a farm even while the livestock is present. With large amounts of livestock DNA circulating, although a deeper read-depth is necessary as up to 6% of reads from the air samples in the pig stable could be attributed to humans and the livestock kept at the farm (pigs, cattle and sheep). Airborne eDNA characterization methods demonstrate that that wild and domestic animal populations could be connected, even in closed farm systems via their shared environment.

We showed that one sample processing workflow, from collection to storage to nucleic acid isolation can be successful in collecting the RNA and DNA used for metabarcoding to identify the vertebrates, bacteria and fungi, as well as shotgun sequencing which found the viruses and AMR genes. Therefore, a single, non-invasive air sample can yield data on a wide diversity of life, a major strength of airborne eNA.

Despite the range of different organisms found by the air samples, we also saw hints that much more could be unlocked in the future. For example, in some samples less than 20% of the ASVs could be taxonomically assigned, and there were instances of potential misidentification due to misclassification in the databases. Improving the quality and coverage of the vertebrate reference databases would dramatically increase the amount of data that is gathered by each sample. These improvements are especially vital if eDNA is to be used as part of disease surveillance, as subtle genetic differences between animals such as domestic pigs (*Sus scrofa domesticus*) and wild boar (*Sus scrofa scrofa*) matter in the context of disease spillover.

Although we had only two sampling timepoints, we noted a significant effect of sampling period on the composition of both the bacterial and fungal communities which aligns with the fluctuation in weather patterns seen during our sampling periods. For example, samples collected in the first period contained a high relative abundance of *Erysiphe* fungi. Fungi in this genus are the causative agents of powdery mildew infection in plants and are known to favor dryer conditions, as we saw in the first week of sampling (0.10mm precipitation) vs the second week of sampling (28.0mm of precipitation) [43]. There was also significant co-structing between the fungal and bacterial communities, indicating that they are shifting in response to the same underlying drivers. This is supported by previous work by Sullivan et al., who reported patterns in detection which matched the timing of pollen and spore releases in the region [44]. Similar findings for seasonal detection of migratory vertebrates show that airborne eDNA may pass though the environment quickly enough to allow for accurate temporal biodiversity monitoring, though future studies

with more timepoints will help to clarify the optimal sampling frequency for One Health surveillance purposes [17].

To help to identify strengths and weaknesses of the airborne eDNA approach, we compared three complementary biodiversity monitoring tools alongside the air samples; camera traps, bioacoustics monitoring and the analysis of touch DNA from the surface of insects. Camera trapping is an established and reliable method for surveying terrestrial animal populations for species above 1 kg body mass, and can provide important temporal and population structure information that cannot be obtained from eDNA alone [45]. At the Oostbroek Estate, in the same two-week sampling period, more wild vertebrate species were detected using eDNA (14) than by the two camera traps (8). Moreover, only three species were found by both methods, indicating that the methods gather different but complementary information. While the eDNA method outperformed the camera traps for the detection of small mammals, the cameras alone detected other species important to disease transmission and conservation such as the roe deer (*Capreolus capreolus*) and pine marten (*Martes martes*). It is important to note that studies using camera traps would typically deploy the cameras for multiple weeks to accurately survey the animal communities [45]. Thus, the camera results in this study act as a kind of 'ground truth' for the validation eDNA results rather than a true side by side method comparison. However, previous work completed in 2024 by Polling et al., extensively comparing camera trapping to airborne eDNA sampling, found that if the whole sampling period was taken together, all species seen in the camera traps, even if only seen once, were also found by the eDNA sampling, at some point, indicating the method's sensitivity [5].

Birds play an important role at the livestock-wildlife interface, as many species of synanthropic, thrive in habitats created by humans are common hosts of viruses, bacteria, fungi and vectors [18,46]. However, due to their small size and behavior, birds may shed less DNA and therefore be underrepresented. To explore the extent of this, we included bioacoustic monitoring in the study, which identified 26 families of birds at the two sites where it was deployed; in contrast, only 10 families were detected via eDNA at the same sites. At the farm site, one of the birds identified solely by the PUCs was the European starling (*Sturnus vulgaris*), a species known to have a role in long distance AMR gene distribution[18]. The omission of such an important species by the eDNA analysis warrants the inclusion of complementary methods such as bioacoustic monitoring. Future addition of a bird specific primer set such as 12S bird (birT), may increase the detection of bird species in the eDNA samples [47].

Lastly, we collected insects nearby the air samplers at Vollenhoven and the Tolakker farm, in order to analyze any potential vertebrate DNA from their body surface. Invertebrates such as biting midges and mosquitoes, interact with multiple vertebrate hosts through blood-feeding and contact with the host skin, blood, and feces, resulting in carriage of host DNA on their bodies [19,20]. Insects are ubiquitous at livestock farms and could be vehicles for indirect contact between wild and farmed animals and thus act as "flying samplers" for the surroundings. In our study, we found DNA from eight vertebrate taxa on the surfaces of the collected insects, including three absent from the airborne eDNA results. While the collection of iDNA did not capture the same diversity as the airborne eDNA, the recovery of vertebrate DNA from this novel "sampler" demonstrates the versatility of the metabarcoding approach and opens the door for other unique sampling targets at the wildlife-livestock interface.

Through the inclusion of these three methods alongside airborne eDNA collection we aimed to demonstrate how a multifaceted approach strengthens capacity for host animal monitoring. Sampling vertebrate eDNA has shown to be a sensitive and versatile tool. Moreover, the additional data which can be derived from the same air samples on microbial communities, as demonstrated by the current study, as

well as invertebrates [12], and plants [11] is unparalleled. In the future, eNA analysis could be part of comprehensive continuous disease monitoring systems leveraging both pathogen specific and host monitoring technologies and are embedded into the infrastructure of high-risk interfaces. Samples could be taken from farm ventilation systems, such as the TI site of this study, or as an added analysis for samples already collected by air quality monitoring networks. Previous studies have shown that eDNA can be reliably extracted from samples collected up to 30 years ago by these networks without changing existing collection or storage protocols [44,48,49]. This time scale allows for analysis of long-term trends to identify possible early warning signals. Additionally, the demonstrated simplicity of the sampling workflow means that collection and metagenomic analysis of airborne eNA could be feasible on a large scale, such has been achieved with wastewater surveillance [50].

The field of eNA analysis is moving quickly to resolve issues such as the unclear relationship between reads and biological abundance, which currently make interpreting eNA findings complicated. Sullivan et al., found that the abundance of eight bird species as reported by traditional surveys explained 60% of the variation in eDNA relative abundances over time [44].Later, in 2026, Jager et al. found a positive correlation between detected zoo species total biomass and the number of reads recovered from air samples. These studies provide evidence that we are moving beyond presence/absence species detection and towards using airborne eDNA to generate quantitative data about changes to biodiversity which can be used to power decision-making about disease risks.

Another open question is that of dispersion. How far a DNA fragment can be carried from its source likely depends on the rate it is shed, particle size, and area characteristics [44]. Catchment area calculations done by Sullivan et al. found that distance from likely origin varied by tens of kilometers between particle sizes [44]. Therefore, for the purposes of local disease risk monitoring, it may be beneficial to exclude smaller particle sizes as they can travel longer distances and complicate interpretation [38].

Finally, it is important to remember that an eNA signal does not equate to organism presence. For instance, DNA from the saltwater fish genus *Sebaste*s was detected in four of the air samples but could have originated from fishmeal added to animal feed or fertilizer. Similarly, in one sample we found two avian associated parvoviruses, however because these viruses are shed in feces, determining whether their presence reflects active host infection or are simply diet associated is difficult, and findings should be interpreted carefully.

Conclusion

The present study highlights the capacity of airborne eNA sampling to monitor the complete living environment. We were able to use a single workflow to gather information on a diverse set of vertebrates and microbes including viruses and AMR genes in and around a livestock farm despite high levels of livestock DNA. This is an important finding, as livestock farms could be an interface for transmission of pathogenic agents between different animal populations and thus form ideal targets for disease monitoring. The ability to detect the presence of organisms from so many branches of life through eNA sampling and sequencing could provide useful information for navigating shifting disease risks although some improvements can still be made. As interest in the field grows, we are rapidly learning more about the ecology of eNA, which will unlock more quantitative data from these readily available samples. These advancements have the potential to ultimately take eNA analysis from a method for species detection to a tool used to inform decisions about mitigating disease transmission risk.

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Author Contributions- CRediT

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Data Availability Statement

The datasets generated and analyzed during the current study are available in the NCBI Sequence Read Archive (SRA) repository, accession PRJNA1520499 .Additionally, the ASV sequences, associated abundance tables, taxonomic annotations, and sample metadata generated in this study have been deposited as phyloseq objects on Zenodo (DOI: https://doi.org/10.5281/zenodo.21919581).

Competing Interests Statement

The author(s) declare no competing interests.

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