

Advancing airborne eDNA sampling methods for monitoring diverse terrestrial vertebrate communities

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript presents impressive and comprehensive work resulting in novel and important findings, validating airborne eDNA sampling as a powerful new method for scalable vertebrate monitoring. After a suite of proof-of-concept studies demonstrating that it is possible to detect vertebrates (and other species groups) using airborne eDNA, Bodawatta et al take the next crucial step. They both validate the veracity of the results - i.e., that detected species indeed represent local and recent species communities, thereby addressing a prominent critique - but also present advances in best practices for airborne eDNA sampling methodology, at least for vertebrates. Here, Bodawatta et al address two key aspects of airborne eDNA sampling: (1) Optimization of filter–fan combinations for optimal collection (a non-trivial optimisation problem), and (2) Comparison of sample storage methods (dry vs. buffer) in terms of DNA recovery efficiency from collected filters.

Overall, the work presented by Bodawatta et al is a significant and timely contribution to the rapidly developing field of airborne eDNA sampling and will undoubtedly help pave the way toward more effective and scalable biodiversity monitoring approaches – which is of critical importance in light of the looming extinction crisis.

Despite the strengths of the manuscript, two critical issues must be addressed before publication:

1. Confounding of Species Richness and Community Dissimilarity

Bodawatta et al use species richness and community dissimilarity as their two main outcome metrics. However, community dissimilarity is calculated using the Jaccard index (i.e., the fraction of shared species), which is inherently confounded with species richness. Specifically, if one method detects more species (even if they are not compositionally different), the proportion of shared species among samples using this method will necessarily be higher compared to a method that recovers fewer species. This leads to the (I suspect erroneous) conclusion that filter type, extraction method, and even filter size not only affect the number of species recovered but also result in detecting different communities. This is especially questionable in the case of changing only filter size while keeping other parameters constant.

There are statistical methods for disentangling species turnover from richness effects (see detailed comments below), and I strongly recommend that these analyses be redone using such methods.

2. Misinterpretation of Filter Efficiency and Particle Capture

Bodawatta et al test various filters and observe differences in detection efficiency. All but one of the tested filters are of similar type (synthetic, progressively woven filter mats), but differ in density and particle size rating. The authors conclude that filters “capturing a larger range of particles” are more efficient, suggesting this as the likely explanatory factor. However, this interpretation is misleading. I believe that the stated particle ranges refer to recommended operational ranges - not the actual size limits of particles that the filters can capture. For instance, it would be implausible for a thick woven filter to have an upper capture limit of only 1 μm . A more plausible underlying factor for differences in detection rates is the total volume of air sampled.

Bodawatta et al measured airflow through the filters, but report only marginal variation and therefore dismiss it as a driving factor. I believe this may be an error stemming from inaccurate airflow measurements. Based on my calculations, the measured airflow rates are 10 times below the rated sensitivity range of the anemometer used, which could explain the unexpectedly small differences in measured flow. If accurate airflow data were available, I suspect that sampled air volume would emerge as the dominant factor affecting collection efficiency.

If these two critical points are adequately addressed, and the results and conclusions are revised accordingly (where

necessary), I recommend the publication of this manuscript.

Below I provide detailed line-by-line comments below for additional suggestions and clarifications. Congratulations once again on this impressive piece of work!

Fabian Roger

L51: Not only vertebrates, also insects as well as non-animals such as fungi and plants. I know the focus here is vertebrates but I think at least in the introduction a wider context would be good (up to you).

L85: Filter mats: The range of the particle size is misleading. The filter mats are rated for a certain retention efficiency (i.e., ISO ePM1 65% nominally retains 65% of PM1 particles), and they might be recommended for certain sizes (0.3–1 μm), but they will capture particles outside this size class - both smaller and, especially, larger.

L101: Filter type is confounded with air volume (i.e., if you forced the same air volume through the different filters, then you would measure type and size; here, you don't know). Also, what model did you run? Please add it in the text (here and elsewhere – you only provide the statistics). It would be good to see the results of a model including airflow and filter type.

L110: It's not possible to assert that the community composition differed significantly by filter type with the Jaccard index if richness also differed. There are methods to try and disentangle the effect (e.g., the nestedness functions in R vegan [<https://rdrr.io/rforge/vegan/man/nestedtemp.html>]) or use null models to account for differences in richness (Raup-Crick dissimilarity – also implemented in vegan). Saying that the community composition differed with filter type asserts that different filter types capture different species - which would be significant. I doubt that's the case here, though.

L123: Same comment about filter type being confounded with air volume.

L125 FF: It would be good to say why you tested whether the amount of chicken reads influenced species detection. The reason would be to test if sequencing depth was high enough, I assume? Or would it inform whether it's worth including chicken-blocking primers (not possible for BirdT I think, we checked once)? Also, some people will argue that chicken DNA is a common contaminant—did you detect any significant amount in your blanks? The results of the blanks need to be mentioned in the main article.

L129: You are testing proportions here. Did you transform the variable before running the LMM?

L140: This is curious. Why would the filter that detects more birds detect fewer (mostly no) mammals?

L178: Amphibians are mentioned for the first time here. You also only detect one amphibian species. I would include it but be clear that it wasn't the target.

L203: I don't know if air sampler numbers are necessary in the figure, as the same points belonging to the same samplers are already connected.

L221 FF: Again, I suspect that total air volume filtered is likely the underlying driver and should be discussed explicitly.

L231: The text here is a bit confusing. It's a new paragraph starting with a new conclusion, but it seems to discuss the same results as the previous paragraph. Also, with respect to community composition, I have the same comments as above - richness and composition are confounded (it would be curious if filter size changed which species were detected).

Figure 4 (and all): Please reduce the vertical jitter—it's not possible to see how many detections a given data point corresponds to. The number of detections is discrete, so the point clusters should be too.

L355 (and elsewhere): Strictly speaking, for assessing sample seasonality, you should have sampled different seasons in the same location and shown turnover; here, you confound location and season. In theory, the presence of seasonal species alone doesn't indicate a seasonal signal. Given how strong the birding community in Denmark is, it would also be interesting to compare airborne eDNA detection vs. eBird / Gbif records and see what proportion of species in-season and out-of-season you detect at every location - which would be a more rigorous test of seasonal signal (that being said, you show turnover on the scale of hundreds of meters - it's quite evident to me that you wouldn't detect species months after they were there).

Analysis suggestion:

For testing temporal and spatial signal bleeding, it occurred to me that you could consider an additional analytical approach that might yield interesting insights. You sampled at three locations with strong spatial replication, two of which were sampled on three consecutive days. In Experiment 2, you assess community similarity relative to sampler distance, but as far as I can tell, you don't account for sampling day - or for wind direction. I believe these data have not yet been analyzed to their full potential.

I would need to look more closely at the methods, but in marine ecology, it is standard practice to estimate connectivity between sites (e.g., for larvae that drift with currents rather than swim against them). A similar approach could be applied here: the distance between samplers is not symmetrical—i.e., a sampler positioned downwind of another is more likely to capture a signal from the upwind location than vice versa. Likewise, a sample taken on Day 2 may be influenced by the

conditions on Day 1, but not the other way around.

You could explore this idea by comparing Mantel tests that incorporate directional and temporal asymmetries with those based on randomized assignments (i.e., a null model). It may even be possible to estimate signal bleeding directly by testing whether signal strength (e.g., read number or prevalence across replicates) is directional. For instance, a strong detection at an upwind site might also appear at a downwind site, albeit at lower strength—whereas detections should not appear in the reverse (upstream) direction.

I'd need to take a bit more time to think through the implementation details, but I believe this line of analysis could be well worth exploring.

L364: Again, the other filters also retain larger particles—it's a bit curious to give an upper bound.

L367: See my concerns on how you measured air volume.

L383: There are two steps: one is collection, the other one is lysis—i.e., getting the particles off the filter again. Could there be differences in lysis efficiency between the filter structures?

L409: Woven air filters have all three filtration mechanisms - impaction, diffusion, and sieving - depending on particle size.

L426: I agree with your conclusion, but doesn't logically follow from the previous statement. The Burkard spore trap has a similar flow rate but a very different collection mechanism (adhesive-coated plastic tape), and it was compared to the Coriolis, which has yet another sampling mechanism (liquid cyclone) and a vastly higher flow rate, but very short collection times. A stronger argument could be that it shows that long collection outperforms high flow rate (the advantage of being 'around' when the signal is there).

L455: It would be helpful to indicate in Figure 7 which habitat was sampled in which season.

L463: There is little evidence that rivers transport eDNA over long distances. See this recent reference: <https://www.nature.com/articles/s41598-023-35614-6>

L493: Not particle size retention but capture efficiency for smaller particles.

L506: I have done this too previously, but I don't think it's very useful. It gives only trajectories, not footprints (particle distribution models do), and the trajectories reach back hundreds of kilometers, while you show that the signal is local to a few tens to hundreds of meters. I would drop it.

L541: 15 min on a laminar flow hood is not much, given the long distance from the light source and the thick filter material. Can you estimate exposure dose (J/m^2)?

L547: How? For accurate velocity measurements, the flow should be approximately laminar, so it would be good to measure inside a longer tube at the end of which the filter and fan are mounted. Also, as far as I understand, rotor blade anemometers are less accurate at low flow rates, and yours is rated at (0.3–45 m/s), if I see it right, while the flow through your filter (max 0.553 m³/hour on the 6x6 filter with 12V) would correspond to 0.043 m/s - i.e., 10x below the sensitivity of your anemometer. This might explain the surprisingly similar flow measurements - it would be crucial to try to get a more accurate measurement, as I suspect that air volume is driving most of your observed patterns. Thermal anemometers (like the Testo 405i) have higher accuracy at low flow speeds.

L571: The information that the filters were first stored in Longmire buffer for 2 weeks and then frozen is missing above—please add it to the results section, too. Also, it's unusual (as far as I know) to freeze samples in Longmire buffer (they are usually stored at 4°C or room temperature). Could this have influenced recovery?

L595: Did the filters homogenize with bead beating? The polyester filters are very resistant to bead beating in my experience.

L598: "adjustment" → adjusting

L607: It's enough to state that you used the sequencing primer for qPCR and describe the primers in the sequencing section. It's a bit confusing in the qPCR section.

L630: Did you use different cycling numbers for different treatment groups? If so, please state that here.

L633: "Six PCR replicates were independently analysed for each DNA extract, negative and positive control." I understand what you mean now but it took me a while. Consider rephrasing.

L648: What concentration measurements does this refer to? You already described qPCR for 142 samples before?

L667: These are quite stringent filtering options. Is the read threshold (5) implemented to account for tag switching? Did this exclude species across the whole dataset or only in certain samples? Same for the PCR replicates? I know this is common practice, but if you recover a read that you can assign to species level and it's distinct from the other reads, why exclude it?

Where should it come from if not from a true detection (if your controls are empty)? It would be interesting to see Figure 7 with all species and the frequency as the ratio of total replicates across samples and/or mean replicate prevalence (n/6). It would also be good to see the analysis done with or without these thresholds to see if the patterns hold (if it's a reasonable effort).

L671: Why clustering to OTUs? Why not use ASVs? The fragments are short and the barcode region is not always very different between species; by clustering to OTUs, you might lose resolution. I would suggest using an ASV inference algorithm, doing the taxonomic assignment, and clustering by that (as you already do, just without previous OTU clustering). (No need to repeat the analysis - just a suggestion.)

L682: What is the threshold for 'similar' here?

L684: Was the local species missing from the database in all cases?

L750: it would be great to make the analysis code available on GitHub (as also recommended by NatComm).

supL34: What concentration of ProtK?

supL89: I am always curious to check what OTUs LULU merges. Did you do the taxonomic assignment before and verify that it didn't merge OTUs with different taxonomy? (I have seen that, although not for vertebrates I think.)

supL87FF: Do you have any sense of where the pig and chicken contamination could come from? (Nearby industrial farms? Manure on fields? Actual wild pigs [wild boar]?)

I don't see the results from the negative controls discussed (did I miss it?). These need to be included in the main text (even just stating that they were blank).

Sup Table S4: I think the occurrence of the excluded fauna needs more explanation. Some are obvious (like chicken, pig, and cow), but others are less so - for example, chimpanzee (15 samples!), *Bos indicus* in 31 samples (was there a herd nearby?), and some of the domestic birds (peacock?). Do you have good estimates for where they came from? Could they also be lab contaminations (like the elephant)? A more detailed discussion would build confidence - right now you seem to only say that they occur in Denmark, which - at least for the gorilla - is a stretch. Does it occur outside of the zoo? How far away is the zoo? When were the samples processed after the last zoo samples? Or could it just be a human sequence that is misannotated as gorilla in the reference database?

Reviewer #2

(Remarks to the Author)

Overall this manuscript provides further advancements in knowledge regarding airborne eDNA and has the potential to be an important contribution in methodological development. The impact of this manuscript would be improved with further clarity about methods, better incorporation of environmental variables and a more tempered and cautious interpretation of results and scope.

1. Methodological ambiguities;

- Contamination controls were underspecified.

- The manuscript does not clearly describe the use of negative field controls, blank filters, or lab air blanks during DNA collection and processing — all of which are essential in airborne eDNA studies where contamination is a major risk.

- Suggest to add explicit details about field blanks, lab controls, and negative extractions used in each experiment. Otherwise, it is difficult to assess the reliability of rare detections.

- Primers

- The study uses 12S (for birds) and 16S (for mammals) primers, but it lacks discussion on primer efficiency across taxonomic groups, any biases for less abundant taxa, or any misassignments of taxonomic identification.

- The results mention only 72 out of 90 taxa identified to species level — what about the remaining 18? Were they low-quality reads, or due to poor database matches?

- Suggest including a primer performance evaluation or reference existing studies to contextualise potential taxonomic blind spots that might occur using these primers.

- Lack of Meteorological Metadata

- Airborne DNA transport is influenced by humidity, wind, precipitation, and temperature. Yet, none of these parameters are

reported or incorporated into the analysis.

- This limits understanding of temporal and spatial variability, and whether DNA movement or degradation confounded the results.
- Suggest that even basic weather metrics (e.g., wind speed/direction, humidity) would help interpret variation in detections.

- qPCR Results

- The study presents qPCR-based concentration data for bird eDNA (BirtT primer), but does not validate whether these concentrations correlate with species richness or read depth, nor discuss if qPCR data support quantitative inference or simply presence/absence.
- This could help calibrate whether airborne eDNA is semi-quantitative, which is an open question in the field.

2. Limitations in experimental design

- Taxonomic range

- The study focuses exclusively on vertebrates, particularly birds and mammals, and one amphibian species. This narrows the applicability of the results and limits generalisability to multi-taxa airborne eDNA studies (e.g., fungi, plants, arthropods).
- Given only vertebrates were targeted, there needs to be clarification that this was an intentional scope limitation and suggest future multi-marker work (e.g., ITS2, COI for insects/plants/fungi).

- Underrepresentation of Mammals and Amphibians

- Mammals were detected in <45% of samples, and amphibians in only ~10%.
- Potential cause: Lower shedding, primer bias, or reduced airborne transport. Unaddressed: The manuscript does not adequately explore why non-avian vertebrate detection is lower (ie Lower shedding, primer bias, or reduced airborne transport), or how to address it.
- Suggest including discussion of these detection biases and how alternative markers or sampling duration could improve results.

- Distance-Decay effect in birds

- A spatial signal was observed for bird communities in Æbelø, but not for mammals. This finding is reported but not explained.
- Could mammals have more dispersed airborne DNA due to larger home ranges? Or does lower detection power mask the effect?
- Suggest including possible ecological explanations for this taxon-specific spatial resolution or acknowledge this as a data limitation.

3. Interpretation of results

- Overgeneralisation About Global Applicability

- The manuscript states airborne eDNA “can be adapted by conservation programs to monitor biodiversity across the globe.” While it is indeed promising, the method is tested only in Denmark, in three well-characterised sites during two seasons. Such claims may overstate transferability, especially to tropical, arid, or urban ecosystems.
- Suggest tempering the generalisation and clearly state ecological and geographic boundaries of applicability.

- Dismissing the use of passive samplers

- In this study, passive samplers yielded very few detections and were concluded to be ineffective. However there could be other explanations for this, ie. sampling duration may have been too short, environmental variables could have been suboptimal.

Passive systems have advantages of being cost-effective and have potential if used over longer periods or with improved surfaces.

- Suggest acknowledging the limitations of passive trial design and suggest refinement, not outright dismissal.

Editorial suggestions:

There was a lot of wordy and awkward passive phrasing throughout the manuscript which could be improved for clarity.

Here are some examples and suggestions:

Line 49: “...to study biodiversity in terrestrial ecosystems and in turn inform conservation efforts”

change to: “...to study biodiversity and inform terrestrial conservation efforts”

Line 63: “...airborne eDNA to provide seasonal and habitat-specific snapshots...”

change to: “...airborne eDNA to yield seasonal and habitat-specific biodiversity profiles...”

Line 125: “...qPCRs results provide information on the total bird amplicons...”

change to: "...qPCR results provide information..."

Line 127: Change to plural 'were' related

Line 134: "highest quantity of bird DNA was the ISO ePM10 65%"

change to: "The filter with the highest bird DNA yield was ISO ePM10 65%"

Line 228: "Passive sampler s thus essentially acted as negative sampling controls"

change to: "Passive samplers functioned effectively as negative controls"

Line 234: "we observed a significant effect of sampler type on the detected number of taxa"

change to: "Sampler type significantly influenced the number of taxa detected"

Line 291: "ecological and behavioural lifestyles"

change to: "ecological and behavioural traits"

Line 319: "such as warblers and swallows, which would have migrated south by the time of sampling"

change to: "such as warblers and swallows, which had likely migrated south prior to sampling"

Line 433: "one caveat with using high air flow is the lasting, portable..."

change to: "One limitation of high airflow is the requirement for a long-lasting, portable power source"

Line 439: "our results validate the use of air filters fitted on fans..."

change to: "Our results support using fan-fitted air filters as an effective eDNA sampling method..."

Line 461: "Together, the findings that airborne eDNA provides a snapshot..."

change to: "Together, these results show airborne eDNA can capture localised, seasonal biodiversity patterns"

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I am satisfied with the revisions the authors have made and have only the following remaining comments.

Airflow measurements

Great that you were able to re-measure! Based on my experience, measuring airflow at the inlet rather than the outlet tends to give more accurate results (likely due to reduced turbulence). Attaching the tube in front of the fan and measuring the flow rate at the tube entrance generally gives the best results (using Volume Flow Duct, Air Type = Supply Air, Input Geometry = Area).

I would also recommend keeping the diameter of the plastic tube constant and large enough to cover the full sensor area of the anemometer (black tip). A 4 cm diameter might be too small, as the two sensors on my Testo 405i are ~5 cm apart.

A good way to check measurement accuracy is to run the fan without a filter and compare the results to the manufacturer's specifications.

Finally, I noticed that the exact fan models are not cited. You cite Lynggaard et al. but in that article only the manufacturer (Sunon Inc.) is listed, not specific models. Please cite the exact models used, as their performance can vary widely.

Line 101

Please include information on the statistical model(s) in the main text; otherwise, the results are not interpretable. From the code, I see you used:

```
lmer(Richness ~ SampleType + (1|SampleSite) + (1|Date), Data)
```

This should be explicitly stated in the text (e.g., "effect of sample type on richness, with SampleSite and Date as random factors").

Figure 4 (and Figure 2)

The reduced horizontal jitter makes the figure clearer, but there is still quite a bit of vertical jitter. I recommend removing the

vertical jitter entirely, since the vertical axis represents the number of species and should be displayed without modification.

Reviewer #3

(Remarks to the Author)

I have reviewed the manuscript titled “ Advancing airborne eDNA sampling results in robust characterisation of vertebrate communities“. This manuscript has already been expertly reviewed. I was specifically asked by the editor to review the quality of edits in response to comments by reviewer 2.

The reviewer’s concerns with the methodology of the study were regarding clear annotation of negative controls, choice of primers (narrow taxonomic range), taxonomic assignment of sequences. The reviewer also pointed to the limitations in the interpretation of results, primarily with use of qPCR results, overgeneralization of global applicability, and dismissing the use of passive samplers. The authors have adequately responded to most of the reviewer’s concerns by making appropriate edits to the manuscript.

However, I disagree with one suggestion by reviewer 2, which was added to the manuscript, “The lack of the spatial trend in mammals might be due to larger home-ranges of mammals, which may result in movement between the sampled microhabitats or because mammals may not shed DNA in a similar way as birds.” If the authors wish to keep this edit, it should be supported by strong evidence that the mammals detected do in fact have larger home ranges than the birds detected. Even in the case of birds, no significant relationship was found when samplers were stored in Longmire buffer. There may be a significant amount of stochasticity in air eDNA sampling and processing, especially since little is known about persistence and transport of air eDNA.

Anish Kirtane

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18th July 2025

Dear editor at Communications Biology,

Thank you very much for handling our manuscript entitled “Advanced airborne eDNA sampling allows robust spatiotemporal characterisation of vertebrate communities” and providing us the opportunity to improve our manuscript based on the reviewer responses.

Upon revisions we have slightly changed our manuscript title to “Advancing airborne eDNA sampling results in robust characterisation of vertebrate communities”. Below we are providing our point-by-point responses for reviewers in [blue font](#). We believe that the revised manuscript has improved substantially.

Thank you very much for considering our manuscript for *Communications Biology*.

Kasun H. Bodawatta & Kristine Bohmann
(for and on behalf of all co-authors)

Section for Molecular Ecology and Evolution,
Globe Institute,
Faculty of Health,
University of Copenhagen,
Copenhagen,
Denmark

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript presents impressive and comprehensive work resulting in novel and important findings, validating airborne eDNA sampling as a powerful new method for scalable vertebrate monitoring. After a suite of proof-of-concept studies demonstrating that it is possible to detect vertebrates (and other species groups) using airborne eDNA, Bodawatta et al take the next crucial step. They both validate the veracity of the results - i.e., that detected species indeed represent local and recent species communities, thereby addressing a prominent critique - but also present advances in best practices for airborne eDNA sampling methodology, at least for vertebrates. Here, Bodawatta et al address two key aspects of airborne eDNA sampling: (1) Optimization of filter–fan combinations for optimal collection (a non-trivial optimisation problem), and (2) Comparison of sample storage methods (dry vs. buffer) in terms of DNA recovery efficiency from collected filters.

Overall, the work presented by Bodawatta et al is a significant and timely contribution to the rapidly developing field of airborne eDNA sampling and will undoubtedly help pave the way

toward more effective and scalable biodiversity monitoring approaches – which is of critical importance in light of the looming extinction crisis.

Response: Thank you for your encouraging comments and recognition of the usefulness of the study.

Despite the strengths of the manuscript, two critical issues must be addressed before publication:

1. Confounding of Species Richness and Community Dissimilarity

Bodawatta et al use species richness and community dissimilarity as their two main outcome metrics. However, community dissimilarity is calculated using the Jaccard index (i.e., the fraction of shared species), which is inherently confounded with species richness. Specifically, if one method detects more species (even if they are not compositionally different), the proportion of shared species among samples using this method will necessarily be higher compared to a method that recovers fewer species. This leads to the (I suspect erroneous) conclusion that filter type, extraction method, and even filter size not only affect the number of species recovered but also result in detecting different communities. This is especially questionable in the case of changing only filter size while keeping other parameters constant. There are statistical methods for disentangling species turnover from richness effects (see detailed comments below), and I strongly recommend that these analyses be redone using such methods.

Response: Based on your suggestion, we reanalysed the community composition data using the Raup-Crick dissimilarity index, which takes the probability of species sharing between samples into account (Lines 798-805). These new analyses did change the statistical outcome of the community compositions (e.g., lack of significant effect of filter type and sampler type on detected community composition: Lines 120-123 and Lines 211-214), but the overall outcome of the findings are not impacted. We have edited the text to reflect this. Specifically, we now say “Principal Coordinate Analysis (PCoA) with the Raup-Crick dissimilarity (based on presence/absence data and the probability of communities sharing species) was used to visualise the community compositional differences with the “pcoa” function in the ape package v.5.8. Raup-Crick dissimilarity was calculated using the function “raupcrick” in the vegan package with the null parameter set to “r1” and nsimul parameter set to 999. Bird and mammal community differences (based on Raup-Crick dissimilarity) were statistically examined using permutational multivariate analysis of variance (PERMANOVA) using adonis2 tests in the vegan package” (Lines 798-805).

2. Misinterpretation of Filter Efficiency and Particle Capture

Bodawatta et al test various filters and observe differences in detection efficiency. All but one of the tested filters are of similar type (synthetic, progressively woven filter mats), but differ in density and particle size rating. The authors conclude that filters “capturing a larger range of particles” are more efficient, suggesting this as the likely explanatory factor.

However, this interpretation is misleading. I believe that the stated particle ranges refer to recommended operational ranges - not the actual size limits of particles that the filters can capture. For instance, it would be implausible for a thick woven filter to have an upper capture limit of only 1 μm . A more plausible underlying factor for differences in detection rates is the total volume of air sampled.

Bodawatta et al measured airflow through the filters, but report only marginal variation and therefore dismiss it as a driving factor. I believe this may be an error stemming from inaccurate airflow measurements. Based on my calculations, the measured airflow rates are 10 times below the rated sensitivity range of the anemometer used, which could explain the unexpectedly small differences in measured flow. If accurate airflow data were available, I suspect that sampled air volume would emerge as the dominant factor affecting collection efficiency.

Response: Thank you for this thorough explanation and for suggesting a more sensitive anemometer. We have now measured the airflow rates with the Testo 405i Smart Probe thermal anemometer and provide a more detailed explanation on the measurement of the volumetric airflow rate (Lines 620-632) where we now say: “Volumetric airflow rates (m^3h^{-1}) of air samplers were measured using a Testo 405i Smart Probe thermal anemometer (order number: 05601405) with an accuracy of $\pm 0.1 \text{ m/s} + 5\%$ of mechanical ventilation (0 to 2 ms^{-1}). The volumetric airflow rates were measured for each of the four tested filter types in Experiment 1 (Fig. 1) and for each of the four air samplers set ups with active filtration in Experiment 3 (Fig. 1). To calculate airflow rates, we first attached the correct filter (e.g., type and size) to the appropriate fan (12 V or 5 V). Then we attached the fan with the filter to a 12 cm long plastic tube with a cross-section area matching the fan (12 V fan: $6 \times 6 \text{ cm}$ and 5 V fan: $4 \times 4 \text{ cm}$). We then placed the sensor of the anemometer at the other end of the tube. We used the cross-section area of the fan to automatically calibrate the airflow measurements using the “volume flow outlet” function. We measured the air flow rate each second for 3 minutes to calculate the average volumetric airflow rate (Table S1)”.

We found that our previous measurements of the airflow rates were highly underestimated (for example the 12V fan with coarse filter has an volumetric air flow rate of $12.3 \text{ m}^3\text{h}^{-1}$) (for updated airflow rates see the Fig 1 and Table S1). Somewhat aligning with your prediction, we do see a slight difference in airflow rates ($1\text{-}2 \text{ m}^3\text{h}^{-1}$) between the best performing filters (ISO ePM 10 65% and ISO ePM 1 65%_FB) and the more poorly performing filters (ISO ePM 2.5 70% and ISO ePM 1 65%_LL). These slight differences lead to around $30\text{-}40 \text{ m}^3$ of additional air filtered by the best performing filters per day (24-h filtration) and this slightly higher amounts of filtered air may have impacted the taxa detections with a positive effect on the detected taxa diversity and robustness of detected communities among filter samples. Now we have changed our discussion to point out observed differences may be caused by these differences in airflow rates. Specifically, in Lines 391-407 we now state: “We found that the operational range of particle sizes of air filters impacted the diversity and homogeneity of the detected vertebrate communities (Fig. 2 & 5). Specifically, the lower grade ISO ePM10 65% filter, which has an operational range of particle sizes between 0.3 and $10 \mu\text{m}$, outperformed higher-grade filters, i.e. filters with narrower operational ranges and designed to be better at

trapping smaller particle sizes (Fig. 2). The size range of aquatic fish eDNA is most abundant in the 1-10 μm particle fraction of filtered water 17 and thereby the similar operational range of particle sizes of the ISO ePM10 65% filter may explain why it performed better. The performance of the lower-grade filter might also be explained by its high volumetric airflow rate (Fig. 1). Lower grade filters are designed to favour capture of coarse particles, thereby providing less resistance to airflow (i.e., lower pressure drop). This in turn results in a higher fan speed, which results in a high and consistent airflow rate. In contrast, higher-grade filters favouring smaller particle sizes have higher resistance to air flow, leading to reduced fan speed and airflow rates. Specifically, the lowest grade filter in the study, the ISO ePM10 65% filter had a volumetric airflow of 12.27 $\text{m}^3 \text{ h}^{-1}$, while the higher-grade filters from the same company, ISO ePM1 65% and ISO ePM2.5 70%, had air flow rates of around 10.79 $\text{m}^3 \text{ h}^{-1}$ and 10.83 $\text{m}^3 \text{ h}^{-1}$, respectively. Thus, the ISO ePM10 65% filtered around 35 m^3 more air per 24 hours, which can explain the increased detection of vertebrates with this filter”.

However, airflow rate alone doesn't seem to impact detections as we observe better performance of ISO ePM 10 65% filters compared to ISO ePM 1 65%_FB. We also now highlight this in the discussion: Lines 409-416 “More factors than volumetric air flow rate impact the detection of vertebrates. For the ISO ePM1 65% we tested a filter from a different company (Filterbyen: FB) and with a different construction than the three other tested filters (Fig. 1F). This filter yielded a volumetric airflow rate of 12.33 $\text{m}^3 \text{ h}^{-1}$, which is comparable to the airflow of the best performing filter (12.27 $\text{m}^3 \text{ h}^{-1}$), the ISO ePM10 65% filter from the company Luftladen (LL). However, the ISO ePM1 65% filter from the FB company yielded significantly fewer vertebrate taxa than the ISO ePM10 65% filter (Fig 2A). This suggests that other factors than the volumetric airflow rate impact a filter’s ability to collect vertebrate eDNA”.

If these two critical points are adequately addressed, and the results and conclusions are revised accordingly (where necessary), I recommend the publication of this manuscript.

Below I provide detailed line-by-line comments below for additional suggestions and clarifications. Congratulations once again on this impressive piece of work!

Fabian Roger

L51: Not only vertebrates, also insects as well as non-animals such as fungi and plants. I know the focus here is vertebrates but I think at least in the introduction a wider context would be good (up to you).

Response: We have changed “vertebrates” to “organisms” and added a few other references from these non-vertebrate groups (Line 51).

L85: Filter mats: The range of the particle size is misleading. The filter mats are rated for a certain retention efficiency (i.e., ISO ePM1 65% nominally retains 65% of PM1 particles), and

they might be recommended for certain sizes (0.3–1 μm), but they will capture particles outside this size class - both smaller and, especially, larger.

Response: We have now changed this to “microfibre air filters with different filter grades (i.e., varying particle capture efficiency profiles)” to avoid misleading information (Lines 95-96).

L101: Filter type is confounded with air volume (i.e., if you forced the same air volume through the different filters, then you would measure type and size; here, you don’t know). Also, what model did you run? Please add it in the text (here and elsewhere – you only provide the statistics). It would be good to see the results of a model including airflow and filter type.

Response: After new calculations of the airflow rates (based on your suggestions) we found that the filter grade was not strongly confounded with the airflow rate of the setup. For example the lower grade filter (ISO ePM 10 65%) has a similar airflow rate (12.27 m³ h⁻¹) to one of our higher-grade filters ISO ePM 1 65%_FB (12.33 m³ h⁻¹). We now added a section in the discussion about this. Please see the response to your second major point.

L110: It’s not possible to assert that the community composition differed significantly by filter type with the Jaccard index if richness also differed. There are methods to try and disentangle the effect (e.g., the nestedness functions in R vegan [<https://rdr.io/rforge/vegan/man/nestedtemp.html>]) or use null models to account for differences in richness (Raup-Crick dissimilarity – also implemented in vegan). Saying that the community composition differed with filter type asserts that different filter types capture different species - which would be significant. I doubt that’s the case here, though.

Response: Thank you for this suggestion. We have now used the Raup-Crick dissimilarity index to measure the community composition. Please see the response to your first major comment.

L123: Same comment about filter type being confounded with air volume.

Response: Thank you for this comment and please see our response regarding the 2nd major comment.

L125 FF: It would be good to say why you tested whether the amount of chicken reads influenced species detection. The reason would be to test if sequencing depth was high enough, I assume? Or would it inform whether it’s worth including chicken-blocking primers (not possible for BirdT I think, we checked once)? Also, some people will argue that chicken DNA is a common contaminant—did you detect any significant amount in your blanks? The results of the blanks need to be mentioned in the main article.

Response: The reason behind testing the association between chicken reads and species detection was to evaluate if the increased number of chicken reads lead to reduced number of detected wild bird taxa and to evaluate if the increased bird DNA correlates with the amount

of chicken reads. Based on this comment, we now we specify this in lines 129-149 where we say: “To evaluate whether there was a difference in the amount of DNA captured by different filter types and whether it was correlated with the number of detected wild taxa, we quantified bird eDNA through qPCR with the BirtT primer set. We chose the BirtT primer set because it only PCR amplifies bird sequences, see Methods for details, and thereby not human and some of the most prevalent domestic animals, such as pig and cow. We found that filter type had a significant effect on the concentration of accumulated total bird DNA (wild and domestic) (lmm: Fdf = 30.613, $p < 0.0001$; Table S7, Fig. S1). Specifically, the ISO ePM10 65% filter accumulated a higher concentration of bird DNA than the two other filter types (Fig. S1). There was a significant association between the quantity of bird DNA within a sample and the number of detected wild bird taxa (lmm: Fdf = 14.881, $p = 0.0003$), indicating that filters that capture a higher amount of bird DNA yield detection of more diverse bird communities. Additionally, we investigated if the amount of domestic chicken sequences within a sample was associated with the amount of bird DNA and impacted the detections of wild bird taxa. Doing so, we found no significant association between the amount of bird DNA as measured by qPCR and the arcsine transformed proportion of chicken sequences detected with metabarcoding (lmm: Fdf = 0.14981, $p = 0.7001$). Further, we found no association between the number of wild bird taxa and the proportion of chicken sequences detected in a sample (lmm: Fdf = 0.65221, $p = 0.4227$). This suggests that the proportion of domestic chicken sequences found within a sample did not impact the recovery of wild bird taxa”.

L129: You are testing proportions here. Did you transform the variable before running the LMM?

Response: This is a good point and now we have conducted the Arcsine Transformation on proportional data and reran the analyses (Lines 141-144). The transformed data did not change the outcome of the statistical analysis: “Doing so, we found no significant association between the amount of bird DNA as measured by qPCR and the arcsine transformed proportion of chicken sequences detected with metabarcoding (lmm: Fdf = 0.14981, $p = 0.7001$).”

L140: This is curious. Why would the filter that detects more birds detect fewer (mostly no) mammals?

Response: This is a good point. One possible explanation for this could be the affinity of the filter material to mammal DNA. Another possible explanation is the construction differences in filter material as the ISO ePM1 65% filter from filterbyen was constructed differently to Luftladen filters.

L178: Amphibians are mentioned for the first time here. You also only detect one amphibian species. I would include it but be clear that it wasn't the target.

Response: We now changed here to avoid mentioning amphibians: “In the mammal dataset, there was also a significant effect of storage method (lmm: Fdf = 11.782, $p = 0.0001$) with dry-

stored filters yielding a significantly higher average number of taxa (1.8 ± 1) than buffer samples (Fig. 3A, Table S10). ” (lines 202-204).

L203: I don't know if air sampler numbers are necessary in the figure, as the same points belonging to the same samplers are already connected.

Response: This is a good point. We have now removed the air sampler names from Fig. 3C. This also helped to reduce the clutter of the Fig. 3C.

L221 FF: Again, I suspect that total air volume filtered is likely the underlying driver and should be discussed explicitly.

Response: Based on the new calculations made based on the earlier comments, we found that filter type is not directly correlated with the airflow rates. As such, we find the 12 V fan fitted with a small filter ($12.07 \text{ m}^3\text{h}^{-1}$) to have a superior airflow rate than the 5 V fan fitted with the large filter ($2.103 \text{ m}^3\text{h}^{-1}$) (Fig 1). It seems like it might be a combination of the airflow, filter area and the filter material that impact the detected communities. Based on the earlier comments, we have now changed the relevant text in the discussion to say: “We found that the operational range of particle sizes of air filters impacted the diversity and homogeneity of the detected vertebrate communities (Fig. 2 & 5). Specifically, the lower grade ISO ePM10 65% filter, which has an operational range of particle sizes between 0.3 and $10 \mu\text{m}$, outperformed higher-grade filters, i.e. filters with narrower operational ranges and designed to be better at trapping smaller particle sizes (Fig. 2). The size range of aquatic fish eDNA is most abundant in the $1\text{-}10 \mu\text{m}$ particle fraction of filtered water and thereby the similar operational range of particle sizes of the ISO ePM10 65% filter may explain why it performed better. The performance of the lower-grade filter might also be explained by its high volumetric airflow rate (Fig. 1). Lower grade filters are designed to favour capture of coarse particles, thereby providing less resistance to airflow (i.e., lower pressure drop). This in turn results in a higher fan speed, which results in a high and consistent airflow rate. In contrast, higher-grade filters favouring smaller particle sizes have higher resistance to air flow, leading to reduced fan speed and airflow rates. Specifically, the lowest grade filter in the study, the ISO ePM10 65% filter had a volumetric airflow of $12.27 \text{ m}^3 \text{ h}^{-1}$, while the higher-grade filters from the same company, ISO ePM1 65% and ISO ePM2.5 70%, had air flow rates of around $10.79 \text{ m}^3 \text{ h}^{-1}$ and $10.83 \text{ m}^3 \text{ h}^{-1}$, respectively. Thus, the ISO ePM10 65% filtered around 35 m^3 more air per 24 hours, which can explain the increased detection of vertebrates with this filter.” (lines 391-407).

L231: The text here is a bit confusing. It's a new paragraph starting with a new conclusion, but it seems to discuss the same results as the previous paragraph. Also, with respect to community composition, I have the same comments as above - richness and composition are confounded (it would be curious if filter size changed which species were detected).

Response: This was a misplacement of text and now we have moved the text to the appropriate location of the manuscript (Lines 253-259). Also redoing the composition analyses with Raup-

Crick dissimilarity lead to lack of statistical impact of sampler type on the detected community composition: “Bird community composition (measured with Raup–Crick dissimilarity) was not influenced by the sampler setup (PERMANOVA: Fdf = 0.76884, R2 = 0.0265, p = 0.6653), but there was a significant effect of the sampling site (PERMANOVA: Fdf = 1.4839, R2 = 0.1153, p = 0.0225). Similarly, mammal communities did not differ significantly between sampler setups (PERMANOVA: Fdf = 1.5634, R2 = 0.0759, p = 0.0521), but the sampling site had significant effect on the detected communities (PERMANOVA: Fdf = 2.3849, R2 = 0.2607, p < 0.0001).”

Figure 4 (and all): Please reduce the vertical jitter—it’s not possible to see how many detections a given data point corresponds to. The number of detections is discrete, so the point clusters should be too.

Response: We have now reduced the width of jitters in all the box plots in Fig 4.

L355 (and elsewhere): Strictly speaking, for assessing sample seasonality, you should have sampled different seasons in the same location and shown turnover; here, you confound location and season. In theory, the presence of seasonal species alone doesn’t indicate a seasonal signal. Given how strong the birding community in Denmark is, it would also be interesting to compare airborne eDNA detection vs. eBird / Gbif records and see what proportion of species in-season and out-of-season you detect at every location - which would be a more rigorous test of seasonal signal (that being said, you show turnover on the scale of hundreds of meters - it’s quite evident to me that you wouldn’t detect species months after they were there).

Response: All these are very good points and we agree that to properly assess the impact of the seasonality we need to perform a more focused study. However, here we are capitalizing on the fact that we conducted our sampling across seasons and habitat types to provide some indications of spatial and temporal trends in biodiversity detections with airborne eDNA. The idea is simply to point out that we can detect some key species of each habitat and that our summer sampling detects more summer active taxa - and that there is no detection of ‘summer taxa’ in our autumn samples.

Analysis suggestion:

For testing temporal and spatial signal bleeding, it occurred to me that you could consider an additional analytical approach that might yield interesting insights. You sampled at three locations with strong spatial replication, two of which were sampled on three consecutive days. In Experiment 2, you assess community similarity relative to sampler distance, but as far as I can tell, you don’t account for sampling day - or for wind direction. I believe these data have not yet been analyzed to their full potential.

I would need to look more closely at the methods, but in marine ecology, it is standard practice to estimate connectivity between sites (e.g., for larvae that drift with currents rather than swim against them). A similar approach could be applied here: the distance between samplers is not symmetrical—i.e., a sampler positioned downwind of another is more likely to capture a signal

from the upwind location than vice versa. Likewise, a sample taken on Day 2 may be influenced by the conditions on Day 1, but not the other way around.

You could explore this idea by comparing Mantel tests that incorporate directional and temporal asymmetries with those based on randomized assignments (i.e., a null model). It may even be possible to estimate signal bleeding directly by testing whether signal strength (e.g., read number or prevalence across replicates) is directional. For instance, a strong detection at an upwind site might also appear at a downwind site, albeit at lower strength—whereas detections should not appear in the reverse (upstream) direction.

I'd need to take a bit more time to think through the implementation details, but I believe this line of analysis could be well worth exploring.

Response: These are good ideas. But given that we didn't measure wind-directions at each sampling point during the time of sampling - where wind can have changed direction - then we cannot test this hypothesis with the given data. Also further analyses on spatial aspects can take the main focus of this study away, which is to identify optimal sampling methods for airborne eDNA. We would also like to highlight that in Experiment 2 we only conducted one 24 hour sampling, thus we didn't collect samples across days in that project. Now we highlight the need for proper examination of fine-scale spatial trends with airborne eDNA in the discussion: "However, more comprehensive examinations are needed to investigate the spatial and temporal resolution of airborne eDNA from vertebrate communities across climate zones and environments, including investigations into the state, transportation and persistence of vertebrate airborne eDNA" (lines 525-528).

L364: Again, the other filters also retain larger particles—it's a bit curious to give an upper bound.

Response: We have changed the use of particle size retention as a reason for our detections. We have now edited the discussion to point out that the observed differences may be a combination of the airflow rate, filter construction and the filter area. We now discuss this in Lines 391-454. We have also added the changed text in the 2nd major point.

L367: See my concerns on how you measured air volume.

Response: This has been amended according to your comments. Please see the response to your second major comment.

L383: There are two steps: one is collection, the other one is lysis—i.e., getting the particles off the filter again. Could there be differences in lysis efficiency between the filter structures?

Response: This is a good point. Now we added this possibility to the discussion (Lines 441-445): "Finally, filter construction and structure could impact the lysis process during DNA extraction. For example, thicker filters with multiple layers may release filter bound particles less effectively, compared to thin filters with fewer layers during the digestion. Thus, the lysis efficiency of different filter types should be tested using a more controlled experimental setting".

L409: Woven air filters have all three filtration mechanisms - impaction, diffusion, and sieving - depending on particle size.

Response: We do agree. Although sieving is only applicable for extremely large airborne objects which are beyond the scope of this study. We are only concerned with aerosols, which have a diameter smaller than a few tens of micrometers.

L426: I agree with your conclusion, but doesn't logically follow from the previous statement. The Burkard spore trap has a similar flow rate but a very different collection mechanism (adhesive-coated plastic tape), and it was compared to the Coriolis, which has yet another sampling mechanism (liquid cyclone) and a vastly higher flow rate, but very short collection times. A stronger argument could be that it shows that long collection outperforms high flow rate (the advantage of being 'around' when the signal is there).

Response: We do Agree. We have modified that and replaced the text with the following (Lines 486-492): "This suggests that our low-cost portable air samplers perform comparably to commercially available samplers, which can be expensive, difficult to manoeuvre and/or need stationary power sources. The volumes of filtered air aside, sampler setups with larger filters yielded more diverse vertebrate communities (Fig. 4), showing that larger filter areas can optimise airborne eDNA sampling. Overall, our results support the use of active air filtration using fans equipped with a low-grade air filter (i.e. a filter with wide operational range and favouring larger particle sizes), with large filter areas for the most efficient airborne eDNA collection."

L455: It would be helpful to indicate in Figure 7 which habitat was sampled in which season.

Response: We agree that season is important to communicate here but prefer to retain this information in the figure caption only (Lines 372-275), thus avoiding an over cluttered figure.

L463: There is little evidence that rivers transport eDNA over long distances. See this recent reference: <https://www.nature.com/articles/s41598-023-35614-6>

Response: We have now removed this comparison from the manuscript.

L493: Not particle size retention but capture efficiency for smaller particles.

Response: Amended accordingly throughout.

L506: I have done this too previously, but I don't think it's very useful. It gives only trajectories, not footprints (particle distribution models do), and the trajectories reach back hundreds of kilometers, while you show that the signal is local to a few tens to hundreds of meters. I would drop it.

Response: At present, nobody knows the lifetime of DNA in the atmosphere with certainty, due both to incomplete knowledge of the mechanisms of DNA degradation, and regarding their relative roles in this specific situation. As we note in the Conclusion, studies are needed regarding the specific impacts of environmental variables (temperature, exposure to sunlight, composition of aerosols e.g. salts, metals, organics, impact of atmospheric reagents (O₃, H₂O₂, radicals like OH, Cl, HO₂, NO, NO₂), pH). In addition, eDNA is present in different forms –

encased in biofilm, adsorbed to soot or clay minerals. And there will be dilution in the atmosphere depending on atmospheric variables – mixing, turbulence, wind speed, terrain.

The back-in-time trajectories are important to show the past history of the air that is being sampled. For example, if the air arrives from high in the troposphere, it would have been significantly colder (below freezing), and the sunlight would have been significantly more intense exposing the material to much more UV light. And, if the air has mainly been at low altitude, it would have been warmer and more humid with less UV, and more chance for addition of local DNA during transport. By showing the path, we also learn something about the likelihood of the observed signal coming from species that might be e.g. more northerly (wind from the north) or southerly (wind from the continent).

L541: 15 min on a laminar flow hood is not much, given the long distance from the light source and the thick filter material. Can you estimate exposure dose (J/m²)?

Response: We believe that the exposure time of UV (15 mins) we used in the study was sufficient to surface sterilize the filters. Supporting this, we don't detect taxa in our filter blanks (which went through the same UV process).

L547: How? For accurate velocity measurements, the flow should be approximately laminar, so it would be good to measure inside a longer tube at the end of which the filter and fan are mounted. Also, as far as I understand, rotor blade anemometers are less accurate at low flow rates, and yours is rated at (0.3–45 m/s), if I see it right, while the flow through your filter (max 0.553 m³/hour on the 6x6 filter with 12V) would correspond to 0.043 m/s - i.e., 10x below the sensitivity of your anemometer. This might explain the surprisingly similar flow measurements - it would be crucial to try to get a more accurate measurement, as I suspect that air volume is driving most of your observed patterns. Thermal anemometers (like the Testo 405i) have higher accuracy at low flow speeds.

Response: Thank you, we have performed additional measurements using a different instrument and confirmed the veracity of the original data, which, as the reviewer points out, was obtained outside the recommended operational range of the previous instrument. Please see our response to the second major comment.

L571: The information that the filters were first stored in Longmire buffer for 2 weeks and then frozen is missing above—please add it to the results section, too. Also, it's unusual (as far as I know) to freeze samples in Longmire buffer (they are usually stored at 4°C or room temperature). Could this have influenced recovery?

Response: This is a valid point, but it is unlikely that freezing would impact the storage capacity of a storage buffer.

L595: Did the filters homogenize with bead beating? The polyester filters are very resistant to bead beating in my experience.

Response: The idea of using the beads was not to homogenise the sample but add an additional layer of disruption to remove particles from the filters during the digestion process. We have now changed the word homogenising with bead beating (Line 679).

L598: “adusmenting” → adjusting

Response: Amended accordingly (Line 684)

L607: It’s enough to state that you used the sequencing primer for qPCR and describe the primers in the sequencing section. It’s a bit confusing in the qPCR section.

Response: We believe that it is ok to introduce these primers here to avoid redundancy within the methods section. Thus, we decided to keep the order of the text the same here (Lines 692-695).

L630: Did you use different cycling numbers for different treatment groups? If so, please state that here.

Response: We tried to use the same cycle numbers within an experiment for all the treatments. This was the case for Experiment 1. In experiment 2, Longmire’s buffer-stored filter halves and buffer extracts required 1-2 cycles extra while in the Experiment 3, passive samples needed around 40 cycles. This information is available in Table S17.

L633: “Six PCR replicates were independently analysed for each DNA extract, negative and positive control.” I understand what you mean now but it took me a while. Consider rephrasing.

Response: We rephrase this with the following: “We conducted six PCR replicates for each DNA extract, including for negative and positive controls.” (Lines 718-719).

L648: What concentration measurements does this refer to? You already described qPCR for 142 samples before?

Response: It is specified in the first sentence of the paragraph: “To assess the quantity of airborne bird eDNA collected with different filter types in Experiment 1, we conducted a new set of qPCRs on all 72 air filter samples.” (Lines 734-735). These qPCRs were conducted to evaluate if the filter efficiency also aligns with the amount of DNA captured by each filter. This information also can be found in the results section (Lines 129-149).

L667: These are quite stringent filtering options. Is the read threshold (5) implemented to account for tag switching? Did this exclude species across the whole dataset or only in certain samples? Same for the PCR replicates? I know this is common practice, but if you recover a read that you can assign to species level and it’s distinct from the other reads, why exclude it? Where should it come from if not from a true detection (if your controls are empty)? It would be interesting to see Figure 7 with all species and the frequency as the ratio of total replicates across samples and/or mean replicate prevalence ($n/6$). It would also be good to see the analysis done with or without these thresholds to see if the patterns hold (if it’s a reasonable effort).

Response: The reason we kept the sequence number threshold to 5 was to capture as much as diversity, while we used the strict 3/6 PCR replicates to test the robustness of the detections, as the focus of the study was to improve the accuracy of airborne sampling and not to capture the full diversity of vertebrates. We believe that for such a methodological exploration, it is important to implement stricter filtering measures. However, future studies that would focus on diversity assessments can be more lenient with the filtering.

L671: Why clustering to OTUs? Why not use ASVs? The fragments are short and the barcode region is not always very different between species; by clustering to OTUs, you might lose resolution. I would suggest using an ASV inference algorithm, doing the taxonomic assignment, and clustering by that (as you already do, just without previous OTU clustering). (No need to repeat the analysis - just a suggestion.)

Response: We thank the reviewer for this suggestion but testing OTU thresholds against ASVs is out of the scope of this work. We intend to work on this in the future.

L682: What is the threshold for 'similar' here?

Response: It is 100% and now we have indicated it in the manuscript (line 767).

L684: Was the local species missing from the database in all cases?

Response: Yes. It seems like the sequence region for all these local species were missing in Genbank.

L750: it would be great to make the analysis code available on GitHub (as also recommended by NatComm).

Response: The code and datasets have been deposited in GitHub (https://github.com/Kbodawatta/Airborne_eDNA_Methods_Paper). Will be permanently archived via Zenodo upon acceptance.

supL34: What concentration of ProtK?

Response: It is 19.3 mg/ml.

supL89: I am always curious to check what OTUs LULU merges. Did you do the taxonomic assignment before and verify that it didn't merge OTUs with different taxonomy? (I have seen that, although not for vertebrates I think.)

Response: Running LULU didn't merge OTUs with different taxonomic groups. In fact only a few OTUs were merged after running LULU. This information can be found in the Supplementary results (Line 82-100).

supL87FF: Do you have any sense of where the pig and chicken contamination could come from? (Nearby industrial farms? Manure on fields? Actual wild pigs [wild boar]?)

Response: Pig DNA is highly abundant in Denmark due high production of pig and the use of manure containing pig waste.

I don't see the results from the negative controls discussed (did I miss it?). These need to be included in the main text (even just stating that they were blank).

Response: Results from negative controls are now included in the main text (Lines 107-109, 189-192, 242-245).

Sup Table S4: I think the occurrence of the excluded fauna needs more explanation. Some are obvious (like chicken, pig, and cow), but others are less so - for example, chimpanzee (15

samples!), *Bos indicus* in 31 samples (was there a herd nearby?), and some of the domestic birds (peacock?). Do you have good estimates for where they came from? Could they also be lab contaminations (like the elephant)? A more detailed discussion would build confidence - right now you seem to only say that they occur in Denmark, which – at least for the gorilla - is a stretch. Does it occur outside of the zoo? How far away is the zoo? When were the samples processed after the last zoo samples? Or could it just be a human sequence that is misannotated as gorilla in the reference database?

Response: We now have added a new column to this table providing potential origin of these sequences. Peacock is potentially an escaped pet, while *Bos indicus* and *Pan roglodytes* are clearly due to mis-annotations of *Bos taurus* and *Homo sapien* sequences. There is also a potential of *Bos indicus* genetic material being in Danish *Bos taurus* due to genetic introgression between these two species. Please see the updated Table S4.

Reviewer #2 (Remarks to the Author):

Overall this manuscript provides further advancements in knowledge regarding airborne eDNA and has the potential to be an important contribution in methodological development. The impact of this manuscript would be improved with further clarity about methods, better incorporation of environmental variables and a more tempered and cautious interpretation of results and scope.

Response: Thank you for your comments on our manuscript. Below we address the comments you provided.

1. Methodological ambiguities;

- Contamination controls were underspecified.

- The manuscript does not clearly describe the use of negative field controls, blank filters, or lab air blanks during DNA collection and processing — all of which are essential in airborne eDNA studies where contamination is a major risk.

- Suggest to add explicit details about field blanks, lab controls, and negative extractions used in each experiment. Otherwise, it is difficult to assess the reliability of rare detections.

Response: This information is specified in the supplementary methods (lines 45 - 49 in the supplementary document). Following this comment, we have now included a sentence about them in the main methods section in lines 685-687 where we have now added: “Moreover, we included an extraction negative control and a vacuum manifold negative control for every 21 extracted samples and a room negative control for every 42 extracted samples (see Extended methods)”. Further, in the methods we have provided information on lack of wild animal taxa in the negative controls in the line 781- 785, specifically we now say: “Negative controls had no wild taxa, and we did not detect positive control taxa in samples. The only detected taxon that did not belong to Danish fauna was *Loxodonta africana* (African bush elephant), which was only found in one sample from Experiment 1 (Table S4) and not in any of the negative or positive controls. We consider this detection a laboratory contaminant”.

- Primers

- The study uses 12S (for birds) and 16S (for mammals) primers, but it lacks discussion on primer efficiency across taxonomic groups, any biases for less abundant taxa, or any misassignments of taxonomic identification.

- The results mention only 72 out of 90 taxa identified to species level — what about the remaining 18? Were they low-quality reads, or due to poor database matches?

Response: We could assign 72 taxa at species level and others we could only assign at genus level (many of these 18 taxa were not found frequently across the samples). The lack of assignments could be due to low-quality reads and poor database matches. To clarify this, we have now edited the text in lines 316-319 to read: “Overall, we detected 90 wild vertebrate taxa of which 72 could be identified to species level, while the rest could be identified at genus level (Fig. 7). Of these, we identified 52 bird species belonging to 11 orders, 19 mammal species belonging to 6 orders and one amphibian species (Fig. 7, Table S15)”.

- Suggest including a primer performance evaluation or reference existing studies to contextualise potential taxonomic blind spots that might occur using these primers.

Response: The purpose of this study was to evaluate different sampling techniques to optimise airborne eDNA sampling and primer biases would equally affect samples collected with the tested sampling and preservation techniques. Therefore, we included a sentence in the discussion to highlight the different inherent metabarcoding biases: Lines 530-533: “The use of airborne eDNA for vertebrate surveys is still in development, and the method shares the same suite of limitations and sources of error as all other eDNA methods, such as analytical errors, primer biases, incomplete reference databases, cost efficiency of sampling, laboratory costs and co-amplification of non-target taxa, such as domestic animals.”

- Lack of Meteorological Metadata

- Airborne DNA transport is influenced by humidity, wind, precipitation, and temperature. Yet, none of these parameters are reported or incorporated into the analysis.

- This limits understanding of temporal and spatial variability, and whether DNA movement or degradation confounded the results.

- Suggest that even basic weather metrics (e.g., wind speed/direction, humidity) would help interpret variation in detections.

Response: The spatio-temporal insights we gain in the current study is a secondary benefit of conducting our three experiments in three different areas in two different seasons. Our attempt is to merely demonstrate that we can detect local animal communities that are expected to be in the sampling areas during the sampled times. We do agree that there should be proper studies examining how these weather conditions truly influence the capture of airborne eDNA. However, that was not the focus of the study. But we agree that the weather is an important factor and so following this comment, we have included data on average daily humidity, precipitation, temperature, air pressure and wind speed for each of the sampling sites for each sampling date. We obtained the data at a provincial level from the Danish weather archives: <https://www.dmi.dk/vejrkiv>. To reflect this, we added the text about daily weather conditions in lines 553-557 (Kalvebod Fælled: “Average daily weather conditions based on Danish

weather archives (<https://www.dmi.dk/vejrarkiv>, accessed June 2025) were stable across the sampling period from 1 - 4 November 2022; temperature $\pm$ SD: 11.37 ± 0.75 °C, wind speed: 6.01 ± 0.68 ms⁻¹, precipitation: 0.04 ± 0.04 mm, humidity: $84.66 \pm 4.61\%$, air pressure: 1011.4 ± 4.3 hPa (Table S16”), 562-565 (Æbelø: “Average daily weather in the sampling area was stable across the two sampling days, however with some rain on 31 August; temperature: 14.89 ± 0.89 °C, wind speed: 2.4 ± 0.57 ms⁻¹, precipitation: 0.23 ± 0.72 mm, humidity: $83.96 \pm 0.91\%$, air pressure: 1005.7 ± 0.23 hPa (Table S16”), and 570-573 (Tofte Skov: “Average daily weather conditions did not vary across the sampling days; temperature: 18.25 ± 1.31 °C, wind speed: 2.93 ± 0.55 ms⁻¹, precipitation: 0 mm, humidity: $86.28 \pm 2.86\%$, air pressure: 1021.9 ± 4.03 hPa (Table S16”). The hourly data can be found in the new supplementary Table 16.

- qPCR Results

- The study presents qPCR-based concentration data for bird eDNA (BirtT primer), but does not

validate whether these concentrations correlate with species richness or read depth, nor discuss if qPCR data support quantitative inference or simply presence/absence.

- This could help calibrate whether airborne eDNA is semi-quantitative, which is an open question in the field.

Response: Based on this comment, we have conducted correlations between the concentration of bird DNA and the number of detected wild bird taxa and the proportion of wild bird sequences (Lines 129-149, Figure S1): “To evaluate whether there was a difference in the amount of DNA captured by different filter types and whether it was correlated with the number of detected wild taxa, we quantified bird eDNA through qPCR with the BirtT primer set. We chose the BirtT primer set because it only PCR amplifies bird sequences, see Methods for details, and thereby not human and some of the most prevalent domestic animals, such as pig and cow. We found that filter type had a significant effect on the concentration of accumulated total bird DNA (wild and domestic) (lmm: Fdf = 30.613, $p < 0.0001$; Table S7, Fig. S1). Specifically, the ISO ePM10 65% filter accumulated a higher concentration of bird DNA than the two other filter types (Fig. S1). There was a significant association between the quantity of bird DNA within a sample and the number of detected wild bird taxa (lmm: Fdf = 14.881, $p = 0.0003$), indicating that filters that capture a higher amount of bird DNA yield detection of more diverse bird communities. Additionally, we investigated if the amount of domestic chicken sequences within a sample was associated with the amount of bird DNA and impacted the detections of wild bird taxa. Doing so, we found no significant association between the amount of bird DNA as measured by qPCR and the arcsine transformed proportion of chicken sequences detected with metabarcoding (lmm: Fdf = 0.14981, $p = 0.7001$). Further, we found no association between the number of wild bird taxa and the proportion of chicken sequences detected in a sample (lmm: Fdf = 0.65221, $p = 0.4227$). This suggests that the proportion of domestic chicken sequences found within a sample did not impact the recovery of wild bird taxa”.

2. Limitations in experimental design

- Taxonomic range

- The study focuses exclusively on vertebrates, particularly birds and mammals, and one amphibian species. This narrows the applicability of the results and limits generalisability to multi-taxa airborne eDNA studies (e.g., fungi, plants, arthropods).

- Given only vertebrates were targeted, there needs to be clarification that this was an intentional scope limitation and suggest future multi-marker work (e.g., ITS2, COI for insects/plants/fungi).

Response: We agree that airborne eDNA provides valuable high-resolution incidence data for insects, plants and fungi. Given the novelty of using airborne eDNA for vertebrate detections, we focus exclusively on these taxa in our study. We avoid redundant discussion of other markers for the detection of non-target taxa in our study.

- Underrepresentation of Mammals and Amphibians

- Mammals were detected in <45% of samples, and amphibians in only ~10%.

- Potential cause: Lower shedding, primer bias, or reduced airborne transport. Unaddressed: The manuscript does not adequately explore why non-avian vertebrate detection is lower (ie Lower shedding, primer bias, or reduced airborne transport), or how to address it.

- Suggest including discussion of these detection biases and how alternative markers or sampling duration could improve results.

Response: We agree that the reduced detections of Mammals could be a result of many factors as the reviewer suggested. Another possibility is that excessive amounts of pig DNA in Danish air may have drowned the amplification of the wild mammal DNA. We believe future work needs to investigate the primer biases and the impact of blocking primers on recovering mammal communities with airborne eDNA.

- Distance-Decay effect in birds

- A spatial signal was observed for bird communities in ~~Bel~~, but not for mammals. This finding is reported but not explained.

- Could mammals have more dispersed airborne DNA due to larger home ranges? Or does lower detection power mask the effect?

- Suggest including possible ecological explanations for this taxon-specific spatial resolution or acknowledge this as a data limitation.

Response: We now include a sentence in the Discussion (Lines 511-514: “The lack of the spatial trend in mammals might be due to larger home-ranges of mammals, which may result in movement between the sampled microhabitats or because mammals may not shed DNA in a similar way as birds.”) addressing this limitation.

3. Interpretation of results

- Overgeneralisation About Global Applicability

- The manuscript states airborne eDNA “can be adapted by conservation programs to monitor biodiversity across the globe.” While it is indeed promising, the method is tested only in Denmark, in three well-characterised sites during two seasons. Such claims may overstate transferability, especially to tropical, arid, or urban ecosystems.
- Suggest tempering the generalisation and clearly state ecological and geographic boundaries of applicability.

Response: Given other research into airborne eDNA across Europe (<https://doi.org/10.1002/edn3.591>, <https://doi.org/10.1111/1755-0998.13840>, <https://doi.org/10.1038/s41598-025-03650-z>), North America (<https://doi.org/10.1002/edn3.388>), and South America (<https://doi.org/10.1002/edn3.385>), we suggest that continued development will render airborne eDNA an important part of the toolkit for global biodiversity monitoring. In the discussion, we now highlight the importance of testing these sampling methods across biomes and ecosystems: “However, more comprehensive examinations are needed to investigate the spatial and temporal resolution of airborne eDNA from vertebrate communities across climate zones and environments, including investigations into the state, transportation and persistence of vertebrate airborne eDNA” (Lines 524-528).

- Dismissing the use of passive samplers

- In this study, passive samplers yielded very few detections and were concluded to be ineffective. However there could be other explanations for this, ie. sampling duration may have been too short, environmental variables could have been suboptimal. Passive systems have advantages of being cost-effective and have potential if used over longer periods or with improved surfaces.
- Suggest acknowledging the limitations of passive trial design and suggest refinement, not outright dismissal.

Response: This is a good point. Now we indicate why passive samplers did not work (Lines 494-501): “We observed negligible vertebrate detections in samples collected with no airflow (passive) (Fig. 4A). This contradicted findings from passive sampling of aquatic eDNA, in which passive filtering provided robust detection of aquatic vertebrates 23. As such, wind and water may not carry eDNA over filters in a similar way, and active filtration of airborne eDNA is crucial, at least for the relatively short sampling durations and the air sampler employed in the present study (24 hours). However, passive sampling may yield better results if the samplers are placed for longer time periods or using a different material. For example, a material which is not designed to purify moving air, i.e. not an aerosol particle filter as used here.”

Editorial suggestions:

There was a lot of wordy and awkward passive phrasing throughout the manuscript which could be improved for clarity.

Here are some examples and suggestions:

Response: Thank you so much for editing our manuscript and providing us with valuable insights to improve the manuscript.

Line 49: "...to study biodiversity in terrestrial ecosystems and in turn inform conservation efforts"

change to: "...to study biodiversity and inform terrestrial conservation efforts"

Response: Amended accordingly (Line 49)

Line 63: "...airborne eDNA to provide seasonal and habitat-specific snapshots..."

change to: "...airborne eDNA to yield seasonal and habitat-specific biodiversity profiles..."

Response: Amended accordingly (Lines 63-64)

Line 125: "...qPCRs results provide information on the total bird amplicons..."

change to: "...qPCR results provide information..."

Response: This sentence has changed following revisions (Lines 129-130).

Line 127: Change to plural 'were' related

Response: This sentence has changed following revisions.

Line 134: "highest quantity of bird DNA was the ISO ePM10 65%"

change to: "The filter with the highest bird DNA yield was ISO ePM10 65%"

Response: We changed this sentence to the following: "Overall, the ISO ePM10 65% filter type yielded the highest number of wild bird and mammal taxa and the highest quantity of bird DNA" (Lines 149-150).

Line 228: "Passive sampler s thus essentially acted as negative sampling controls"

change to: "Passive samplers functioned effectively as negative controls"

Response: This sentence has changed following revisions. "Furthermore, we show that active sampling is important for collection of vertebrate airborne eDNA. Specifically, the setup with small filters and no active filtration did not yield wild vertebrate taxa, while the setup with large filters and no active air filtration yielded detection of five wild vertebrate taxa with detections in only four of the 60 analysed samples (Fig. 4A). Samples from these two passive setups thereby essentially functioned as negative controls, supporting the authenticity of results from active filtering." (Lines 271-277).

Line 234: "we observed a significant effect of sampler type on the detected number of taxa"

change to: "Sampler type significantly influenced the number of taxa detected"

Response: This sentence has changed following revisions (Line 247).

Line 291: “ecological and behavioural lifestyles”

change to: “ecological and behavioural traits”

Response: Amended accordingly (Line 320)

Line 319: “such as warblers and swallows, which would have migrated south by the time of sampling”

change to: “such as warblers and swallows, which had likely migrated south prior to sampling”

Response: Amended accordingly (Lines 350-351)

Line 433: “one caveat with using high air flow is the lasting, portable...”

change to: “One limitation of high airflow is the requirement for a long-lasting, portable power source”

Response: This section has been changed due to responses to reviewers.

Line 439: “our results validate the use of air filters fitted on fans...”

change to: “Our results support using fan-fitted air filters as an effective eDNA sampling method...”

Response: This sentence has been changed to the following: “Overall, our results support the use of active air filtration using fans equipped with a low-grade air filter (i.e. a filter with wide operational range and favouring larger particle sizes), with large filter areas for the most efficient airborne eDNA collection” (Lines 490-492).

Line 461: “Together, the findings that airborne eDNA provides a snapshot...”

change to: “Together, these results show airborne eDNA can capture localised, seasonal biodiversity patterns”

Response: This sentence has changed to the following: “Together, these results indicate that airborne eDNA can capture seasonal localised vertebrate community patterns” (Lines 523-524).

24th January 2026

Dear editor at Communications Biology,

Thank you very much for handling our manuscript entitled “Advancing airborne eDNA sampling results in robust characterisation of vertebrate communities” and providing us the opportunity to finalise our manuscript based on the reviewer responses. Following the editorial suggestion we have now changed the manuscript title to the following: “Advancing airborne eDNA sampling methods for monitoring diverse terrestrial vertebrate communities”.

We apologise for taking a long time to resubmit the final revisions. Below we are providing our point-by-point responses for reviewers in **blue font** (line numbers are based on the clean manuscript file).

We have also gone through the *Communications Biology* guidelines for final revisions. Please find the document attached along with the manuscript files.

Thank you very much for considering our manuscript for *Communications Biology*.

Kasun H. Bodawatta & Kristine Bohmann
(for and on behalf of all co-authors)

Section for Molecular Ecology and Evolution,
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Denmark

Editor's comments

Thank you for your patience during the peer review process. We are very sorry about the delay in obtaining referee input on your revision. Unfortunately, some of the original reviewers were not available to re-review, so we had to invite an alternative reviewer.

Your revised manuscript entitled "Advancing airborne eDNA sampling results in robust characterisation of vertebrate communities" has now been by our referees, whose comments appear below. In light of their advice I am delighted to say that we are happy, in principle, to publish a suitably revised version in *Communications Biology*.

Please allow a few days for our journal staff to provide further details on our requirements for the final version of the manuscript prior to formal acceptance -- this will be sent as an attachment with specific editorial guidance for the final requested revisions and acceptance checklist for your manuscript.

In the meantime, you may wish to already work on incorporating the latest suggestions from reviewers. **Specifically, please include the exact model of fan used and more information on the statistical models in the Methods section of the main text.** Please note that we do not impose word limits and all methods should be described in enough detail so that they may be reproduced. We will be sending the acceptance checklist soon; you do not need to supply an updated manuscript with these minor revisions before receiving the final editorial requests.

Congratulations on an excellent study!

Response: Thank you very much for handling our manuscript and your encouraging words. We have now gone through the final reviewer comments and adjusted the manuscript accordingly. Specifically, we now include the exact models of the fans (Lines 549-551) and provide brief introductions to statistical models in the results section (e.g., Lines 103-105, 122-124, 138-141, 186-187, 243-244).

REVIEWERS' COMMENTS:

Reviewer #1:

Remarks to the Author:

I am satisfied with the revisions the authors have made and have only the following remaining comments.

Response: Thank you very much for reviewing our manuscript and providing us with valuable feedback.

Airflow measurements

Great that you were able to re-measure! Based on my experience, measuring airflow at the inlet rather than the outlet tends to give more accurate results (likely due to reduced turbulence). Attaching the tube in front of the fan and measuring the flow rate at the tube entrance generally gives the best results (using Volume Flow Duct, Air Type = Supply Air, Input Geometry = Area).

I would also recommend keeping the diameter of the plastic tube constant and large enough to cover the full sensor area of the anemometer (black tip). A 4 cm diameter might be too small, as the two sensors on my Testo 405i are ~5 cm apart. A good way to check measurement accuracy is to run the fan without a filter and compare the results to the manufacturer's specifications.

Response: We have now measured both inflow and outflow airflow rates using a tunnel with an area of 6 X 6 cm. We have decided to report both airflow rates in our study now (Supplementary table 1, Fig. 1 and 4, and Lines 592-614).

Finally, I noticed that the exact fan models are not cited. You cite Lynggaard et al. but in that article only the manufacturer (Sunon Inc.) is listed, not specific models. Please cite the exact models used, as their performance can vary widely.

Response: This has been added (Lines 549-551)

Line 101

Please include information on the statistical model(s) in the main text; otherwise, the results are not interpretable. From the code, I see you used:

```
lmer(Richness ~ SampleType + (1|SampleSite) + (1|Date), Data)
```

This should be explicitly stated in the text (e.g., “effect of sample type on richness, with SampleSite and Date as random factors”).

Response: Throughout the results section we have now briefly mentioned the model parameters (e.g., Lines 103-105, 122-124, 138-141, 186-187, 243-244).

Figure 4 (and Figure 2)

The reduced horizontal jitter makes the figure clearer, but there is still quite a bit of vertical jitter. I recommend removing the vertical jitter entirely, since the vertical axis represents the number of species and should be displayed without modification.

Response: We have decided to keep the figure as it is, as the vertical jitter allow to see individual datapoints. We are unsure what reviewer referred to as “modifications” as we have not done any modifications to the distribution of the data.

Reviewer #3:

Remarks to the Author:

I have reviewed the manuscript titled “ Advancing airborne eDNA sampling results in robust characterisation of vertebrate communities“. This manuscript has already been expertly reviewed. I was specifically asked by the editor to review the quality of edits in response to comments by reviewer 2.

The reviewer’s concerns with the methodology of the study were regarding clear annotation of negative controls, choice of primers (narrow taxonomic range), taxonomic assignment of sequences. The reviewer also pointed to the limitations in the interpretation of results, primarily with use of qPCR results, overgeneralization of global applicability, and dismissing the use of passive samplers. **The authors have adequately responded to most of the reviewer’s concerns by making appropriate edits to the manuscript.**

However, I disagree with one suggestion by reviewer 2, which was added to the manuscript, “The lack of the spatial trend in mammals might be due to larger home-ranges of mammals, which may result in movement between the sampled microhabitats or because mammals may not shed DNA in a similar way as birds.” If the authors wish to keep this edit, it should be supported by strong evidence that the mammals detected do in fact have larger home ranges than the birds detected. Even in the case of birds, no significant relationship was found when samplers were stored in Longmire buffer. There may be a significant amount of stochasticity

in air eDNA sampling and processing, especially since little is known about persistence and transport of air eDNA.

Response: We have decided to remove the sentence from the manuscript.

Anish Kirtane