

ORIGINAL ARTICLE OPEN ACCESS

Airborne Plant eDNA Reflects Local Biodiversity:
A Multi-Method Assessment in a Botanical Garden

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Received: 31 December 2025 | **Revised:** 9 June 2026 | **Accepted:** 10 July 2026

Keywords: aerobiology | amplicon sequencing | botanical | environmental DNA | metabarcoding | particle size | shotgun sequencing | Viridiplantae

ABSTRACT

Airborne environmental DNA (eDNA) is emerging as a powerful tool for noninvasive assessment of terrestrial biodiversity. We evaluated the effectiveness of three analytical pipelines for characterizing plant diversity in a botanical greenhouse, with a particular focus on how particle size and sampling height influence detection. Air was sampled for up to 4 days using active Total Suspended Particulate samplers, a 14-stage impactor, and a vertical filter stack, all equipped with quartz filters. To assess methodological variability and identify optimal approach, three analytical pipelines were applied: (A) Illumina amplicon sequencing with a QIIME-based workflow, and (B) Illumina and (C) Nanopore shotgun sequencing processed with Kraken2. Of ~2100 plant genera recorded in the greenhouse and adjacent garden, ~86% were considered detectable based on *rbcl*, *trnL*, *ITS1*, and *ITS2* barcode coverage. Pipeline A (32 samples) detected 149 genera, 88% of which were known from the garden; 55% of species-level assignments matched cataloged species, with ~11% attributable to misannotations or contamination. Pipelines B (6 samples) and C (2 samples) detected 76 and 88 genera, respectively, with 81%–82% corresponding to garden taxa and 50%–51% confirmed at species level. The two shotgun pipelines produced highly similar taxonomic profiles despite samples being collected 7 weeks apart, and both were strongly complementary to the amplicon results. Size-dependent sampling yielded elevated detections in the 2.5 μm impactor fraction, indicate substantial contributions from sub-pollen particles, while larger fractions ($> 40 \mu\text{m}$) also contained plant eDNA, suggesting multiple particle carriers or aggregates. Overall, airborne eDNA provided robust detection of local plant biodiversity and yielded new insights into particle-size distributions relevant to eDNA transport and deposition.

1 | Introduction

Environmental DNA (eDNA) has rapidly become a transformative tool in biodiversity monitoring, enabling the detection of organisms through genetic material shed into the environment (Deiner et al. 2017; Taberlet et al. 2012). While initially focused on aquatic systems, recent advances have demonstrated that eDNA captured from the air can provide valuable insight into terrestrial biodiversity (Clare et al. 2022; Lynggaard, Bertelsen, et al. 2022). Airborne eDNA has now been successfully applied

to detect a range of taxa, including plants (Banchi, Ametrano, Greco, et al. 2020; Banchi, Ametrano, Tordoni, et al. 2020; Johnson et al. 2021; Thuillet et al. 2024), mammals (Garrett et al. 2023; Lynggaard, Frøslev, et al. 2022), insects (Roger et al. 2021), fungi (Abrego 2024), and even at country scales using existing air-monitoring stations (Littlefair et al. 2023; Tournayre et al. 2025). Such studies suggest that airborne eDNA may revolutionize terrestrial biomonitoring by offering noninvasive, scalable, and taxonomically broad detection capabilities (Blackman et al. 2024).

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Despite these advances, several challenges remain. Key questions concern the mechanisms governing the transport, persistence, and ecosystem scale relevance of airborne eDNA, including the influence of particle size, source proximity, and sampling conditions (Klepke et al. 2022; Zinger et al. 2025). Traditional pollen-focused aerobiology has offered insights into airborne particle behavior (Després et al. 2007; Skjøth et al. 2007) and has been complemented by recent metabarcoding studies targeting plant and fungal taxonomic groups (Banchi, Ametrano, Greco, et al. 2020; Banchi, Ametrano, Tordoni, et al. 2020; Krinitsina et al. 2023; Polling et al. 2022; Tegart et al. 2024). However, pollen typically represents only one component of the airborne biological particle spectrum. Pollen grains are relatively large (10–100 µm) and often seasonal, and their detectability is strongly influenced by phenology, plant reproductive strategy, and meteorological conditions (Baksay et al. 2020; Plaza et al. 2022). Importantly, many airborne eDNA studies, including those conducted during winter or outside peak flowering seasons, have reported substantial detection of plant taxa despite minimal airborne pollen (Johnson et al. 2019; Lennartz et al. 2021). This suggests that a proportion of airborne plant eDNA may reside in sub-pollen particle sizes, including (pollen-derived) cellular fragments or extracellular DNA (Barnes and Turner 2016; Taylor et al. 2002). Therefore, it is important to focus more on analyzing smaller particle sizes and their role in detecting different plant taxa, especially during times when pollen levels are naturally low.

Comparative studies are therefore crucial to understand how technical factors, such as sampler type, particle fractionation, and sequencing platforms, affect airborne biodiversity assessments (Berelson et al. 2025; Lin et al. 2025). To date, systematic evaluations of airborne eDNA sampling strategies and analytical pipelines for plant detection under controlled conditions remain rare. Johnson et al. (2019) demonstrated that airborne plant eDNA captures higher diversity than conventional vegetation surveys, but their study did not disentangle the impacts of particle size or vertical sampling gradients. Moreover, while practical frameworks for airborne eDNA sampling have been discussed (Thuillet et al. 2024; Tulloch et al. 2025), empirical data on how particle size influences plant taxa detection remain limited. Airborne eDNA studies deploying novel sequencing approaches (like shotgun) remain rare and only appeared recently, showing very promising results (Giolai et al. 2024; Mikko et al. 2025; Nousias et al. 2025; Sullivan et al. 2025).

In this study, we evaluate the effectiveness of three analytical pipelines for characterizing plant diversity in a controlled botanical greenhouse. We explore the effect of particle size on eDNA detection using active air samplers, evaluate two sampling heights, and we compare taxonomic outputs from three bioinformatic pipelines: (A) amplicon sequencing via Illumina MiSeq + QIIME, (B) short-fragment shotgun sequencing via Illumina NextSeq + Kraken2, and (C) long-fragment shotgun sequencing via Nanopore MinION + Kraken2.

The short- and long-read sequencing approaches both have their potential advantages and disadvantages. A potential drawback of shotgun sequencing is that species-level identification can

be unreliable due to incomplete reference databases, particularly for taxa lacking complete genomes such as plants (Bell et al. 2021). Whilst for amplicon sequencing, the typical target regions (i.e., ITS, rbcL, and trnL) may not provide enough resolution to allow for accurate species-level identification. In addition, similar to aquatic environment (Bylemans et al. 2018; Saito and Doi 2021) airborne eDNA may be degraded and fragmented providing more basis for assessments using shorter read versus longer read strategies. To identify the most effective analytical approach for characterizing plant biodiversity from airborne eDNA, we compare both short- and long-fragment shotgun approaches with a targeted amplicon sequencing method using four molecular markers (primers).

By benchmarking recovered taxa against a curated list of approximately 2100 plant genera and 9100 plant species known to occur within the greenhouse and surrounding gardens, and by assessing their theoretical detectability through *in silico* screening of genomic reference data, we evaluate the robustness and ecological relevance of airborne eDNA-based monitoring. This study aims to expand the methodological foundation for airborne plant eDNA and to provide insight into how airborne particle size distributions can shape detection patterns and inform models of eDNA transport and deposition.

2 | Materials and Methods

2.1 | Sampling Sites and Equipment

The experiment was conducted in the tropical greenhouse at the Utrecht Botanical Garden over a 4-day period in both December 2024 and February 2025. Based on a previous pilot study that detected substantial external DNA (data not published), the greenhouse was used primarily as a sheltered, powered location enabling extended sampler deployment. We deployed automatic air sampling equipment comprising three different sampling approaches: (i) a Total Suspended Particulates (TSP) sampler developed inhouse by TNO; (ii) a Dekati Low Pressure impactor (DLPI) sampler, and (iii) a “Stack Sampler” designed inhouse by TNO (see Figure 1).

The TSP and DLPI sampler were run during the same time in late December 2024 and the Stack Sampler was run in early February 2025. In addition to the actual samples a field blank was also collected for the TSP and Stack Sampler setups by placing a filter and removing it from the device before pumps were activated. Below is a more detailed explanation of each setup and Table 1 provides an overview of the sampling setups.

2.1.1 | TSP Sampler

In total, 18 filters were sampled using two vacuum pumps, each connected to nine sampling heads fitted with 47-mm quartz filters (Whatman QM-A; Cytiva, Marlborough, MA, USA). QM-A filters are quartz fiber depth filters with a nominal particle retention of approximately 2–2.5 µm. Sampling heads were mounted on tripods at heights of 1.5 and 3 m, with each sampler operating at a flow rate of approximately 8.3 L/min. The quartz filters were collected after 89.5 h having sampled 44.5 m³ of air

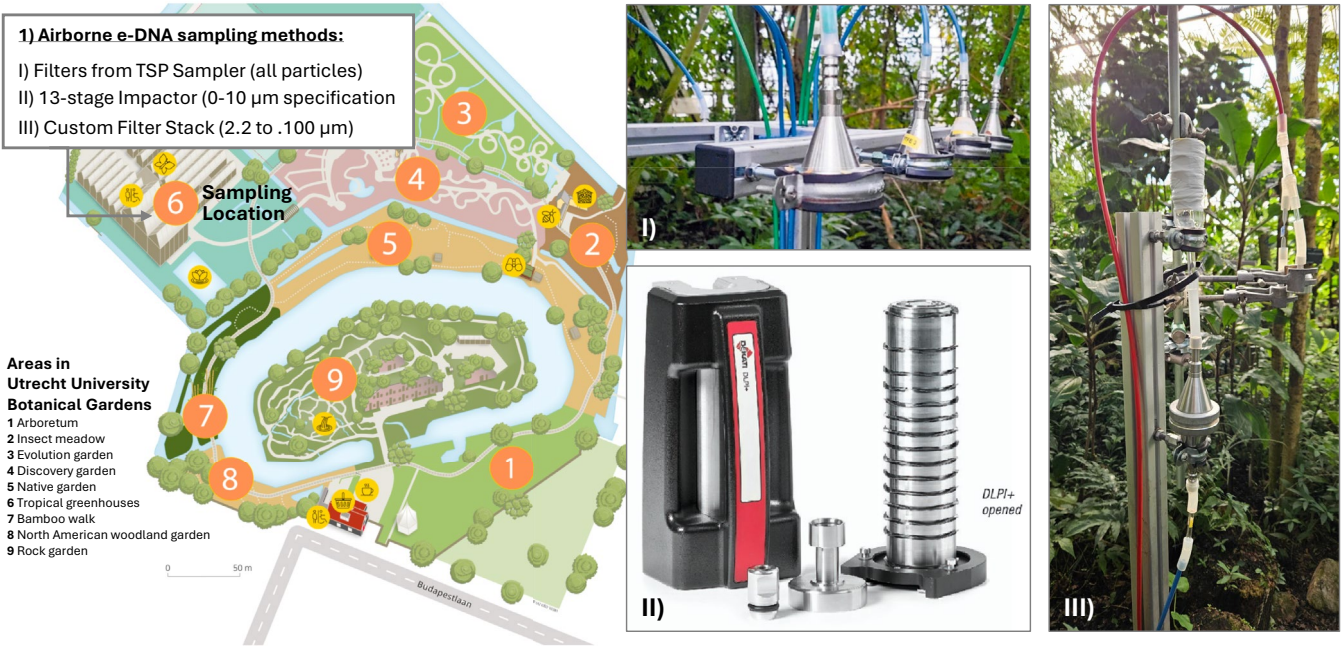


FIGURE 1 | Sampled location (6) in botanical garden greenhouse and pictures of the three sampling methods deployed in this study.

TABLE 1 | Overview of the different sampling methods and key parameters, including the analysis pipeline used.

Equipment	Filter type	Particle size (μm)	Flow (L/min)	Time (min)	Start sampling date	Number of samples	Analysis pipeline
TSP Sampler	Quartz filter	> 2.2	8.3	5370	17 December 2024	18 (+1 field blank)	A
TSP Sampler	Quartz filter	> 2.2	1–3	5370	17 December 2024	2	C
13-Stage impactor	Impactor collection plates	< 0.030 to 10	10	5370	17 December 2024	14	A
Filter stack	Quartz filter and EasyStrainer mesh filter	> 2.2, > 40, > 70, and > 100	1, 2.4, and 15.8	4287	03 February 2025	4 in the stack, + 2 separate (+1 field blank)	B

and submitted for analysis by pipeline A. A third vacuum pump was set up to collect backup samples and for other analyses (not reported here) but also sampled two quartz filters at 16.3 L/min for 41 h (total sampled volume 41 m³) which were submitted to analysis by pipeline C.

2.1.2 | DLPI Sampler

In total, 14 samples were sampled by the DLPI (Dekati Ltd., Tampere, Finland), which separates particle capture from 0.030 to 10 μm as well as a final filter capturing the remainder (<0.030 μm). The DLPI collects particles on 25 mm collection substrates and was sampling from a height of 1.5 m and was pumping air at a rate of 10 L/min. The filters were collected after 89.5 h having sampled 53.7 m³ of air and submitted for analysis by pipeline A.

2.1.3 | Stack Sampler

This setup comprised a 100, 70, and 40 μm EASYstrainer mesh-filter (Greiner Bio-One B.V., Alphen aan de Rijn, the Netherlands) set on top of a quartz-filter providing a single stack with filters to capture particles of different sizes. The stack was run for 71.5 h, sampling at 15.8 L/min and thus having sampled about 67.7 m³ of air. Next to the stack setup, there were two other TSP samples with quartz-filters running at 1 and 2.4 L/min resulting in 10.3 and 67.7 m³ air sampled respectively. The six samples were submitted for analysis in pipeline B.

2.2 | Analytical Pipelines

To assess analytical variability, three distinct sequencing and bioinformatic pipelines were applied at different laboratories,

TABLE 2 | Overview of different analysis pipelines and key parameters.

	 4 × Amplicon 32 Samples	 Short Shotgun 6 Samples	 Long Shotgun 2 Samples
DNA fragmentation	Yes, by targeted forward and reverse primers	Yes, by tagmentation	Yes, by Rapid PCR barcoding Kit
DNA target region	4 primers: ITS1, ITS2, <i>rbcl</i> , and <i>trnL</i>	All	All
Sequencer	Illumina: MiSeq	Illumina: NextSeq	Oxford Nanopore Technologies: MinIon Mk1C
Laboratory	SGS Lisbon, Portugal	TNO, Leiden, the Netherlands	TNO, Rijswijk, the Netherlands
Sequencer reads	300–600 bp; first 300 bp from each end of a fragment	150–300 bp; first 150 bp from each end of a fragment	Range of 20 bp to 4 Mb (average fragment length for Rapid PCR barcoding Kit is ~3 kb)
Key rationale	Reference database coverage of common target regions may be better	Degraded eDNA may contain more shorter sequences	Longer DNA fragments may yield higher accuracy
Bioinformatics pipeline/database	QIIME2/PLANIITS & NCBI	Kraken2/PlusPFP	Kraken2/PlusPFP
Number of samples	32 (+1 field blank)	6 (+1 field blank)	2
DNA yield (range)	0.15–0.64 ng/μL	~22–199 ng/μL (NanoDrop, total nucleic acid)	< Qubit HS detection (pre-PCR), 0.23–1.41 ng/μL (post-PCR)
Read count	2 × 8,490,998 (total)	~800 million paired end (total)	3902–60,569 per sample

to which filters were transported under cold-chain conditions following air sampling.

The choice of approaches (Table 2) reflected a trade-off between the expected fragmentation of airborne eDNA, which favors short target regions, and the need for longer genetic markers to achieve reliable taxonomic resolution in plants.

Full laboratory and bioinformatic protocols for all three pipelines (including reagent details, primer sequences and references, PCR conditions, control samples, and software versions and parameters) are provided in Appendix S1. The descriptions below summarize the design and the parameters relevant to interpretation. To limit underrepresentation of pollen-bound DNA, each extraction included a disruption step: zirconium bead-beating for Pipelines A and B, which mechanically ruptures pollen exines, and the DNeasy enhanced (gram-positive) enzymatic lysis protocol for Pipeline C. None of the samples were excluded due to too low DNA yield or too few reads.

2.2.1 | Pipeline A: Illumina MiSeq With QIIME Bioinformatics

DNA was extracted using a CTAB-based lysis, bead-beating and enzymatic steps; followed by magnetic bead-based purification. Four plant-targeting primer sets were used, spanning markers with differing levels of conservation and taxonomic resolution: ITS1, ITS2, chloroplast *trnL*, and chloroplast *rbcl*. Amplification of ITS regions was performed using primer sets targeting ITS1/

ITS2 regions (ITS2-S3R/ITS2-S2F/ITS4) originally described in White et al. (1990) and adapted in Yao et al. (2010) and Chen et al. (2010). Amplification of the chloroplast *rbcl* gene was done using the RBCL_1353F/RBCL_1502R primer pair targeting a short fragment suitable for degraded DNA, following established plant DNA barcoding and mini-barcode approaches (Hollingsworth et al. 2009; Kress and Erickson 2007). The chloroplast *trnL* (UAA) intron was amplified using the TRN_186F/TRN_617R primer pair, based on the universal *trnL* system described by Taberlet et al. (2007).

For each DNA sample and marker, a single Illumina sequencing library was prepared. Target regions were amplified using a two-step PCR protocol adapted for Illumina sequencing. In the first PCR, locus-specific primers carrying Illumina overhang adapters amplified the target region (35 cycles; annealing 52°C for ITS and 56°C for *rbcl* and *trnL*); a second PCR was performed to attach dual indices and Nextera XT indexing primers. An unexposed filter blank, negative controls (molecular-grade water) and positive controls (pure plant DNA) were included and processed in parallel; no taxa were detected in the blanks and negative controls, and the positive control was identified as *Ficus* to at least genus level across all four markers. Libraries underwent standard quality control at each processing step prior to sequencing. Sequencing was performed on an Illumina MiSeq platform using paired-end 300 bp chemistry, generating a total of 2 × 8,490,998 reads.

Raw sequencing data were processed using a metabarcoding pipeline implemented in QIIME2. Primer sequences were trimmed, reads lacking expected primer motifs were removed,

and amplicon sequence variants (ASVs) were inferred and filtered based on expected amplicon length. Classifiers were trained on the full PLANiTS (Banchi, Ametrano, Greco, et al. 2020; Banchi, Ametrano, Tordoni, et al. 2020) database for ITS1/ITS2 and on NCBI references (Benson et al. 2018) trimmed to the amplified subregion for *rbcL*/*trnL*; taxonomic assignment used classify-sklearn at 95% confidence.

2.2.2 | Pipeline B: Illumina NextSeq With Kraken2 Bioinformatics

DNA was extracted from air filter samples using (zirconium) bead-beating followed by magnetic bead-based purification. Quartz filters and a field blank were aseptically sectioned prior to extraction, with a subset of the field blank spiked with the ZymoBIOMICS Microbial Community Standard (ZymoMix) as a process control; an additional ZymoMix sample was included to validate extraction and sequencing performance. Extracted DNA from replicate filter sections was pooled and quantified using a NanoDrop spectrophotometer.

Shotgun metagenomic libraries were prepared using the Illumina DNA Prep Kit following the manufacturer's protocol, employing bead-linked transposome chemistry for simultaneous fragmentation and adapter ligation. Libraries were amplified using the limited-cycle indexing PCR of the Illumina DNA Prep protocol (68°C 3 min; 98°C 3 min; 5 cycles of 98°C 45 s/62°C 30 s/68°C 2 min; 68°C 1 min). Libraries were then normalized, pooled and quality-checked prior to sequencing. Sequencing was performed on an Illumina NextSeq platform using the NextSeq 550 system high-output kit (2×150 bp), generating a total of 800 million paired-end reads.

Raw sequencing data were quality controlled and processed for taxonomic classification using Kraken2 v2.1.3 (default k-mer length 35) with the PlusPFP database (release date: 23 May 2025) applying a confidence threshold of 0.6, selected to suppress false-positive plant assignments from short reads while retaining full recovery of the ZymoBIOMICS control. Species-level abundance estimates were generated using Bracken. Using the ZymoBIOMICS standard of known composition, we selected the lowest read-count cutoff that removed spurious low-count taxa while retaining all expected community members. This cutoff defined the microbial dataset well but was deliberately not applied to plant taxa as it filtered all plant detections (details in Appendix S1: S1.2).

2.2.3 | Pipeline C: Oxford Nanopore MinION With Kraken2 Bioinformatics

DNA was extracted from two quartz-filters using the DNeasy Blood & Tissue Kit (Cat. No. 69504; QIAGEN). Prior to extraction, the filters were cut into smaller pieces using the UniCore Punch Kit 6.0 mm (Cat. No. WB100040; QIAGEN) and transferred in DNA low-binding tubes to minimize DNA loss. Extraction was carried out following the manufacturer's gram-positive extraction protocol. An unexposed filter blank and a reagent-only control were processed and sequenced alongside the samples. The concentration of eDNA extracted was quantified using

the Qubit 4 Fluorometer (Cat. No. Q33238; Invitrogen) with a dsDNA HS Quantification Kit.

Shotgun metagenomic libraries were prepared using the Rapid PCR Barcoding Kit 24 V14 (Cat. No. SQK-RPB114.24; Oxford Nanopore Technologies) according to the manufacturer's protocol (30-cycle barcoding PCR). Read length, a proxy for fragment length after Rapid-PCR preparation, averaged ~2500–3900 bp for the samples (up to ~15.6 kb) and was much shorter in the reagent blank (mean ~590 bp; median ~80 bp). Sequencing was carried out on the MinION Mk1C system using Flongle Flow Cells (R10.4.1) with MinKNOW software (Oxford Nanopore Technologies, Oxford, UK).

Raw sequencing data from samples were basecalled using Dorado v7.9.8 with the SUP model. Input files were concatenated, and per-read statistics were generated using Fastcat v0.20.0 to provide a detailed overview of the quality and composition of the basecalled reads. Reads below mean quality Q10 were removed. EPI2ME v5.2.5 was used to run the metagenomics v2.13.0 workflow. Taxonomic classification was performed using Kraken2 v2.1.3 (default k-mer length 35) with the PlusPFP database (release date: 23 May 2025), applying a confidence threshold of 0.0, as a higher confidence threshold yielded no plant detections. Bracken estimated species-level abundances (species-level detections only), and taxa supported by fewer than 10 reads were excluded.

2.3 | In Silico Analysis of Detection Potential

In silico reference capability was assessed by comparing all genera and species represented in the classifier databases with the inventory of plants present in the botanical garden. This assessment focused specifically on sequences corresponding to the ITS1, ITS2, *trnL*, and *rbcL* markers used in the amplicon-based Pipeline A. Taxa in the inventory were classified as “detectable” or “non-detectable” depending on whether at least one reference sequence was available for taxonomic assignment. The same coverage assessment was performed for the shotgun PlusPFP database, comparing its taxonomy to the garden and greenhouse inventories to provide a detection baseline for Pipelines B and C.

2.4 | Botanical Garden Collection Lists

The Utrecht Botanical garden keeps a curated list of the plants present in the garden. The total garden list can be found on the website (<https://utrecht.gardenexplorer.org/>) from which a list of species, genera, and families was extracted. In addition, the botanists from the Utrecht Botanical garden were also asked to review species and genera that could not be linked to known occurrences, resulting in some further refinement as some species were detected which could have originated from saplings that did not mature and enter the official collection.

2.5 | General Workflow

For this study, the read data from each sample were loaded in Microsoft Excel allowing easy manipulation and comparison to be made between sample groups and add various taxonomic

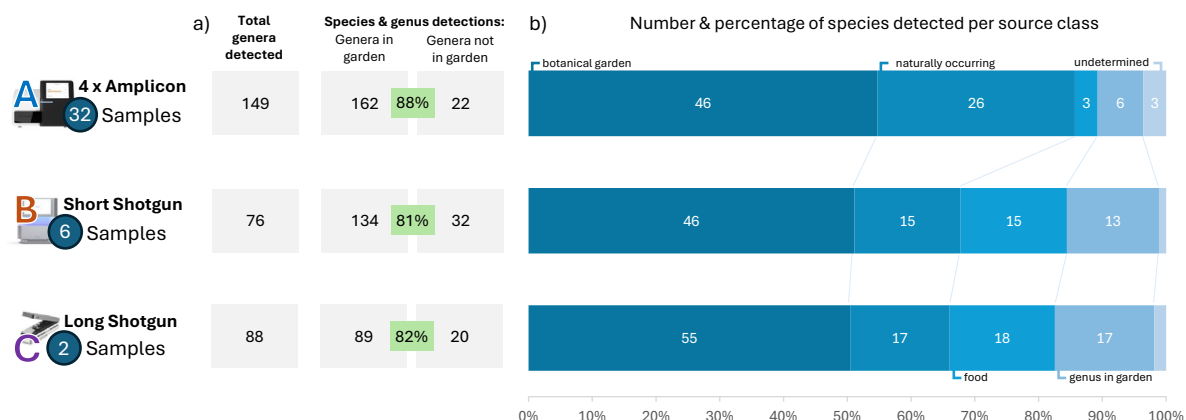


FIGURE 2 | Comparison of inferred source categories for plant taxa detected using three analytical pipelines (A–C) at (a) the genus and (b) the species level. In panel (a), gray bars (left to right) indicate total genera detected, taxa (at species and genus detection) belonging to genera listed in the botanical garden, and taxa belonging to genera not listed in the garden; percentages are shown in green. In panel (b), white numbers indicate total species detections per source category, while blue shading shows normalized percentages, representing species found in the botanical garden, naturally occurring taxa, food-related species, species belonging to garden-listed genera, and taxa of undetermined origin.

levels or read cutoffs. Taxon-presence thresholds differed by pipeline and were each set against a known reference: Pipeline A required ≥ 10 reads per taxon with tag-jump correction ($< 0.1\%$ of total observations); Pipeline C excluded taxa with < 10 reads; for Pipeline B the ZymoBIOMICS-derived cut-off was applied to the microbial community only and deliberately not to plant taxa, which were interpreted conservatively at genus level. No single uniform threshold was applied across pipelines.

To compare sample groups, the results from individual samples were pooled and compared to list of taxa from the botanical garden via automatic comparisons on species or genus name. After this step, taxa not existing in the garden were manually curated to identify potential issues due to synonyms and to determine the most likely source. Non-land-plant (algal) detections were excluded from the analysis. We determined if the species or genus could also naturally occur (also as crop), or if it was commonly used as (an imported) food or lastly, if a species belonged to a genus that did exist in the garden. In the latter case, it was assumed that the likelihood of a misannotation or read error leading to an incorrect species identification was high. To highlight the importance of detecting such cases, we categorized these species calls separately as “genus in garden.” Overall, the data analysis and comparative studies initially focused on the genus level, but as more than 80% of the genera could be matched to those in the garden a further comparison to species level was conducted.

3 | Results

Across all samples (40) and analytical pipelines (3), we detected a total of 192 different species, 209 genera, spanning 85 families. As of October 2025, the botanical garden documented 8136 plant species (9080 cultivars), representing 2123 genera across 324 families. In silico evaluation of the amplicon and shotgun pipeline (the combined marker set ITS1, ITS2, *rbcL*, and *trnL*; and the plusPFP database) could theoretically classify 3039

species in the garden (37%) and 1818 genera (86%); for the greenhouse collection, this is 34% of the species and 78% of the genera.

3.1 | Identifying the Likely Sources of Airborne Plant eDNA

After manual curation of taxonomic assignments, Pipeline A (amplicon-based) detected the highest number of different genera (149), followed by Pipeline C (long-read shotgun; 88) and Pipeline B (short-read shotgun; 76). Most taxonomic detections (species and genera) across all pipelines could be attributed to genera present in the botanical garden, with attribution rates of 88% for Pipeline A, 81% for Pipeline B, and 82% for Pipeline C (Figure 2a). When pooling the detections of all samples, Pipeline A detected 128 different genera from the garden, Pipeline B 61, and Pipeline C 71.

At the species level, all pipelines showed similar source distributions (Figure 2b). Approximately half of detected species corresponded to taxa documented in the garden's living collection, with the remaining detections primarily attributable to naturally occurring Dutch flora, food-related species, or species belonging to genera present in the garden. Only a small number of species across pipelines could not be assigned to a plausible source.

3.1.1 | Detected Food-Related Species

Several of the food-related species detected correspond to commonly consumed raw vegetables or greens, including *Cucumis sativus* (cucumber), *Cucurbita pepo* (pumpkin/zucchini), *Solanum lycopersicum* (tomato), *Brassica oleracea* (cabbage/broccoli), *Spinacia oleracea* (spinach), and *Vigna radiata* (mung bean sprouts). We also detected species typically encountered in more processed food products, such as *Manihot esculenta* (cassava), *Cicer arietinum* (chickpea), *Vigna angularis* (adzuki bean), *Mucuna pruriens* (a legume widely used in dietary supplements),

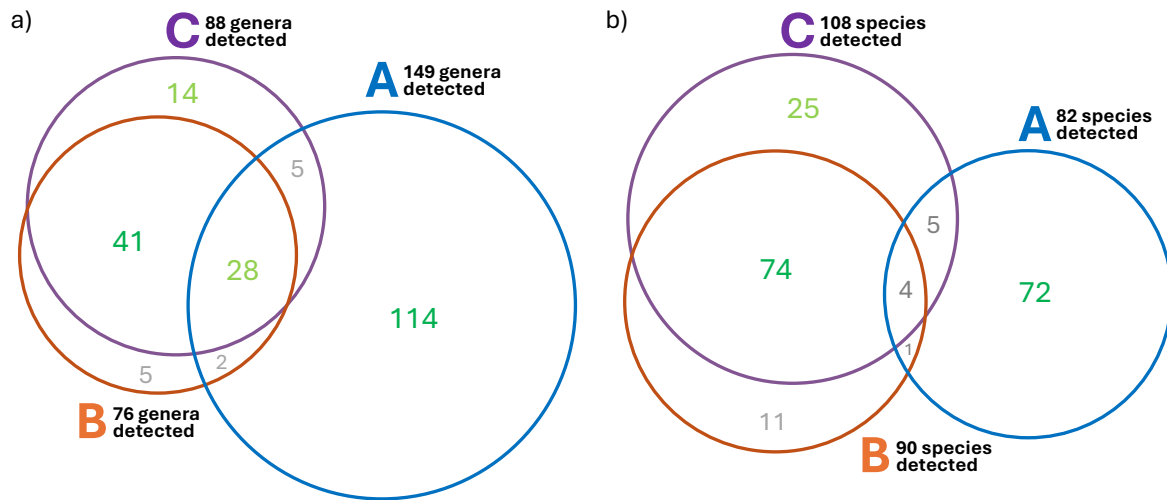


FIGURE 3 | Venn diagrams showing overlap in detected taxa across pipelines at (a) genus level and (b) species level (circle and overlap areas are schematic and not drawn to scale). At the genus level, 28 genera were shared across all pipelines, with substantial overlap between the shotgun approaches (41 genera shared by Pipelines B and C). Pipeline A uniquely detected 114 genera, whereas Pipelines B and C detected 5 and 14 unique genera, respectively. A similar pattern was observed at the species level: only four species were detected by all pipelines, 74 were shared between Pipelines B and C, and unique detections comprised 72 species for Pipeline A, 25 for Pipeline C, and 11 for Pipeline B.

and *Triticum aestivum* (wheat). In addition, an exotic ornamental species commonly used in floral arrangements, *Telopea speciosissima*, was also placed in this category. Most food-related taxa were detected in more than one sample, indicating these were not isolated contaminations or detections.

3.1.2 | Manual Review of Unknown Detections

To investigate unknown detections, the five unassigned species (*Tetrataenium yunnanense*, *Mucuna macrocarpa*, *Phanera variegata*, *Hevea brasiliensis*, and *Rhodamnia argentea*) were reviewed with botanical staff. Three of these taxa were subsequently explained:

- *Phanera variegata* is a synonym of *Bauhinia variegata*, and *Bauhinia* is present in the garden but not listed on the public website.
- *Mucuna macrocarpa* had been cultivated in the garden until July 2022, suggesting the detection may reflect older or residual DNA.
- *Hevea brasiliensis* may have been present as a seedling, which did not survive to be added to the formal collection.

3.2 | Comparison of Taxonomic Recovery Across Analytical Pipelines

To compare the performance of the three analytical pipelines, we assessed the overlap in detected taxa at both genus and species levels (Figure 3a,b). Overall, the two shotgun-based pipelines (B and C) showed broadly similar detection profiles and exhibited strong complementarity with the amplicon-based pipeline (A). PlusPFP's land plant content is small (~100 genera,

~130 species, overwhelmingly crops) and largely a subset of the amplicon barcode references reviewed here, sharing 94 genera and 109 species. It can theoretically classify 78 garden genera and 62 garden species, compared to 1818 genera and 3035 species detectable via the amplicon pipeline.

3.3 | Sampling Height and Particle Size Distribution of Airborne Plant eDNA

Samples collected at 1.5 and 3.0 m using the TSP sampler and analyzed with Pipeline A showed no substantial differences in total or average taxonomic detections. Consistent with this, PCoA based on species and genus-level detections (Figure 4a) showed only weak separation between height groups, with extensive overlap and low explanatory power (< 5%–10% variation on each axis).

To examine the size fractions contributing to airborne plant eDNA at our study site, we used (i) a 13-stage impactor covering the 0–10 µm range and (ii) a filter-mesh stack designed to capture particles from ~2.2 to > 100 µm.

3.3.1 | Fine Particle Fractions (0–10 µm) From the 13-Stage Impactor

Analysis of impactor samples using the amplicon pipeline (Pipeline A) revealed a pronounced peak in taxonomic detections in the 2.5 µm fraction (Figure 4b). Elevated detections were also observed in neighboring stages (1.6 and 4.4 µm).

The taxonomic composition of the 1.6–4.4 µm fractions generally matched the taxa detected by the concurrently collected quartz-filter samples at 1.5 m and also clustered closely to the 3 m samples (Figure 4a).

3.3.2 | Coarse Particle Fractions From the Filter-Mesh Stack

To evaluate contributions from larger particles, we analyzed a filter-mesh stack targeting > 2.2, > 40, > 70, and > 100 μm fractions (Figure 5a). Plant DNA was consistently detected on the 40 and 100 μm meshes and, to a lesser extent, the 70 μm mesh (Figure 5b).

Particles < 40 μm contributed substantially to the airborne plant eDNA signal. When compared to the average genera detected in

the two control filters (no mesh), approximately 63% of genera were recovered in the < 40 μm fraction alone.

Reviewing the taxa detected in the < 40 and > 40 μm fractions did not reveal strong differentiation between potential source classes (e.g., greenhouse vs. outdoor garden vs. natural flora vs. food-related). However, a subset of taxa was detected in the control filters and at least one coarse mesh fraction, but not in the final quartz filter of the filter-stack, suggesting attribution to particles > 40 μm and a robust signal detected in three sampling heads. These taxa included the species *Helianthus annuus*,

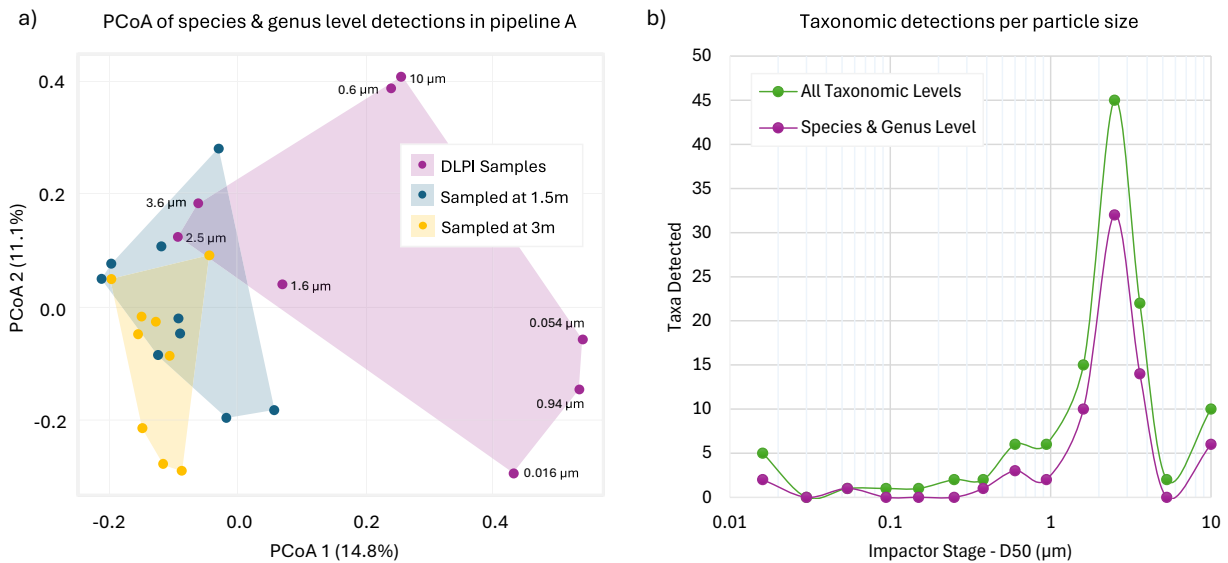


FIGURE 4 | (a) Principal coordinate analysis (PCoA) based on species and genus-level detections (Pipeline A; combined data from all four primer sets), showing separation among three sample groups indicated by shaded colors. DLPI samples are annotated with their corresponding D50 particle size fractions. (b) Taxonomic detections (all and species and genus level) across D50 particle-size classes collected by the 13-stage impactor and analyzed using the amplicon pipeline (also Pipeline A). A clear peak is observed at ~2.5 μm .

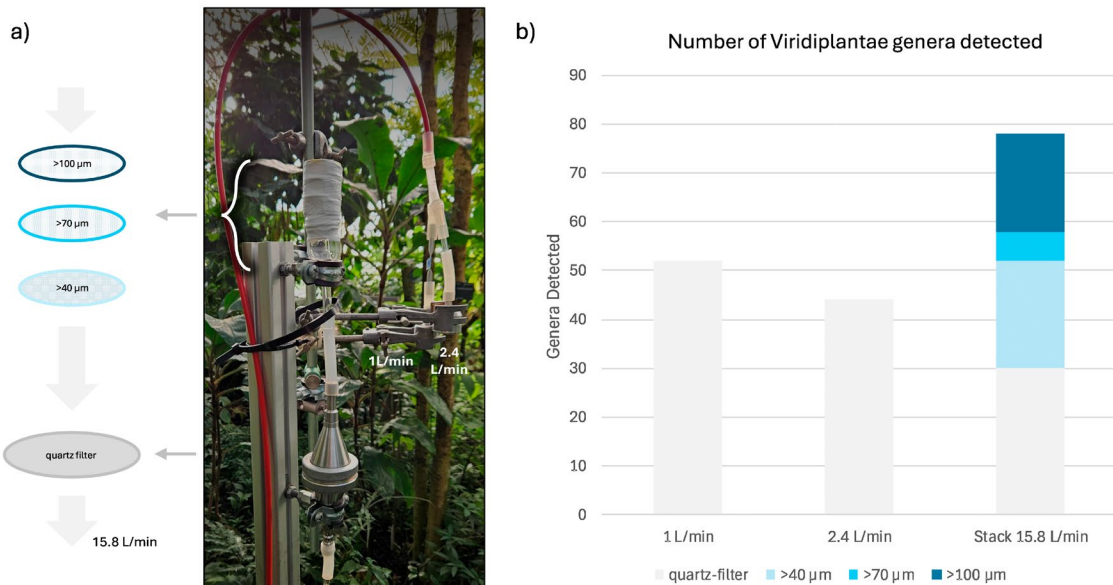


FIGURE 5 | (a) Schematic and photograph of the filter-mesh stack. (b) Genera detected across the mesh stack (four fractions combined) compared with two control filters. Genera represent total taxa per sample; the stacked bar thus aggregates data from all four laboratory analyses and does contain duplicate taxonomic classifications.

Cucurbita pepo, *Diospyros lotus*, *Gossypium hirsutum*, *Papaver somniferum*, *Sorghum bicolor* (which were all outdoor) and the genera *Phoenix* (two species outdoors; one greenhouse species), *Lotus* (*L. maritimus* outdoors) and *Medicago* (three species outdoors).

4 | Discussion

This study demonstrates that airborne eDNA can reliably recover a meaningful proportion of local plant biodiversity within a complex botanical setting, despite a restricted sampling window outside of pollen season, limited air volumes, and heterogeneous laboratory methods. Below, we discuss the performance of our approach, the complementarity among analytical pipelines, and the implications for understanding particle sizes, transport dynamics, and source attribution.

4.1 | Expected Performance and Practical Constraints

Global reference availability is an important factor in determining the taxonomic resolution achievable with eDNA-based plant monitoring. Although hundreds of thousands of land plant species and roughly 13,000 genera are known (Freiberg et al. 2020; Govaerts et al. 2021), and about a quarter of species currently have at least one barcode for metabarcoding (Kartzinel et al. 2025). Our in silico evaluation (Section 2.3) indicated that 86% of all genera present in the botanical garden could, in principle, be detected using the four amplicon markers deployed, whereas species-level resolution remained restricted at 37% coverage. Our in silico analysis thus provided a similar order of magnitude for species-level coverage reported by Kartzinel et al. (2025). We extended the same assessment to the shotgun pipelines by screening the PlusPFP database. In contrast to the amplicon markers, PlusPFP contained a reference for only 78 garden genera (3.7%) and 62 garden species (0.8%). Shotgun detection of local flora is therefore far more severely reference-constrained than amplicon sequencing, the binding limit being the availability of complete reference genomes, which exist for only a small and economically biased set of plants (i.e., crops).

Within this constraint, airborne eDNA performed well. Across the three pipelines, we detected 171 of 1818 detectable botanical garden genera and 88 of the 3039 detectable species based on the in silico analysis (combining the four amplicon primers and PlusPFP). Thus capturing 9% or 3% of the theoretically detectable and local plant biodiversity at genus or species level, respectively. While these proportions are modest, plant eDNA shedding is likely asynchronous, and highly seasonal, and winter sampling likely represents a conservative scenario for airborne plant eDNA detection. Given the short sampling window, the modest air volumes, and the single indoor sampling location (with open windows), these proportions thus represent a promising baseline for future work.

The plants in the greenhouse had slightly lower in silico reference capability compared to the overall garden (78% of genera and 34% of the species were potentially detectable) reflecting slightly poorer barcode representation of its predominantly

tropical families (e.g., Araceae, Bromeliaceae, Orchidaceae). Detectability is therefore likely to be shaped more by reference completeness, than by proximity to the sampler. Because the shotgun database adds very limited plant coverage beyond the amplicon markers, combining pipelines broadens which taxa are recovered (Figure 3) rather than raising the ceiling of what is theoretically detectable. In addition, we expect that more extensive biodiversity recovery will require broader spatial coverage, increased sampling volume or samplers, and temporal replication, particularly across seasons.

4.2 | Source Attribution and the Role of Food-Derived DNA

For Pipeline A, 88% of detected genera and 55% of detected species corresponded to taxa known from the botanical garden, with an additional 26% attributed to flora naturally occurring outside the garden. Pipeline B and C have slightly lower values as 80%–81% of detected genera and 50%–51% of detected species corresponded to taxa known from the botanical garden. For all pipelines, a subset of detections originated from dietary plants, a pattern also observed in earlier airborne eDNA studies from human-modified environments (Clare et al. 2022; Lynggaard, Bertelsen, et al. 2022). These are likely to reflect food handling or visitor activity and emphasize the need to consider anthropogenic DNA contributions in public settings. This food signal is amplified by the database itself: PlusPFP is dominated by crop genomes, so the garden taxa the shotgun pipelines can resolve are disproportionately food plants (e.g., *Vigna unguiculata*, *Mangifera indica*, *Cucumis sativus*), so their prominence ($\approx 15\%$ – 18% of shotgun detections versus $\approx 3\%$ for amplicon; Figure 2) partly reflects this bias rather than a proportionally higher airborne abundance.

In addition to living plants and food-derived inputs, garden management practices may represent a secondary source of airborne plant eDNA. The botanical garden uses organic management in which large volumes of leaf litter from the surrounding area are processed into mulch and redistributed across the garden, including within the greenhouse. This mulch may retain residual DNA from diverse plant taxa and contribute to airborne eDNA through resuspension of fine organic particles. Such recycling of biomass complicates detailed assessment of source distance for specific species present in the garden.

Fewer than 20% of species-level detections lacked an immediately obvious origin, and most of these belonged to genera known to occur in the garden but were likely affected by misreadings, misannotations, or synonymy. Of particular interest is the detection of *Mucuna macrocarpa*, which was not on the official garden species list but for which the garden had a live specimen up to 2022. Detecting this plant's DNA in the garden air suggests that a plant's airborne DNA emissions can persist for several years after the presence of a live plant in an area. However, we cannot exclude incomplete removal of plant debris (mulch) or misclassification with the closely related *Mucuna pruriens*, a species used as a food supplement.

When naturally occurring flora, food-related taxa, taxonomic corrections, and curator insights (e.g., unlisted accessions or

removed plants) were incorporated, only three species across all pipelines could not be linked to a plausible source. This high rate of source attribution underscores the robustness of airborne eDNA for identifying local plant biodiversity, especially at the genus level. It also stresses the importance of manual curation and review of detected taxa to determine the accuracy of detections.

4.3 | Analytical Pipeline Considerations and Complementarity

Accurate characterization of airborne plant eDNA presents inherent analytical challenges. Many plant lineages exhibit limited sequence divergence in commonly used barcode regions, while hybridization and recent speciation can result in shared or near-identical sequences among closely related taxa. These biological constraints are further compounded by incomplete reference databases and by the short, fragmented DNA molecules typical of airborne eDNA, limiting species- and, in some cases, even genus-level resolution regardless of sequencing platform.

Against this background, clear qualitative differences emerged among the three analytical pipelines. The amplicon-based Illumina MiSeq workflow (Pipeline A) recovered the broadest range of genera, driven in part by the larger number of samples analyzed but also likely reflecting the sensitivity of targeted amplification and the extensive use of curated plant barcode markers. However, this broader genus-level recovery did not translate into consistently higher species-level resolution, likely due to conservative, confidence-based taxonomic assignment and the limited discriminatory power of short barcode regions for closely related plant taxa.

The two shotgun pipelines (Illumina NextSeq, Pipeline B; and Oxford Nanopore MinION, Pipeline C) provided complementary insights to Pipeline A (Figure 3). Despite differences in sequencing technology and read length, both shotgun approaches produced strikingly similar taxonomic profiles. The much smaller number of shotgun samples (six and two) limits direct quantitative comparison, and detected diversity also reflects DNA input, read depth, dilution of plant DNA within a large microbial background and library complexity. Yet garden-genus recovery already approaches the ceiling set by reference coverage as Pipelines B and C detected 61 and 71 of the 78 garden genera with a PlusPFP reference. This indicates currently the main constraint on recovering local flora using shotgun sequencing is reference completeness rather than sampling effort. Although the sampling events were separated by approximately 7 weeks, both occurred during the winter season, suggesting that airborne plant eDNA signals may be relatively stable during this period and robust to methodological variation. This convergence may also reflect the shared reliance on the PlusPFP reference database and the recovery of overlapping components of the airborne plant community, even when individual taxa differed among pipelines.

Importantly, the shotgun pipelines required platform-specific analytical adjustments. For example, standard Kraken2 confidence thresholds commonly applied in metagenomic studies (0.3–0.5) yielded no plant detections, whereas lower thresholds

produced largely plausible genus- and species-level assignments for the Nanopore-based pipeline, while a higher confidence threshold (0.6) was appropriate for the Illumina-based shotgun data. This discrepancy reflects the fact that Kraken2 confidence scores depend on read length and sequencing error profiles and are therefore not directly comparable between Illumina and Nanopore data. Similar trade-offs between strong taxonomic recovery and elevated false positives in plant/pollen shotgun sequencing have been reported previously when using Kraken-based classification with public plant genome references (Bell et al. 2021). In addition, read-count thresholds derived from the ZymoBIOMICS microbial control were suitable for microbial taxa but eliminated nearly all plant detections when applied directly, underscoring the extremely low abundance and high fragmentation of plant-derived airborne eDNA. Consequently, plant detections were interpreted separately and conservatively, with primary emphasis placed on genus-level patterns and secondary evaluation of species-level calls.

Because Pipelines B and C share a common shotgun/Kraken2 basis, their parameters follow the same logic and differ only where the platforms require it, whereas Pipeline A is amplicon-based; each key threshold was set against a known reference (the positive control, the ZymoBIOMICS standard, or standard Kraken2 behavior), and a full multiparameter sensitivity analysis across all datasets is identified as a priority for future work.

While airborne plant eDNA has previously been studied using targeted amplification and metabarcoding approaches (Baksay et al. 2020; Banchi, Ametrano, Greco, et al. 2020; Banchi, Ametrano, Tordoni, et al. 2020; Polling et al. 2022; Tegart et al. 2024) and shotgun-based approaches have only recently emerged (Giolai et al. 2024; Mikko et al. 2025), this study is, to our knowledge, the first to directly compare amplicon-based and shotgun sequencing approaches for airborne plant eDNA within a controlled environment and against known species occurrences. Although sample sizes were limited, our results indicate that untargeted shotgun sequencing can attribute a comparable proportion of its detections to locally present flora (Figure 2), while yielding partly distinct taxonomic detections (Figure 3). Differences among pipelines therefore likely reflect complementary analytical perspectives on the same underlying signal, constrained primarily by bioinformatic classification strategies and reference database coverage rather than by sequencing technology alone.

Overall, these findings highlight that airborne plant eDNA analysis benefits from the use of multiple sequencing approaches and from tailoring analytical workflows to the properties of both the sample and the sequencing platform, rather than applying uniform thresholds or classification parameters. Platform-specific confidence settings and control-informed filtering are critical for balancing sensitivity and reliability, and for maximizing the biological interpretability of airborne eDNA data.

The final considerations are costs and computational requirements. Amplicon sequencing is typically the most cost-efficient approach (~€80–200 per sample: extraction ~€30–60, PCR/library prep ~€20–80, sequencing ~€20–100, bioinformatics ~€20–50). Long-read shotgun ranges from €175–380 per sample

(extraction ~€10–30, library prep ~€20–130, sequencing ~€70 for a Flongle Flow Cell, bioinformatics ~€75–150). Short-read shotgun is more costly (~€200–600 per sample), dominated by library preparation (~€80–200) and sequencing (~€100–400), with similar bioinformatic demands (~€75–150). All pipelines require a one-time hardware investment. Computationally, Kraken2/PlusPFP classification requires a high-memory server, the QIIME2 amplicon workflow runs on a standard workstation, and long-read basecalling benefits from a GPU.

4.4 | Particle Size Classes Carrying Airborne Plant eDNA

Identifying the particle size fractions that transport airborne plant eDNA is central to understanding dispersal, deposition, and source attribution. Traditional aerobiology emphasizes pollen grains (typically 10–100 μm) as the dominant airborne plant propagules, yet pollen rupture and fragmentation can generate smaller pollen-derived particles below 10 μm (Fragola et al. 2022; Mampage et al. 2022; Taylor et al. 2002). Consistent with emerging airborne eDNA studies, our data demonstrate that plant eDNA is not restricted to intact pollen grains but is frequently associated with finer particle fractions, possibly not directly associated with pollen.

Impactor sampling revealed a pronounced peak in taxonomic detections in the sub-pollen size range, particularly around 2.5 μm , with elevated detections also observed in adjacent fractions (1.6–4.4 μm). These fractions largely mirrored the taxonomic composition detected on concurrently collected quartz filters, indicating that fine particles broadly represent the dominant airborne plant eDNA signal. Together, these results support the hypothesis that airborne plant eDNA is often associated with fragmented plant material or extracellular DNA rather than intact pollen alone, consistent with previous metabarcoding and airborne eDNA studies (Banchi, Ametrano, Greco, et al. 2020; Banchi, Ametrano, Tordoni, et al. 2020; Klepke et al. 2022).

Filter-stack data further showed that particles <40 μm contributed substantially to the detected plant eDNA pool, recovering approximately 63% of genera observed in control quartz filters without size selection. Larger particle fractions (>40 μm) also yielded plant DNA, including detections on meshes up to 100 μm , suggesting that aggregated debris or larger biological particles may contribute to airborne plant eDNA. However, detections in coarse fractions did not indicate preferential capture of taxa located closest to the sampling point or restricted to the greenhouse interior, and the limited sample size precludes firm conclusions regarding source proximity or transport distance for large particles.

Sampling was conducted during winter (December–February) to minimize direct pollen emission and focus on background airborne plant eDNA. While some taxa (e.g., *Corylus avellana* and *Alnus glutinosa*) may flower as early as February, only *A. glutinosa* was detected in the >100 μm mesh fraction, with concurrent detection on downstream quartz filters indicating that smaller particles (<40 μm) contributed to this signal. Detection of this species in December samples and across multiple pipelines further suggests that direct pollen emission was not the

dominant source. To ensure that pollen-derived DNA was not systematically underrepresented, extraction protocols included mechanical and enzymatic disruption steps known to lyse the pollen exine and release DNA (Edwards et al. 1991; Kraaijeveld et al. 2015).

Notably, up to 16% of detected plant taxa were attributable to food sources, providing additional evidence that airborne plant eDNA includes particles not directly associated with pollen. Food-derived plant material is frequently processed, fragmented, and abraded during handling, preparation, and consumption, generating fine particulates that can become aerosolized. Such processes likely contribute plant tissue fragments and extracellular DNA to the airborne eDNA pool and may also occur in nature caused by animals and wind and thus offer a plausible mechanistic explanation for the prominence of sub-pollen particle size fractions in our data.

4.5 | Effect of Sampling Height on Airborne Plant eDNA Composition

Sampling height did not differentiate inferred source categories nor support the hypothesis that lower samplers preferentially captured nearby vegetation while higher samplers reflected more distant or well-mixed sources. The vertical separation in our setup (1.5 m) was limited and primarily intended to inform future sampling design, being constrained by the greenhouse dimensions; larger vertical gradients may therefore be required to detect height-dependent effects. In this dataset, sampling height had no detectable influence on airborne plant eDNA composition, consistent with (i) a well-mixed indoor air column, (ii) evidence that multiple particle size fractions contribute to a broadly similar plant eDNA signal, and/or (iii) sampling below the vertical resolution at which stratification driven by particle size-dependent settling is expected to occur (Rojo et al. 2019). Airborne plant eDNA likely entered the greenhouse via multiple pathways, including ventilation, doors, and human movement, suggesting substantial air mixing, although the alternative explanations cannot be excluded.

4.6 | Implications for Transport Modeling and Biomonitoring

Our results indicate that airborne plant eDNA is frequently associated with particle sizes smaller than intact pollen grains, suggesting that pollen-based dispersal models may not fully capture the transport dynamics of plant-derived DNA in air. Particles in the ~2.5 μm size range are known from aerosol and particulate matter research to remain suspended for extended periods and to be transported over longer distances than larger particles, although most empirical work in this size range has focused on inorganic particulates or evaporative aerosols rather than biological material. How closely plant-derived particles conform to these transport behaviors remains poorly constrained.

At the same time, pollen itself is capable of long-distance dispersal under suitable meteorological conditions, and long-range transport of pollen has been well documented (Skj  th et al. 2007; Sofiev et al. 2013). Within the spatial scale of the botanical

garden and given the limited temporal and spatial replication in this study, our data do not allow robust inference of transport distances or source proximity as a function of particle size. For these low-biomass aerosol samples we also did not treat read counts or repeat detections as quantitative proxies for biomass, as they are also affected by marker copy number, primer affinity, genome size, and reference completeness. The dominance of taxa known from the immediate surroundings therefore cannot be directly interpreted as evidence for short-range transport, nor do detections of nonlocal taxa necessarily imply long-distance dispersal.

Rather, our findings suggest that airborne plant eDNA integrates signals across multiple particle size classes, potentially combining contributions from both locally abundant sources and more broadly transported material. This highlights the need for future transport models of airborne plant eDNA to consider a wider range of particle sizes than intact pollen alone, while also accounting for the distinct physical properties and biological origins of plant-derived particles. Additional empirical data linking particle size, atmospheric residence time, and taxonomic composition will be required before airborne plant eDNA can be reliably interpreted in a spatially explicit biomonitoring framework and additional spatially distributed samplers could help disentangle biomass, particle size, and transport distance in future work.

5 | Conclusion

This study provides a comprehensive assessment of airborne plant eDNA within a complex botanical garden setting and demonstrates that airborne eDNA can recover a meaningful and ecologically interpretable subset of local plant biodiversity, even under constrained sampling conditions. Across three distinct analytical pipelines, the majority of detections could be plausibly linked to known local sources following manual curation, highlighting the robustness of airborne eDNA for qualitative biodiversity assessment at the genus and, to a more limited extent, species level.

Comparison of amplicon-based and shotgun sequencing workflows revealed strong complementarity among approaches. Targeted metabarcoding recovered the broadest range of genera, while untargeted shotgun methods captured overlapping but partly distinct taxonomic signals, despite differences in sequencing technology and analytical parameters. These results underscore that no single workflow fully captures the airborne plant eDNA signal and that multi-method strategies provide a more complete and resilient representation of airborne biodiversity. Notably, the shotgun pipelines' recovery of garden taxa is bounded by reference-genome availability, so within that ceiling detection was efficient, and their apparent food-plant emphasis partly reflects this crop-biased reference coverage rather than airborne abundance alone.

A key finding of this study is that airborne plant eDNA is frequently associated with fine particle size fractions ($<10\mu\text{m}$), including sizes substantially smaller than intact pollen, while larger particles also contribute to the overall signal. This multi-size distribution suggests that pollen-centric frameworks alone

may not fully describe the physical carriers of airborne plant DNA and that fragmented plant material and extracellular DNA play an important role. Consequently, sampling and the interpretation of airborne plant eDNA require consideration of multiple particle classes with potentially different, release, transport, deposition, and sampling characteristics.

Although the original experimental scope focused on the greenhouse interior, the detection of numerous taxa known from the surrounding botanical garden revealed that airborne plant eDNA integrates signals beyond the immediate sampling location. This unanticipated result prompted an expansion of the interpretive scope to include both indoor and nearby outdoor sources, while remaining cautious about inferring transport distances given the limited spatial and temporal replication.

Taken together, these findings position airborne plant eDNA as a promising, noninvasive tool for plant biodiversity monitoring that bridges aerobiology, molecular ecology, and atmospheric science. Future work incorporating expanded spatial and temporal sampling, improved and regionally curated reference databases, and particle-resolved transport modeling will be essential to fully resolve source–distance relationships and to integrate airborne eDNA into quantitative biomonitoring and ecological forecasting frameworks.

Author Contributions

Willem J. van Strien: data curation, writing – original draft, visualization, writing – review and editing. **Joyce Fabriek:** methodology, investigation (sampling and lab work), writing – review and editing. **Ilka M. F. Marissen:** formal analysis, investigation (sampling and lab work), writing – review and editing. **Nicole D. Plomp:** formal analysis, investigation (sampling and lab work), writing – review and editing. **Daniel Pinto-Neves:** investigation (sampling and lab work), data curation, writing – review and editing. **Vanessa Zuzarte-Luís:** investigation (sampling and lab work), writing – review and editing. **João A. Rodrigues:** data curation, writing – review and editing. **Hans C. van Leeuwen:** writing – review and editing. **Henrik C. van de Ven:** conceptualization, methodology, investigation (sampling and lab work), writing – review and editing, supervision, project administration, funding acquisition.

Acknowledgments

We thank the staff of the Botanical Garden for providing access to greenhouse facilities and supporting the setup of sampling equipment. We also acknowledge the TNO and SGS technical and research staff for assistance with sampler deployment and sample processing.

Funding

This work was privately funded by TNO (The Netherlands Organisation for Applied Scientific Research). No public or external grant funding was used.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data supporting the findings of this study, including the raw FASTQ files and the full list of taxa detected in each sample (Excel format), have been made publicly available on Zenodo via <https://doi.org/10.5281/zenodo.18086271>.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Appendix S1:** Supplementary methods.