Contents lists available at ScienceDirect

Science of the Total Environment

Review

Airborne DNA: State of the art – Established methods and missing pieces in the molecular genetic detection of airborne microorganisms, viruses and plant particles

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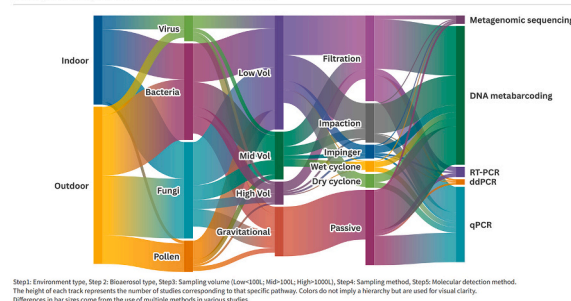 E-mail address: clara.pogner@ait.ac.at (C.-E. Pogner).

HIGHLIGHTS

- Combined systematic and scoping review approach
- Evaluation of studies using molecular genetic analysis for bioaerosols
- Comprehensive overview of used and appropriate techniques for biological targets
- Identification of gabs in knowledge, data publication and reference datasets
- Compilation of requirements for publication of data and easy access of results

GRAPHICAL ABSTRACT

Overview of bioaerosol sampling and molecular detection methods
Sankey flow diagram based on 178 worldwide studies

Step1: Environment type, Step 2: Bioaerosol type, Step3: Sampling volume (Low<100L; Mid>100L; High>1000L), Step4: Sampling method, Step5: Molecular detection method. The height of each track represents the number of studies corresponding to that specific pathway. Colors do not imply a hierarchy but are used for visual clarity. Differences in bar sizes come from the use of multiple methods in various studies.

ABSTRACT

Bioaerosol is composed of different particles, originating from organisms, or their fragments with different origin, shape, and size. Sampling, analysing, identification and describing this airborne diversity has been carried out for over 100 years, and more recently the use of molecular genetic tools has been implemented. However, up to now there are no established protocols or standards for detecting airborne diversity of bacteria, fungi, viruses, pollen, and plant particles. In this review we evaluated commonalities of methods used in molecular genetic based studies in the last 23 years, to give an overview of applicable methods as well as knowledge gaps in diversity assessment. Various sampling techniques show different levels of effectiveness in detecting airborne particles based on their DNA. The storage and processing of samples, as well as DNA processing, influences the outcome of sampling campaigns. Moreover, the decisions on barcode selection, method of analysis, reference database as well as negative and positive controls may severely impact the results obtained. To date, the chain of decisions, methodological biases and error propagation have hindered DNA based molecular sequencing from offering a holistic picture of the airborne biodiversity.

Reviewing the available studies, revealed a great diversity in used methodology and many publications didn't state all used methods in detail, making comparisons with other studies difficult or impossible.

To overcome these limitations and ensure genuine comparability across studies, it is crucial to standardize protocols. Publications need to include all necessary information to enable comparison among different studies and to evaluate how methodological choices can impacts the results.

Besides standardization, implementing of automatic tools and combining of different analytical techniques, such as real-time evaluation combined with sampling and molecular genetic analysis, could assist in achieving the goal of accurately assessing the actual airborne biodiversity.

1. Introduction

Monitoring of biological particles in the air has been performed for >160 years (Marple, 2004) and its importance has been widely pointed out. Research interests include various fields from the environmental, ecological and agronomic point of view (Fröhlich-Nowoisky et al., 2016; Aguilera and Ruiz-Valenzuela, 2019; Bogawski et al., 2019; Stern et al., 2021) as well as from a human health perspective (Marchetti et al., 2017; Kim et al., 2018; Tao et al., 2022) due to airborne allergens and pathogens such as pollen, fungi, bacteria and viruses. Relevant environments include indoor and outdoor air, ranging from private over public and occupational settings and from ground level into the higher atmosphere. Recent publications have even showed the possibility to collect animal DNA through air sampling, providing insight into macroorganism diversity (Yoo et al., 2017; Lear et al., 2018; Clare et al., 2022; Roger et al., 2022; Johnson et al., 2023). Given its health implications, a primary focus of aerobiological and allergological studies is the analysis of pollen grains and fungal spores in the air, with particular emphasis on allergenic taxa (Buters et al., 2012). Modern epidemiological studies from various countries indicate that currently 15–20 % of the population suffers from allergic diseases (Holst et al., 2020). Among those with allergies, approximately 50 % are sensitized to pollen allergens, while around 30 % are sensitized to fungal spores, with even higher rates observed in children (Dharmage et al., 2019; Gutowska-Ślesik et al., 2023).

One of the leading causes of worldwide allergies are grass pollen, though not all species are allergenic. Presently only using DNA analysis makes it possible to accurately distinguish different grass pollen species in air samples (Brennan et al., 2019; Krinitsina et al., 2023).

Another important application of aerobiological sampling is agriculture, and the relative plant health, in particular food production security. Fox example, monitoring the abundance of *Olea* pollen is important in relation to crop production (Orlandi et al., 2020) and monitoring of *Ambrosia* pollen is an efficient tool in relation to pest control of the invasive ragweed (Schaffner et al., 2020). Besides, a large range of fungal spores are known to have a devastating impact on both agricultural crops (Dean et al., 2012) and forestry (Gomdola et al., 2022), and a similar impact is seen for bacteria (Mansfield et al., 2012). Some of these pathogens are transmitted through the air and are opportunistic while others are host specific. Monitoring is therefore of importance in programmes mitigating further spread of diseases (Isard et al., 2011). However, monitoring airborne fungi and bacteria can be very challenging, as many are very difficult to identify accurately.

1.1. Analytical methods of bioaerosols

Off-line and on-line methods are available for the analysis of bio-aerosols (pollens, spores, viruses, bacteria), as it has been already described in previous reviews (Ghosh et al., 2015; Huang et al., 2024). All these analytical methods have advantages and disadvantages, and some limitations can be overcome by analysis of the molecular genetic information. The methods used include optical, spectroscopic, immunological, culture based and molecular genetic approaches.

The standard microscopic morphological identification approach is widely used in aerobiological networks (Buters et al., 2018). It relies on visual identification of morphological species-specific features such as size, form, apertures, and cell wall ornamentation allowing taxonomic identification of pollen to the family or genus level. However, it is time-consuming, requires a trained palynologists and struggles to distinguish key allergen producing taxa like *Phleum* or *Dactylis* (Huffman et al., 2020). Identification of fungi, even for experienced mycologists, is limited due to the lack of evident morphological differences between fungal spores. Moreover, without staining, small biological particles such as bacterial cells may be unrecognizable using a light microscope, and the latter is ineffective for detecting viruses, visible only using electron microscopy. Recent advances in automated digital image recognition have improved the throughput of pollen and fungal spore identification (Benyon et al., 1999; Ranzato et al., 2007; Holt and Bennett, 2014). Image-based automatic identification is now operational (Sevillano et al., 2020) and real-time automatic pollen capture and recognition using laser scattering, image processing and machine learning are used in monitoring networks (Oteros et al., 2015; Sauvageat et al., 2020; Buters et al., 2022; Erb et al., 2023).

While these systems provide immediate data, they do not identify small bioaerosols. In contrast, off-line molecular genetic identification approaches offer high accuracy, reliable identification on the family, genus, and species level (de Vere et al., 2012). In addition, the methods can be used to quantify fungi or bacteria in whole samples.

Spectral properties and biochemical composition may also be used to discriminate between genera or species (Zimmermann et al., 2015). Techniques like fluorescent spectroscopy (Forde et al., 2019; Zhang et al., 2019), Fourier transformation infrared spectroscopy (FTIR; Muthreich et al., 2020; Zimmermann et al., 2015), and RAMAN spectroscopy (Wang et al., 2015a) have been tested for automatic identification of pollen, fungal spores, and bacteria. Auto-fluorescence has been used for the online detection, identification and quantification of pollen and fungal spores (Huffman et al., 2020) and can be combined with other detection methods for improved classification of bioaerosols (Erb et al., 2024; Pogner et al., 2024). While promising, these techniques need further validation for routine bioaerosol detection and in contrast to molecular genetic identification methods, lack high taxonomic resolution at the species level.

Immunological techniques, such as enzyme-linked immunosorbent assay (ELISA), use allergen-specific antibodies to detect and quantify antigens in bioaerosols, but do not identify species (Sander et al., 2012; Buters et al., 2015; Grewling et al., 2020). These methods are limited by the need for available antibodies, and pre-identified targets (Grewling et al., 2023). They also require intact allergens, which can be degraded by environmental factors like UV radiation and oxidizing agents, whereas DNA for molecular genetic identification and quantification is more stable. Despite these limitations, immunological methods are valuable for assessing allergenic exposure, as they detect only intact allergens related to possible health effects (Buters et al., 2012).

Culture-based methods, one of the oldest bioaerosol identification techniques (King et al., 2020) grow fungi, bacteria, and viruses under controlled conditions for species identification based on colony traits (King et al., 2020). Further analysis of colonies may use microscopic methods, MALDI-TOF or molecular genetic identification for improved discrimination. Comparing different detection techniques, most methods have a higher detection limit than culturing since processing of the sample is necessary. However, as they are based on viable species able at forming colonies on artificial media, non-culturable species that may still affect health are overlooked (King et al., 2020). As culture-based techniques have been used to establish a baseline for airborne microbial concentrations and diversity knowledge about airborne diversity is biased towards culturable species in research. Molecular genetic identification and quantification may help filling the knowledge gap of unculturable species leading towards a more complete picture of airborne diversity.

Usually, non-culture methods are faster than culture-based analysis, they cover a greater biodiversity and achieve high accuracies and analytical sensitivity (Amann et al., 1995; Macneil et al., 1995).

1.2. Lack of harmonization

Overall, the used types of analysis provide different kinds of information on airborne biological particles, each with distinct advantages and drawbacks and finally need to be used complementary to enable a complete picture. The development of molecular genetics and biotechnology tools since the 1990s has brought more rapid and accurate methods for detecting and classifying organisms, especially for non-culturable microorganisms and helped to improve our knowledge on the structure and function of airborne microbial communities.

As the use of molecular genetic tools for identification and quantification of complex airborne samples is increasing, a great diversity of research was published in the last decades.

Standards have been developed for sampling and analysis of ambient air, pollen grains and fungal spores by conventional light microscopy (EN 16868, 2019) and for the indoor detection and enumeration of moulds by cultivation (ISO 16000-17, 2008). However, standards for sampling and analysis for DNA detection and identification for pollen, fungal spores, bacteria, and viruses are still missing.

In this review we screened scientific articles describing molecular genetic based analyses of airborne diversity in the last 23 years. We evaluated the works to provide an overview of common approaches and agreed methodologies, as well as identifying the knowledge gaps and lack of documentation and provide recommendations for filling them in the future.

However, the evaluation shows that there are still gaps in knowledge about airborne diversity, as not all aspects are covered by the studies. Additionally, harmonization of used methods and approaches is needed to ensure high quality and robustness of data. Furthermore, not all information needed for comparison of various studies is given in all research papers, hindering comparison of studies or meta-analysis.

2. Methodology of paper review

We reviewed the existing literature to create a comprehensive overview of existing eDNA methodologies and their application for aerobiological samples. A systematic review combined with the scoping review approach following the framework by Arksey and O'Mally, as described by Daudt, van Mossel and Scott, was used to map all potential eDNA aerobiological studies (Arksey and O'Malley, 2005; Daudt et al., 2013). The scoping review approach was employed to ensure a thorough comprehensive review of a broad subject.

In the present work, the literature search involved a general Google search and several electronic databases, including Google Scholar. In Web-of-Science, the search used the string: "*(pollen and DNA and air) or (pollen and metabarcoding and air) or (pollen and metagenomics and air) or (Spores and DNA and air) or (Spores and metabarcoding and air) or (Spores and metagenomics and air) or (Bacteria and DNA and AIR) or (Bacteria and Metabarcoding and AIR) or (Bacteria and metagenomics and AIR)*". The search and review of papers began in Spring 2023 and the last addition of papers was completed in December 2023. An article from 2024 included in the review (Tegart et al., 2024) was available online in December 2023. Only articles available in English were considered.

In total 2575 potential studies were found, and the number was reduced to 207 after filtering the studies for aerobiological content using SCOPUS. This list was then extended with two additional approaches: *i)* extension with a hand-search of key journals: Aerobiologia and Science of The Total Environment, and *ii)* scanning the reference lists from relevant papers to identify others that may not have been found in the initial search.

The concept of using DNA barcoding for samples from air, soil or water was proposed in 2003 and has since expanded dramatically, leading to the development of environmental DNA (eDNA) analysis (Hebert et al., 2003). Hence, we restricted the literature search back to the year 2000. This resulted in 178 studies, focusing on detection of viruses, bacteria, fungal spores and pollen from air and dust samples, using molecular genetic tools for taxa identification directly on the sample. Studies collecting colony forming units and using molecular genetic tools for identification of the cultures were excluded from the review.

In most cases, sampling was performed at fixed locations (Supplementary Table 1) and in 11 studies with mobile sampling (Supplementary Table 2). These tables present the information extracted from the reviewed papers including general features, target organisms, bioaerosol sampling approach and the type of molecular analysis implemented. The general features of the papers recorded were: *i)* main sampling sites with countries and coordinates given in decimal degrees; *ii)* environmental characteristics, including urban (homes, hospitals, buildings, offices, etc.,) suburban (industrial areas, urban surroundings), rural (countryside, agriculture, etc.), natural areas (mountains, forests, etc.) and specific natural locations (Greenland, Artic, Antarctic, etc.); *iii)* sampling placement, such as outdoor and/or indoor. Target organisms included viruses (V), bacteria (B), fungi (F), and viridiplantae (PL, pollen included).

Information collected on bioaerosol sampling included: *i)* Passive and/or Active Sampling (Volumetric), *ii)* sampling methods, such as Filtration; Impaction; Cyclone; Liquid Impinger; Passive sedimentation; Swab or Vacuum collection of dust; *iii)* collection surface, *iv)* device brands; *v)* flow rate L/min (intended as low <100 L/min; medium ≤100–<1000 L/min; high ≥1000 L/min); and vi) sampler placement (roof and/or ground, or meters above ground or floor for indoor sampling).

Molecular analyses included the types of DNA or RNA extraction methods (kit and customizations) and their amplification using quantitative PCR (qPCR), digital droplet PCR (ddPCR), and reverse transcriptase-PCR (RT-PCR). The studies also considered different sequenced regions (primers for sequencing or whole genome shotgun (WGS)) and the application of DNA metabarcoding. This also included Targeted Amplicon parallel Sequencing (TAS), and shotgun metagenomic sequencing, across various platforms, as well as the construction of reference sequence databases. Details that were not described were marked as "ND" in the tables, while "NA" was used for not-applicable categories. "Yes" and "No" were represented with "1" and "0", respectively, to simplify summarization.

The information gathered was used to complement knowledge on suitable sampling techniques, processing methods, and molecular genetic tools for the assessment of aerobiological diversity (see Sections 5 to 9). Furthermore, an overview of sampling devices, a world map indicating the sampling locations, and tables summarizing analysed organisms used DNA extraction Kits and molecular genetic analysis methods were composed based on the collected studies.

3. Spatial distribution of studies

Considering the spatial distribution of studies, our data show that most sample collections using subsequent molecular genetic analysis have been conducted in Central Europe, followed by the USA and the South-Eastern Asiatic coast. Data from the centre of the Eurasian continent are completely missing, possible due to the limitation on articles written in English. Africa, South America, and Australia are represented only by a few sample collections (Fig. 1).

There are some initiatives – e.g. the Global Spore Sampling Project (GSSP) – that aim at systematically sampling fungal spores on varying latitudes and altitudes in both urban and natural environments (Ovaskainen et al., 2020), or the continental-scale microbiome study of indoor dust collected in hotel rooms (Fu et al., 2020).

4. Target organisms

In most of the analysed studies, only one environment was considered: either indoor (33 %) or outdoor (60 %), with just 7 % examining both. For fungi and bacteria, about 65 % of studies focused on outdoor air, while 35 % looked at indoor air. The evaluation of virus particles was more prevalent indoors (56 %), whereas plant material was primarily assessed outdoors (90 %).

Most of the reviewed studies only targeted one group, either fungi or bacteria (45 and 50 out of 178 papers, respectively). The next most common focus was on both fungi and bacteria together (31 publications), followed by plants (including pollen) or virus individually (16 and 14 papers respectively, see Table 1). Studies targeting three or even all four types of organisms were rare; combining fungi and bacteria with plants was more often done than combinations with viruses (seven versus three times). In five studies all four groups were targeted, including viruses, plants, fungi, and bacteria, using metabarcoding in four of these studies, a metagenomic approach in three, and combining both methods in two studies.

5. Sampling of bioaerosols for DNA detection

Important requirements to meet when selecting the methodology for sampling bioaerosols are the efficiency in collection of the target organism diversity (bacteria, viruses, fungus, pollen, insects), the size of the target particles, the sampling medium for further processing - if viability needs to be determined-, and the sampling site. Additionally, the objective of the sampling and the procedure, e.g. single or multiple species detection, qualitative or quantitative analysis, needs to be considered (Mbareche et al., 2018; Mainelis, 2020).

Many devices are available for sampling bioaerosols. They can use either passive methods, relying on gravitational sedimentation onto a horizontal surface or active suction directing particles to a collection medium. Passive samples are quantified by the deposition rate, which is based on the surface size and exposure time. Active (volumetric) sampling measures bioaerosol concentrations in air, knowing the volume of the sample. Samplers can be designed as stationary sampling devices, usually bigger and more heavy, light personal sampling devices or be installed in a moving object.

In previous and recent reviews, an overview of bioaerosol sampling methods is already given (Ghosh et al., 2015; Huang et al., 2024) but with no particular insight on the applicability for molecular genetic methods, therefore, we give an overview of suitable or frequently used sampling methods here.

These methods include techniques where the sample can be obtained in a liquid (without or after processing), whereas sampling on culture media is not suitable for subsequent DNA analysis. Suitable devices use filtration on filters or membranes, impaction onto surfaces like semi-solid media or tapes covered by petroleum jelly or silicone, electrostatic precipitation using electrodes, thermal precipitation onto temperature-sensitive media, impingement sampling into liquids, and cyclones using wet or dry tubes (Waldmann and Schmitt, 1966; King et al., 2020).

Sampling devices can be positioned outdoors or indoors, with the location of the sampling points influencing the quality and representativeness of the sample. Samplers can be fixed (on or near the ground, on a rooftop, etc.) or mobile, moving along a trajectory, on an object (car, boat, airplane, etc.) or on a person (Mayol et al., 2017; Li et al., 2018;

Fig. 1. Overview of sampling locations of molecular genetic based field studies summarized in Supplementary Table 1; the search parameters only included papers written in English; only studies that indicated the location in the publication could be represented in this map © OpenStreetMap (gpsvisualizer.com assistance).

Table 1
Summary of targeted organism groups in molecular genetic based field studies summarized in supplementary table 1, indicating the number of publications targeting from one up to four organism groups and the type of organism analysed.

number of organisms	1	2	3	4	Sum of publications
Number of publications	125	38	10	5	178

	Fungus	Bacteria	Plants	Virus	Plants + Virus	Sum of publications
Fungi	45	31	5			96
Bacteria		50		2		98
Plants			16			33
Viruses				14		24
Fungi + Bacteria			7	3	5	

Sánchez-Parra et al., 2021). Sampling in indoor environments is often linked to health-related questions, in occupational environments (industrial, educational, and residential office and building environments) hospitals, or residences of vulnerable people. Outdoor bioaerosol sampling is used to compare with indoor data as well as in surveys of outdoor environments, such as urban infrastructure, agricultural areas,

composting sites, wastewater treatment plants, or natural environments (Masclaux et al., 2013).

In the reviewed articles, nearly 28 % of the reviewed papers used deposition of particles for sample collection. The active sampling flow rates range between 1.5 and 3300 L per minute. Low-volume samplers (flow rate < 100 L/min) were used in 68 % of the studies with active

sampling, medium-volume samplers (≤100- <1000 L/min) in 22 %, and high-volume samplers (≥1000 L/min) only in 10 % of the papers.

The most frequently used sampling approaches were impaction, followed by filtration, impingement, and dry and wet cyclone devices

(Fig. 2). Dust sampling, using swabs or vacuum cleaners were also used (20 %) frequently. In indoor environments, the samples were collected either by deposition using passive samplers or active sampling, while in outdoors active samplers were predominant. In 11 % of papers more
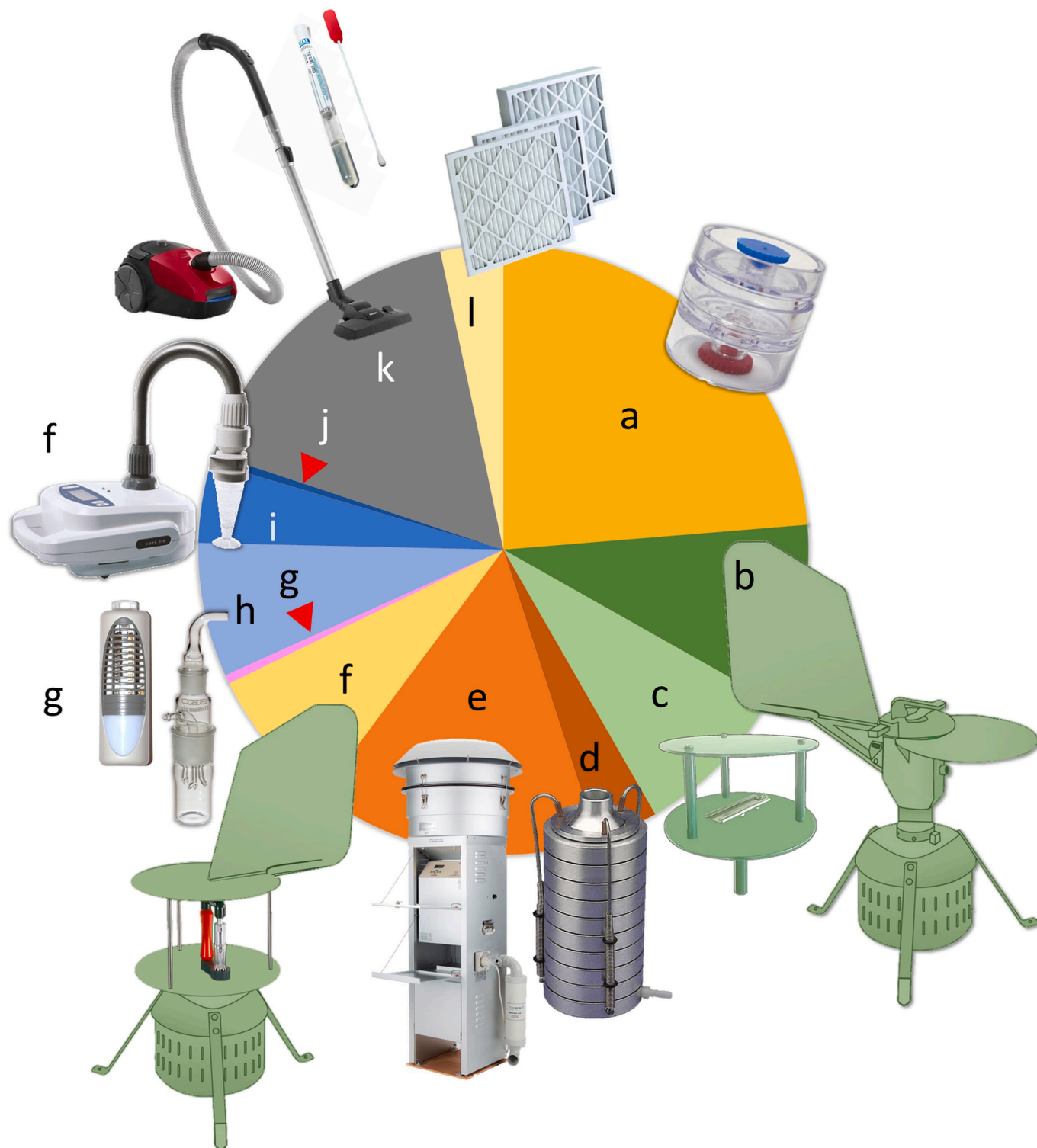
Fig. 2. Percentage of the types of volumetric air sampling devices used (209) in molecular genetic based field studies (177) summarized in Supplementary Table 1. In 11 % of the studies more than one type of device was used. a: filters (*N* = 49), b: Hirst-type impactors (*N* = 20), c: passive samplers (*N* = 18, being EDC the most frequently used one, in 28 %), d: cascade impactors (*N* = 6), e: other impactor types (*N* = 31), f: dry cyclones (*N* = 15), g: electrokinetic devices (N = 1), h: liquid impingers (N = 15), i: wet cyclones (N = 10), j: not determined (N = 1), k: HVAC filters (*N* = 7), l: dust samplings (*N* = 36; dust collected with vacuum cleaners and swabs in 56 % and 39 % of the studies, respectively). Note: in non-metagenomic molecular studies, other impactor types (d) are more frequently used, mostly with growth media filled petri dishes.

than one type of device was used for sample collection.

5.1. Gravitational settling

This method involves sampling particles that naturally settle on a horizontal surface (Grinshpun et al., 2007; Ghosh et al., 2015; Seidl, 2021). The surface can be an artificial matrix placed in a specific location or an existing surface. Sampling may either track a known settling time (placing of a clean collection matrix, cleaning prior to the deposition) or collect existing dust (Varotto et al., 2021). In the studies reviewed, sampling time varied widely and was often not specified (Supplementary Table 1). This method was originally used to account for colony-forming units (CFU/m^2/h) using petri dishes containing a specific medium for sampling. Whenever surfaces suitable for washing or particle extraction are used, DNA extraction and analysis can also be conducted. The reviewed papers frequently utilized cloth dust collection (in petri dishes or fixed on other transport surfaces), swab sampling or vacuum for dust collection. The main advantage of gravitational settling is its practicality and cost-effectiveness (Crook, 1995; Ghosh et al., 2015).

In indoor environments, this method allows simultaneous sampling at different locations but has key drawbacks. It shows a bias towards larger particles, which settle faster and easier than smaller ones, despite smaller particles having greater health impacts (Crook, 1995). Its success also depends on the airflow in the room and exposure time, raising questions about whether settling samples accurately represent ambient air particles (Pasquarella et al., 2000; Ghosh et al., 2015).

The use of passive samplers outdoors is less common, but devices like the Durham Sampler (collecting on a slide) or Tauber traps (collecting in fluid-filled containers), and dust collectors offer potential for geographical and biodiversity assessment due to their simplicity, low cost and ability to operate in remote areas without electricity or solar power (Tauber, 1974; Barberán et al., 2015; Leontidou et al., 2018, 2021). These methods are recommended only for qualitative measurements. However, our review shows, that these methods are not widely adopted yet.

5.2. Filtration

Filtration is a widely used method for capturing aerosols by drawing air through a filter, where particles are retained based on pore size. Our review identified 49 studies using various filter-based sampling methods. The used filter type, size, and porosity depend on the sampling method and sample processing techniques. Filters come in different pore sizes and can be fibrous, membrane, or capillary. Common materials include cellulose ester, polyvinyl chloride, PTFE (membrane filters), and polycarbonate (capillary filters), as well as glass fibre, quartz, and nylon and the choice of material is based on the analysis carried out subsequent to sampling.

For DNA analysis the most suited are PTFE filters, although other types can be used such as cellulose ester, polycarbonate, glass fibres, polyvinyl chloride membranes or gelatine filters (Lindsley et al., 2017; Mainelis, 2020).

The advantage of filtration is, that the sampling efficiency is high, as the whole airstream passes through the membrane. Additionally, if polycarbonate or gelatine filters are used, they can be dissolved, enabling the acquisition of the whole sample in liquid form. However, the sample volume after filter washing or dissolving may be high, and the recovery of the total DNA may be labour-intensive.

A disadvantage of filtration is that filters can get saturated or clogged-up, which impacts the filtration efficiency and air flow. Furthermore, co-extraction of the samples with the filters can inhibit DNA in downstream analyses (Uhrbrand et al., 2018).

5.3. Impaction based samplers

Impaction-based samplers were the most used devices in bioaerosol sampling in the reviewed studies. A pump creates an airstream that passes through nozzles, where particles are separated by inertia and accumulate on an impaction surface. For DNA analysis suitable surfaces can include, filters, or smooth surfaces like plates, petri dishes, slides, or tapes coated with greasing products as silicone or petroleum jelly. Impactors vary by flow rate, cut-off diameters, nozzle number, and collection stages, and are classified into types such as impactors using petri dishes, spore traps, and rotating arm collectors (Lindsley et al., 2017; Mainelis, 2020).

Cascade impactors use multiple impaction stages, with each stage collecting progressively smaller particles due to smaller nozzles and cut-off diameters, enabling size-segregation of bioaerosol particles. Using high air sampling volumes, this method allows for easier detection of rare bioaerosols and with special filters offers high efficiency for capturing under 1 μm. Several studies employed high-volume sampling with subsequent DNA analysis to measure bioaerosol concentrations in extreme environments, such as the Artic or deserts (Stern et al., 2021) (Tawabini et al., 2017; Wex et al., 2019; Stern et al., 2021). A drawback of cascade filtration sampling on filters is the increased labour and consumables costs for analysis. Additionally, efficiency of filter washing to extract bioparticles is not always achieved.

The Andersen impactor (Andersen, 1958), is one of the most widely used cascade samplers, operating at a flow rate of 28.2 L/min, with up to six impactor stages, sampling on petri dishes (Lindsley et al., 2017; Mainelis, 2020). Another example of impactors is the Hirst spore trap, designed for airborne pollen and fungal spores (Hirst, 1952), which uses an airflow of 10 L/min and a rotating drum covered by petroleum jelly or silicone coated tape. The bioaerosols deposited on the tape can extracted and subsequently used for DNA analyzation.

Other common impactor samplers include the Rotorod and the Surface Air System samplers (SASS). The Rotorod collects particles on small surfaces (plastic strip, petri dish, slide) fixed between the rotating arms, without active air suction. Samples can be processed for DNA extraction (Mandroli et al., 1998). The SASS can operate at flow rates between 100 and 180 L/min and the particles are impacted directly onto a plate, which can be coated with petroleum jelly for subsequent DNA extraction (Sánchez-Parra et al., 2021).

In some environments the sampling volume of devices with low flowrate may be insufficient for DNA analysis, on the other hand in very polluted environment, the impaction surface may be saturated with particles.

5.4. Cyclones

Cyclonic samplers feature a chamber where the air stream enters through tangential nozzles (Hering, 2001) and spirals within. These samplers use a centrifugal impaction system that causes particles to settle on the dry walls by inertia.

Most studies using cyclones, used the Burkard Multi-vial dry cyclone that deposit particles into eight small Eppendorf tubes (Pashley et al., 2012; Abrego et al., 2018; Brennan et al., 2019; Rowney et al., 2021; Apangu et al., 2022, 2023). The tubes can be automatically replaced daily and have proven to be robust for larger networks (Ovaskainen et al., 2020; Rowney et al., 2021), offering long-term operation with weekly tube manipulation (Hanson et al., 2022a). Direct sampling into laboratory-ready containers, minimizes processing steps and microbial growth due to absence of liquid. However, they sample at 16–100 L/min, which may be insufficient for DNA bases analysis. Additionally, the samples face issues like varying efficiency, particle buildup in the cyclone above the collection tube, and liquid entering from rain or fog (West and Kimber, 2015).

Single stage and two-stage NIOSH cyclones, can be used for low volume stationary or personal sampling, also using standard laboratory

containers for sampling. They have been shown to be efficient in capturing samples for DNA analysis using a 10 L/min flow rate (Verreault et al., 2011; Turgeon et al., 2014; Schuit et al., 2021).

Wet cyclones, unlike dry cyclones, are suited for shorter sampling periods (10 min to 6 h), collecting samples by creating a vortex in the sample liquid, allowing airflow to pass through. They operate at flow rates of 100 to 1000 L/min (Grinshpun et al., 2015), including Coriolis samplers that function at 100 to 300 L/min (Lin et al., 2018). This review found ten studies using wet cyclones, including the SAS2300 (Dybwad et al., 2014), self-designed samplers, wet vacuum cleaners, and commercial Coriolis samplers. The Coriolis sampler detects pollen, fungal spores, bacteria, and viruses (Carvalho et al., 2008; Kumar et al., 2021), with bacteria and fungal spores identified via qPCR and sequencing (Unterwurzacher et al., 2018; Watt et al., 2020). The SAS2300 detects aerosols within the size range of fungal spores and bacteria but is less efficient for smaller bacteria (1 μm) than the Coriolis (Dybwad et al., 2014). Reviewed articles mainly report its use for virus collection and genetic analysis.

5.5. Liquid impingers

Impingers are comparable to impactors, but collect particles in a liquid, such as mineral oil or a buffer, depending on the sampling goal (Kesavan et al., 2010; Šantl-Temkiv et al., 2018). Unlike impactors, they resist saturation and preserve the biological integrity of the particles. However, they have lower sampling efficiency than cyclones for some organisms (Kumar et al., 2021). Additionally, some liquids evaporate easily, limiting their use to shorter campaigns, and may cause microorganisms to float and escape again or are incompatible with DNA extraction.

In the reviewed studies, 14 used liquid impinger including the Bio-Sampler (12.5 L/min and 20 mL of liquid), the DS 5600 high flowrate sampler (800–1000 L/min) and the portable BioCapture 650 (sampling up to 200 L/min into 10 mL cassettes), which were also described in previous studies (Fahlgren et al., 2011; Mainelis, 2020) (Mainelis, 2020). with the gold standard. The All-Glass Impinger (AGI) (12.5 L/min, 20 mL of liquid), described as the standard sampler for culture-based analysis, was not used in the reviewed studies.

5.6. Alternative sampling methods

Alternative bioaerosol samplers referred to in the literature include planes, generating the sampling airflow by their movement (Zweifel et al., 2012) and car cabin filters or HVAC systems using their filtration system for sampling (Hurley et al., 2019). While the airflow varies and is not controlled, these systems can offer a qualitative overview of the bioaerosols present in the vehicle's route or airstream of a building. In our review, 11 studies were found and summarized in Supplementary Table 2.

Two other collection principles are electrostatic and thermal precipitation. Electrostatic precipitation uses electric force to collect particles from an airstream, including smaller ones, but is limited in collection rate an area (Knutson and Whitby, 1975; Ghosh et al., 2015). Thermal precipitation, one of the oldest methods, relies on a temperature gradient perpendicular to the airflow to deposit particles on a cooler surface (Waldmann and Schmitt, 1966). This method is effective in capturing small particles, although the system complexity is considered a major drawback (Ghosh et al., 2015).

6. Sample transport and storage

After collection, the preservation of the samples, regarding sample integrity, stability and concentration and composition of the target organisms, until their processing (e.g. washing of filters) and subsequent DNA extraction is essential. Samples may need to be transported to the laboratory for analysis and in some cases, storage (for a short or longer time) of the samples till processing and DNA extraction is needed. Stability of the sample may be influenced by i) destruction of the cells during the sampling procedure, making DNA more vulnerable to degradation, ii) presence of DNAse in the sample, which may be active under higher temperatures, iii) microbial growth of intact cells, altering the composition of the sample. Depending on the type of sample and group of species to be detected, various procedures are used for stable transport and storage of samples. In the reviewed papers, the storage and transport conditions were only seldom and or insufficient described. However, in laboratory studies, various procedures were tested and described and summarized in the following section.

6.1. Transport and short-term storage

Pollen are relatively stable, while viruses, fungi or bacteria can grow in or on the sampling media (Wu et al., 2000; Viegas et al., 2020), and DNA might degrade with time, on the sampling media or during transport and storage (Espinel-Ingroff et al., 2004; Webb et al., 2018). Environmental samples in general contain a mixture of many microorganisms and as such, a compromise is often needed for processing and storage. Samples from remote locations or sampled by third parties may take several days in transit before reaching the laboratory and therefore, it is recommended to store samples in appropriate temperature (e.g. 4 to 8 °C) and storage materials while in transit, to avoid degradation of DNA or change in sample composition (Clasen et al., 2020). The analysis of 16S rRNA sequences from a number of different sampling media under various conditions has shown no significant differences for short-term storage temperatures (3–14 days) ranging from +20 °C to −80 °C (Lauber et al., 2010). On the contrary, freeze drying (lyophilization) is controversial, with known negative impact on later extracted fungal DNA concentration and integrity (Bainard et al., 2010). However, it was successfully used in multi-site studies for birch pollen allergens (Buters et al., 2012). Storage of bacterial cultures often follows protocols recommending 4 °C for short period, matching guidelines from a review on environmental DNA sampling (Lear et al., 2018). It is therefore suggested that mixed samples of bioaerosols are, ideally, cooled to 4 °C before leaving the sampling site and transferred to freezers afterwards. Alternatively, if the sampling approach allows this, then samples can be kept unfrozen for a few days before being transported to a storage location before freezing. Thawing cycles should generally be avoided or at least minimized.

6.2. Long term storage

Freeze drying of fungal spores and bacteria and storage in liquid nitrogen prior to DNA extraction is generally considered the best overall approach for maintaining integrity, noting that there is no universal procedure for all species (Webb et al., 2018). However, a general recommendation for long-term storage is to use the lowest storage temperature available. Freeze drying of samples with mixed bioaerosols should be avoided due to the risk of reducing DNA extraction efficiency for certain species unless the sampling approach requires this. Within the UK funded PollerGEN project (Brennan et al., 2019) and the Global Spore Sampling Project (Ovaskainen et al., 2020) cyclones were used for continuous sampling into dry collection tubes over a long time. They were frozen at −20 °C locally before shipment to the laboratory using ordinary mail, unfreezing the sample in the process. This is not recommended, as thawing can lead to moisture, microbial growth and change in the sample composition. Contrary, long-term storing of samples without thawing showed good stability of the samples. Storing material at −80 °C has successfully been used for airborne samples for a full season (Pashley et al., 2012) or even four years in the UK. The extraction of samples collected by Hanson et al. (2022b) resulted in much lower DNA extraction rates after storage for about 9 months −20 °C (Skjøth, 2024; Apangu et al., 2024). The temperature difference in long-term storing of bacteria isolates showed little difference between −70 °C

and −80 °C (Sunarno et al., 2021).

A range of other methods have previously been reviewed for storing DNA such as using chemicals or liquid nitrogen for extreme long-term storage (Nagy, 2010), but these methods are generally not applied to bioaerosols. Currently, only dry samples of bioaerosols have shown high DNA extraction rates after long term storage. Therefore, studies need to address how to distribute and store bioaerosol samples to optimize DNA extraction efficiency, particularly in relation to large networks that utilize a large number of sampling sites (e.g. globally). Nevertheless, a minimum requirement for long-term bioaerosol samples storage for DNA extraction should be set to −70 °C. It is recommended that the stored samples should be kept in a dry state until other studies find better approaches. Freeze drying of the bioaerosol samples should be avoided, if possible, on samples with mixed bioaerosols, as freeze drying may impact DNA extraction rates of some species.

7. Sample processing, DNA/RNA extraction and storage

Airborne biological particles are captured using various devices (see Section 5 and Fig. 2) and on a variety of surfaces that may need special processing steps to retrieve the sample. The most common collecting surfaces are represented by sticky tapes, filters, or dry sampling vessels. Beside sampling on surfaces, sampling in liquids is also common, here no processing to get the sample into a liquid stage is necessary. The subsequent DNA extraction protocol(s), either employing a commercial kit or not, usually perform differently according to how well the amount of aerobiological material has been removed from the collecting surfaces. Consequently, the amount and quality of recovered DNA will be determinant for the next PCR amplification and sequencing steps.

7.1. Removal of the sample from the sample matrix and processing

When using dry sampling onto a surface or into a vessel, the processing step includes washing of the vessel, using enough buffer to retrieve all material and minimizing dilution of the sample is essential.

In the case of sampling performed on sticky tapes, for example, when using a Hirst-type spore trap, these samples can also be used for subsequent DNA extraction and PCR analysis (Calderon et al., 2002). Sample processing of these tapes was tested using chemical and mechanical methods for retrieving the sample and fungal DNA subsequently (Calderon et al., 2002). Extraction of the tapes can be done by cutting the tape to fit it into a 1.5 mL microcentrifuge tube, with the sticky surface facing inward, freezing and subsequent grinding with metal beads and a bead-beater for 5 min and adding lysis buffer (Banchi et al., 2020b; Polling et al., 2022). If multiple tubes are used for parts of the tape, the sample can be pooled after DNA extraction. Commonly, the Hirst type trap use wax and petroleum jelly which may reduce the efficiency of DNA extraction (Quesada et al., 2018). For processing of samples coming from sticky and resinous surfaces or supports, some protocols have been established in the forensic field. Forsberg et al. (2016) suggested a pre-extraction step using acetone, hexane, or chloroform to remove most of the resinous material (Forsberg et al., 2016). Subsequently, such steps have also been implemented for processing of aerobiological samples (Leontidou et al., 2018; Polling et al., 2022).

Sampling on filters is a common approach and was found to be suitable to collect and extract fungal DNA (Olsson et al., 1996; Frohlich-Nowoisky et al., 2009; Basapathi Raghavendra et al., 2023). After sampling onto a filter surface, the sample must be separated from the sample matrix by washing (with buffer, solvent, or others) or dissolving the filter. In the literature, filter types like gelatine, polytetrafluoroethylene, and polycarbonate filters have generally provided good results. However, although polycarbonate filters have been found to be the best option for fungal spores and bacteria (Wang et al., 2015b), the choice of filter substrate may result in extra steps during the DNA extraction procedure (Stern et al., 2021) and impact overall DNA extraction and amplification efficiency. Although gelatine filters can be

dissolved in liquid buffer, enabling the retrieval of the whole sample, depending on the size of the filter 1 to 20 mL of buffer is necessary. As the volume cannot be reduced, without coagulating the gelatine, the subsequent DNA extraction can only be performed on an aliquot or is very labour intensive. On the contrary, polycarbonate filters can be dissolved in a lower volume of phenol-chloroform and the whole sample processed for DNA extraction. Furthermore, Teflon (PTFE), polycarbonate or foam filters can be washed to retrieve the particles (Buters et al., 2012; Pogner et al., 2019).

After processing of the sample, or sampling into a large volume of liquid, it may be necessary to increase the concentration of the sample (Lear et al., 2018). Filtering of Coriolis μ samples as well as freeze drying or use of a vacuum concentrator have been published (Unterwurzacher et al., 2018; Wessely, 2020).

In general, steps reducing the concentration of the sample should be avoided and a low amount of liquid for washing of sample surfaces or vessel should be used. The use of wax, grease, oil, or petroleum jelly should be avoided, and dry sampling vessels are suitable for retrieval of the whole sample. However, the used sampling method must fit to the sampling purpose (short term, long term, daily sampling, …) of the campaign and the volume of air possible to collect.

7.2. DNA/RNA extraction

Airborne DNA or RNA extraction has been performed both by standardized extraction kits and following customized procedures, regardless of the target organisms to be studied (see Table 2). In seven of the reviewed studies the method for DNA extraction was not given, and in 22 studies own lab procedures instead of commercial kit were used. As for customization 77 of 178 analysed papers altered the procedures in some way, they sometimes include the physical lysis of bioparticles by beating beads, chemicals denaturing proteins (e.g. SDS, proteases, Nonidet P-40, Phenol:Chloroform:Isoamyl Alcohol mixture, resins), and or CTAB methods to remove membrane lipids (Abrego et al., 2018; Serrano-Silva and Calderón-Ezquerro, 2018). Most of the papers considered in the review performed the DNA or RNA extraction step using commercial extraction kits, even if none has been nowadays specifically designed for airborne DNA. The choice of the diverse authors

Table 2
Overview of used types and targets of DNA extraction kits and performed customizations in the protocols. Customizations include variation in the lysing matrix as well as changing amount or types of buffers.

Kit group	Number of kit types/ vendors	Number of papers	Customization		
			ND	No	Yes
Bacteria	2	2			2
Blood/tissue	1	1			1
DNA	13	26	3	11	12
Extraction buffer	1	1			1
Feaces/soil	2	2		2	
Feaces	1	1		1	
Microbes	3	5		3	2
Miniprep	1	1		1	
NA		1			
ND		5			
No kit		23			
PCR	2	5		3	2
Plant	9	16		8	8
RNA	2	2		2	
Soil	12	69	6	43	20
Sputum	1	1		1	
Stool	1	1	1		
TRItidy	1	1			1
TRIzol	1	2		1	1
Virus	5	7		6	1
Water	3	10		5	5
Yeast	1	1		1	

has been therefore directed towards extraction kits either specific to the target organisms (plants/pollen, fungi, viruses, bacteria) or intended for complex matrices (e.g., soil, water, tissue).

To define a scientific-oriented pipeline for the choice of the DNA or RNA extraction kit to be utilized for specific samples and target organisms, some papers focused on comparing the DNA yield obtained by different DNA extraction kits in various sampling conditions. For pollen various extraction kits have been compared (Leontidou et al., 2018) and for fungi extraction kits as well as beat beating matrixes and conditions were evaluated (Aguayo et al., 2018; Unterwurzacher et al., 2018). For the extraction of bacteria important in occupational air samples, three different DNA extraction kits were tested (Schäfer et al., 2017). Interestingly, DNA extraction kits, advertised for soil samples, seem to work well for bioaerosol samples, as the mentioned studies receive satisfactory results with these kits and kits designed for this matrix were most often used in the reviewed articles (69 of 178). Other commercial extraction kits (e.g. AllPrep DNA/RNA Micro Kit and PowerWater DNA isolation kit, Qiagen) that allow simultaneous co-extraction of DNA and RNA from bacteria, fungi or viruses have proven successful for water and filter samples (Ankley et al., 2022; Erkorkmaz et al., 2023) but are yet to be extensively employed in bioaerosol studies.

The vast variety of used kits shows the need for harmonization of DNA extraction methods for bioaerosol eDNA samples. Based on the information available, and sometimes lack of details on sample treatment, a meta study comparing the suitability might not be able yet. The first step therefore is including all information about sample treatment in publications. Further, if a complex matrix is evaluated regarding multiple organisms (bacteria, fungi, pollen) a beat-beating step should be included. A review of environmental samples (Lear et al., 2018), recommends extracting DNA using DNA extraction kits, in contrast to non-commercial methods, as this reduces the lab-to-lab variation.

7.3. DNA/RNA storage

During storage of extracted DNA or RNA degradation may occur, influenced by several mechanisms: *i)* oxidation and hydrolysis of the phosphate backbone or the base from the sugar (depurination), *ii)* temperature, which in general is positively correlated to enzyme reactions degrading the DNA, *iii)* pH, which is, on the other hand, the key factor for improving DNA stability against degradation. Indeed, both acidic and basic conditions enhance the hydrolysis rate of DNA by either increasing the electrophilicity of the DNA or the nucleophilicity of water. Therefore, DNA or RNA, when stored in a solution, is usually stored in a neutral or weakly basic buffer (pH 8). In fact, even changes in the pH from 6 to 5 were estimated to increase the DNA degradation rate by an order of magnitude (Matange et al., 2021).

During lab work, DNA and RNA samples can be kept at 4 °C or room temperature and recurrently used to set up PCR amplifications. If stored in a dry state at room temperature, the DNA tends to degrade more rapidly. Short-term storage is performed at −20 °C instead, and the DNA or RNA can be either kept in a buffer solution and frozen or lyophilized. In this case, the DNA or RNA will undergo freeze-thawing processes each time it is needed to set up PCR amplification. Freeze-thawing processes tend to compromise the stability of the molecule and should be avoided or reduced to a minimum. In the case of long-term storage, when the DNA or RNA is not needed for experiments /amplifications, it should be preserved at −80 °C (Matange et al., 2021). Either short- or long-termed stored should be thawed gradually, usually in ice or at 4 °C to reduce the fractioning of the molecule during the thawing.

8. DNA/RNA detection and analysis methods

As for most analytical approaches only a small amount of DNA or RNA is necessary, a sequence of different molecular genetic analysis can be performed from the same sample. Indeed, in 61 of the reviewed studies multiple analytical methods were implemented (see Table 3).

Table 3

Overview of used molecular genetic methods used in the reviewed publications; PCR -use of genus or species specific assays; MBC – Meta-Barcoding; MGS – Metagenomic Sequencing; DGGE profiles – Denaturing Gradient Gel Electrophoresis; RT-PCR – reverse transcriptome PCR; qRT-PCR – quantitative reverse transcriptome PCR; Pass – passive sampling; Vol – volumetric/active sampling; HVAC – use of existing HVAC systems for sample collection.

Analytical method	HVAC	Pass	Vol	Vol/HVAC	Vol/pass	Total
ddPCR		1	1		1	3
ddPCR; MBC		1				1
MBC	3	10	65	1	3	82
MBC; MGS			1			1
MGS		1	5			6
PCR			2			2
PCR; DGGE profiles; MGS		2				2
qPCR		1	13		4	18
qPCR; MBC		23	25	1	3	52
qPCR; MBC; MGS		1	2	1		4
qRT-PCR; MBC		1				1
RT-PCR			2		4	6
Total	3	40	117	3	15	178

In most cases, PCR assays require a priori knowledge of the expected organism, as specific primers are needed for its detection. With sequencing, it is possible to get an overview of species, with no or low prior knowledge of the expected genera but have barely been applied in aerobiology compared to other research fields (King et al., 2020). In public databases, the universally applicable ribosomal DNA sequences (rDNA, such as ITS for fungi, 16S for bacteria, and COI for plants) of various described species have been stored and can be used for sequence comparison. Very few studies compared the results of molecular and culture-based approaches (Simoni et al., 2011; Urbano et al., 2011; Unterwurzacher et al., 2018; Tordoni et al., 2021). Based on the results of Urbano et al. (2011) the methodologies resulted in a substantially different list of species, whereas Tordoni et al. (2021) recovered fairly comparable results in their study of airborne fungal particles. In particular, sequencing results demonstrated a surprisingly broader diversity of fungal communities in air samples than previously estimated using traditional assessment methods (Rittenour et al., 2014; Unterwurzacher et al., 2018). Although highly significant advances have been achieved in this area in the past few years, available genetic information is still incomplete and for many species even totally missing (Horner, 2003). Still, rDNA sequences are easier accessible compared to other more specific gene sequences (i.e., protein coding sequences; Ward et al., 2004) where the lack of available databases makes the identification uncertain.

Classical molecular approaches as Polymerase chain reaction (PCR) combined with gel electrophoresis to visualize the amplified DNA fragments, is often used as quality control and is often the first step of checking the samples. The overview of papers showed that qPCR was often used additionally to metabarcoding and metagenomic approaches in the recent years (see Table 3).

Nowadays, different high throughput sequencing (HTS) technologies have been used intensively to analyse eDNA with metabarcoding procedure and describe biological diversity in a comprehensive way. In the reviewed articles analysing bioaerosols, 141 analysed the samples using metabarcoding, making use of different target regions, organisms, and sequencing technologies. For short read sequencing, Illumina, Ion Torrent and GenapSys technology were most frequently used.

Metagenomic approaches, including third generation sequencing have emerged in the past decade and were used in ten studies. Shotgun sequencing of the whole samples was employed by most of the studies and Illumina Miseq and Hiseq, Ion Torrent and 454 pyrosequencing was used.

8.1. Primer selection

In nearly all modern molecular detection approaches, primers are used to amplify a specific sequence of DNA. In this way, the quality of the selected primers is of great importance to have reliable results. A primer is a short segment of RNA or DNA that forms complementary base pairs with the template via Watson–Crick base pairing (Basu, 2022). DNA primers are more stable and less susceptible to chemical degradation than RNA-based primers (Basu, 2022). For PCR, qPCR, ddPCR and dPCR the depth of taxonomic resolution of detected taxa is dependent on the specificity of the primers used, which also depends on the length and the GC-content of the primer (Basu, 2022). Wu et al. (2002) examined 53 different primer pairs for fungi and bacteria, for their selectivity, using PCR. For fungi, ten universal primer pairs were found, where one set allowed amplification of most fungi except *Aspergillus fumigatus*, *Aspergillus niger*, and two species of *Paecilomyces*, emphasising the influence of primer selection on the detection of taxa. The test of published genus-specific primers revealed amplification of non-target species, showing the importance to validate every primer pair on a wider range of species and isolates to avoid false positives. Additionally, PCR conditions should be optimized to improve specificity, usually by setting the annealing temperature (Wu et al., 2002). Based on these findings, a new set of universal primers for quantification of the total number of fungi, the FungiQ assay was developed and is now widely used (Liu et al., 2012). Few airborne fungal species have selective primers already designed for their detection. For instance, Unterwurzacher et al. (2018) designed a set of primer-pairs to distinguish the most relevant fungal taxa. Primer development on airborne pollen is less advanced compared to airborne spores. One study presented specific primers for eight different airborne grass pollen (Rowney et al., 2021), but there are hundreds of flowering grasses showing that this is not straightforward. Similarly for trees developing specific primers will be challenging as some genera such as *Quercus* (Gömöry et al., 2001) contain species with very little genetic difference.

In high-throughput sequencing (HTS) universal primers are used, to amplify a DNA sequence present in most taxa of interest (fungi, bacteria, eucaryotes), so called universal barcodes. For detection of fungi in barcoding and metabarcoding, the Internal Transcribed Spacer (including the two parts ITS1 and ITS2) region of the ribosomal RNA cistron is the universal marker normally targeted due to its high copy number, optimal species-level resolution in most groups, and the ability to map both fungus-specific and universal primers (Man et al., 2010; Schoch et al., 2012; Tedersoo et al., 2015). Meanwhile, the 16S small subunit ribosomal RNA is the universal genetic region commonly considered for primer design and later used for amplification and next-generation sequencing of bacterial species in bioaerosols (Lane et al., 1985; Ogier et al., 2019). For identification of pollen, the plastid DNA genes ribulose-bisphosphate carboxylase (*rbcL*) and Mat-kinase (*matK*) are the universal barcode regions being used for designing primers for amplification and identification of land plants as well as intergenic sequence *trnH-psbA* and the ITS as the supplementary genes (CBOL Plant Working Group; Hollingsworth et al., 2009; Kang et al., 2017; Frisk et al., 2023).

However, caution should be taken when using primers targeting the rbcL region for metabarcoding as they have poor taxonomic resolution at species level (Kang et al., 2017). Previous studies produced a list of different pairs of primers for fungal metabarcoding and reported potential biases in taxonomic identification (Tedersoo et al., 2015; Nilsson et al., 2019a; Banchi et al., 2020b). For grass pollen it was shown that complementary information can be achieved by combining the rbcL and ITS2 regions as each had reduced taxonomic detail (Brennan et al., 2019). Moreover, the use of a preamplification step, to enhance the sensitivity of the detection may influence the detected alpha diversity of fungal air samples (Wessely, 2020). Various primer sets were tested for coverage of bacteria and archaea, revealing that none of the primer pairs was able to cover the whole taxonomic diversity (Klindworth et al.,

2013).

Since there is no universal protocol to cover the entire range of bioaerosols present in a sample, an intensive work in the design of primers remains essential. There are four major steps normally considered when designing primers: *(i)* target identification, *(ii)* definition of assay properties, *(iii)* characterization of primers, and *(iv)* assay optimisation (Bustin and Huggett, 2017). Evaluation steps should not only include new designed primers but also established ones, verifying their target coverage and specificity on sequences available in databases. Different databases, such as NCBI-Genbank, are powerful tools for mining sequences for primer design and primer evaluation, as the number of available sequences is increasing daily. However, it is important to have absolute clarity on the target amplification region and have curated sequence datasets during mining of the databases to avoid false positives of results (for specific primers) or missing out specific taxa (for universal primers). Having downloaded the sequences from the databases, they can be aligned and analysed using readily available bioinformatics pipelines such as MUSCLE, De Novo, DADA2, QIIME, mothur, Galaxy, Geneious etc. (Nilsson et al., 2019b).

Parameters of primers such as the length of nucleotides (10–50 bp), melting temperature (depending on the type of PCR, e.g. standard PCR, nested PCR, qPCR, dPCR, ddPCR or library preparation for next-generation sequencing), buffer properties, GC content (40–60 %) and annealing temperature should be considered to choose and design, specific and sensitive primers. On the other hand, studies suggest that modification of PCR conditions by lowering the annealing temperature of primers can favour amplification of target taxa that have one or more primer pattern mismatches, but may also enhance non-specific priming, resulting in the amplification of random genomic fragments or non-targeted taxa.

8.2. DNA amplification: Polymerase chain reaction

8.2.1. Polymerase chain reaction (PCR) and quantitative PCR (qPCR)

By using the polymerase chain reaction (PCR) technique a specific target DNA region is amplified and further analysed (Saiki et al., 1985). Conventional PCR is usually combined with gel electrophoresis to visualize the amplified DNA fragments and identification of targeted (micro)organisms in the collected air samples (Williams et al., 2001). The method is fast, and depending on the primer sets used, the results can be either general, genus- or species-specific. The readout is qualitative or semi-quantitative at most.

For quantifying the amplification of the target DNA, quantitative PCR (qPCR) technique uses a fluorescent dye, such as SYBR green which binds to double stranded DNA. Through the bonding the intensity of its signal increases by 1000-fold, independent of the nucleotide sequence (Cruz-Perez et al., 2001). Standards, with known quantity, that are measured in parallel with the samples, enable quantification of the DNA content. The TaqMan qPCR technique is a modified version of the qPCR, where additionally to the primer set, a specific probe is designed, resulting in increased specificity of the assays (Stetzenbach et al., 2004).

Both techniques are established in the field of aerobiology and used with general and specific primers (Haugland et al., 1999; Williams et al., 2001; Meklin et al., 2004; Bellanger et al., 2009; Unterwurzacher et al., 2018). The analysis has been used in combination with several sampling techniques, like impingement (Haugland et al., 1999), cyclone sampling in laboratory tubes (Williams et al., 2001), filtration on Teflon filters (Bellanger et al., 2009) or wet wall cyclone sampler (Unterwurzacher et al., 2018).

In the reviewed studies qPCR was used in 78 out of 178 papers. The number for the use of PCR is not certain as often PCR is used as quality control or to get a rough estimation of the DNA content of a sample before using sequencing techniques. However, in most studies no such step is mentioned and can be assumed to not be documented in detail in the papers as it may not influence the end result.

8.2.2. Digital droplet PCR (ddPCR) and digital PCR (dPCR)

A newer and promising technology is digital droplet PCR (ddPCR) offering higher precision and removing biases caused by calibration curves used with the qPCR methods (Gao et al., 2018; Nyaruaba et al., 2019; Wang et al., 2022). In a review on ddPCR (Nyaruaba et al., 2019), the technology is described as making an emulsion of 20,000 or more miniature droplets, in which the PCR reaction takes place, being able to detect bacteria, parasites, and viruses. The absolute concentration is then based on statistics and the number of positive PCR reactions in the droplet. For environmental air samples, the multiplexing option in ddPCR can be relevant, where different targets are analysed simultaneously. ddPCR has been applied only a few times on air samples, mentioned only in four of the reviewed studies. The technology was used on air samples with ultra-low concentrations of SARS-CoV-2 (Liu et al., 2020; Truyols Vives et al., 2022), bioaerosols from compositing sites (Gao et al., 2018), and for bacteria from indoor air samples (Middelkoop et al., 2023). The increased precision and sensitivity of ddPCR can be particularly useful in cases where the viral load is low or for detecting residual viral RNA in patients who have recovered from COVID-19. Several studies have demonstrated that ddPCR can detect low levels of SARS-CoV-2 RNA, sometimes even in the absence of detectable viral RNA using qPCR (Kim et al., 2021; Zhang et al., 2022).

By using ddPCR to quantify the total amount of pollen DNA or RNA in the collected and processed samples, it is possible to analyse pollen abundance and seasonality patterns. This information can be useful for tracking vegetation phenology, understanding the timing and duration of pollen seasons, and assessing the impact of environmental factors on pollen production (Banerjee et al., 2022). The downside is that costs of instruments and consumables can be higher than for qPCR, a higher risk of contamination and a limited reaction volume mixture (Nyaruaba et al., 2019). These are probably reasons why the technology is currently less available worldwide, in particular in developing countries (Nyaruaba et al., 2019).

dPCR in contrast to ddPCR uses microfluidic wells as reaction sites instead of droplets. As no generation of droplets is needed, this makes the technology simpler to use, less prone to errors and more cost effective. dPCR is already used in various fields for analysis of DNA in different types of samples. The method has also been tested for environmental air samples. However, no publications presenting the results of analysing air samples were found with our search parameters (Quan et al., 2018; Cao et al., 2020; King et al., 2020).

8.3. Sequencing technologies and reference databases

With high throughput sequencing technologies and bioinformatics analyses it is possible to describe the biological diversity of environmental samples (mostly complex communities) at different levels (Deiner et al., 2017). Different technologies have been used intensively to detect and analyse airborne microbial communities in various aerobiological samples in the past decades. Samples from indoor and outdoor environments were analysed regarding a broad-spectrum of aerodispersed plant pests and human pathogens (Yooseph et al., 2013; Kováts et al., 2016; Prussin et al., 2016; Banchi et al., 2020c). Beside community information, also additional information such as possible metabolic processes within the communities and the interaction with the environment can be deciphered (Segata et al., 2014).

8.3.1. DNA metabarcoding

DNA metabarcoding (MBC), also known as targeted amplicon parallel sequencing, uses universal primer sets. This technique can amplify a specific DNA region across multiple species at the same time to examine the present sequences, i.e. "barcodes", and address them to specific taxa. The method can be used to simultaneously determine species presence and diversity, sometimes even enabling the detection of novel species in mixed samples. Besides, these fragments are also useful to detect genetic alterations, such as single nucleotide polymorphisms

(SNPs) or insertion/deletions (INDELs). In the reviewed papers, metabarcoding was the most prominent analytical method and used in 141 (out of 178) studies as the only method or in combination with PCR or metagenomic sequencing.

Regarding sequencing technologies, the so-called next generation sequencing or second-generation sequencing were employed in most studies. The short-read sequencing is based on Illumina platforms, which are currently the most widely used in the laboratories (Cantu et al., 2022). Still, some Illumina instruments show low precision in SNP and INDELs detection compared to other short read platforms. Other short read sequencing instruments are the HiSeq $10\times$ and the NovaSeq (150-bp and 250-bp paired-end chemistries) by Illumina, the BGISEQ-500 and MGISEQ-2000 by the Beijing Genomic Institute Group (BGI), and its subsidiary MGI Tech Co., Ltd. (MGI), respectively, as well as the GS111 by GenapSys (Drmanac et al., 2010; Goodwin et al., 2016; Cantu et al., 2022). BGISEQ-500 showed low mapping efficiency and high multiple-mapping rate when compared to other short read sequencing platforms, likely due to SR lengths. BGISEQ-500 and MGIDEQ-2000 both had lower duplicate and unmapped reads, which may be attributed to the pattern array of the flow cells used by the instruments (Cantu et al., 2022).

In comparison to traditional Sanger sequencing, only a small amount of DNA is needed as a template to achieve accurate and reliable data. The sequence time is significantly reduced, and multiple samples can be run in parallel, by attaching an identifier sequence. DNA metabarcoding allows the detection of low frequency variants, quantitative analysis of mixed populations, and scalable analysis of many samples. Although this is a powerful approach, it still only shows a part of the real diversity, as it is prone to amplification bias, such as species abundance is influenced by the selected primer set, the used database (completeness) and quantification is only relative to the number of sequenced reads (Lindahl et al., 2013; Tedersoo et al., 2018; Nilsson et al., 2019a).

There are also cases in which species identification cannot be performed at all, as the sequence has not yet been deposited in a database or the barcode is not unique. The amount of sequence data, used as reference and representing the species barcodes, derive from previous sequencing efforts. These are based on traditional Sanger sequencing (Sanger et al., 1977), where individually DNA of species is extracted, amplified, and sequenced for genetical characterization. This is followed by significant editing and quality controls that are necessary before sequences are taxonomically annotated (reliable taxonomic assignment) and become publicly available. However, up to date, many organisms still have no published sequence information, i.e., their own barcode, to allow for identification. Consequently, most metabarcoding studies can only confidently characterize communities to the genus or family level (Nilsson et al., 2019a).

8.3.2. Metagenomic sequencing

In contrast to metabarcoding, no amplification of a specific region is used in metagenomic sequencing (MGS). Environmental samples are processed, the DNA is extracted, and the complex mixture is analysed. This methodology enables a wide and profound characterization of genome wide mRNAs, small RNAs, chromatin structure and DNA methylation patterns, transcription factors, microbial communities, and metagenomics (Ansorge, 2009). In the reviewed studies MGS was only used in 13 out 178 studies. This low number, compared to MBC, is probably due to the higher costs of the analytical method as well as the higher amount of DNA needed, as the analysis is ideally performed without a multiplication step (e.g. PCR). As volumes of air samples are limited and the concentration of organisms relatively low compared to other matrixes like soil, sufficient amounts of DNA are not easy to achieve.

In the early 2000s, shotgun sequencing was predominately used for this approach. In 2005 the first high-throughput sequencing and metagenomic analysis of environmental samples was published, using massive parallel 454 pyrosequencing. Sequencing technologies also employed in metabarcoding approaches are used for metagenomic

analysis, namely Illumina MiSeq and HiSeq, SOLiD, Ion Torrent and 454 pyrosequencing. A new wave of sequencing technologies has emerged, and third generation sequencing is mainly used for metagenomics analyses (Taberlet et al., 2012).

Compared to short-read (SR) sequencing up to a few hundreds of base pairs in length, third generation sequencing generates reads much longer (i.e., long reads, LR). Although they have rarely been used in aerobiological studies so far, it is worth mentioning them here as potential next approaches to pursue in the analyses of aerodispersed organisms. The long fragments are up to several thousand nucleotides in length and can also detect larger structural alterations in the DNA regions (e.g., translocations, inversions, duplications). LR sequencing makes it able to resolve difficult-to-sequence regions of the genome, especially when large stretches of repetitive sequences are present and represent a challenge to align to the correct portion of the reference genome (Cantu et al., 2022).

Instruments using this sequencing technology are devices from Oxford Nanopore Technology (ONT), such as the PromethION (R9.4 flow cell) and MinION (Flongle and R9.4 flow cells) and Pacific Biosciences (PacBio) circular consensus sequencing. Ion Torrent and GenapSys technology use sequencing chips that detect changes in pH and electrical impedance, respectively, as nucleotides are incorporated during sequencing (Buermans and den Dunnen, 2014). Comparing the platforms, PromethION provides the highest throughput, capable of large population-based studies; they have the lowest mapping rate out of all platforms and capture the lowest proportion of INDELs. PromethION outperformed all short-read instruments in RepeatMasker regions and was comparable to PacBio. MinION data cannot be compared to other platforms in repetitive regions or SNV/INDEL detection due to the low coverage. A considerable advantage with the ONT instruments is the lower capital costs compared to e.g. PacBio and the real-time analysis of the ONT as the nucleotides pass through the nanopore. Furthermore, the MinION can be used as a portable devise in the field or in a laboratory using scalable flow cells to accommodate smaller data sets (Flongle) and larger data sets (R9.4). However, in technology comparison, ONT instruments had the highest error rates in repetitive regions but performed very consistently across samples. PacBio on the other hand offered the highest mapping rate and best performance in repetitive regions, outperforming the other platforms. Compared to short read sequencing, error rates were lower for long read sequencing with comparable mismatch rates to short read sequencing (Cantu et al., 2022).

Third generation sequencing based on Oxford Nanopore Sequencing (MinION) was applied in a metabarcoding analysis, describing the diversity of aerodispersed fungi and bacteria associated with wax drops on ancient manuscripts (Pavlović et al., 2022). The analysis was complemented with culture isolation of fungi and bacteria (which were tested for their enzymatic activities) and studying the mechanism of colonisation of wax drops by the airborne microorganisms (Pavlović et al., 2022). Recently, nanopore technology using the Nanopore Grid-ION benchtop device was selected for a campaign DNA analysis, producing the first high-resolution bioaerosol composition dataset in Northern Europe (Sofiev et al., 2022). Here, although a variety of samplers were employed and enough DNA was always yielded for the metabarcoding sequencing technology, the results highlighted the deficiency of generic DNA databases for their applications to atmospheric biota. Indeed, about 40 % of the samples were not identified with standard bioinformatics methods (Sofiev et al., 2022). Like in metabarcoding analysis, third generation sequencing, is based on annotation of sequences to respective species. Therefore, the usefulness of new sequencing technologies is dependent on matchable datasets, which further stresses the importance of the richness and accuracy of the available database.

8.3.3. Databases

DNA metabarcoding databases for fungal (i.e. UNITE, Nilsson et al., 2019b) and bacterial (i.e. SILVA; Quast et al., 2013) sequences have

been launched already since 2003 (Kõljalg et al., 2013) and progressively updated. Nowadays, ad hoc databases dedicated to certain group of taxa specifically associated to certain ecologies and/or environments are created to avoid misleading conclusions such as a database for all flowering species found in Wales (de Vere et al., 2012). For example, aerobiological studies have assisted in the creation of the database for cereal rust fungi (nuclear ITS2 locus, CR-ITS2-refDB; Chen et al., 2022), and one for plants (entire nuclear ITS region of pollen DNA; PLANiTS) by Banchi et al. (Banchi et al., 2020a). The CR-ITS2-refDB (Chen et al., 2022) presents a curated ITS2 reference database for a selection of rust species. It was created to evaluate intraspecific variation for the suitability of ITS2 as a marker in rust diagnostic. In silico assessment of taxonomic coverage and specificity of nine forward and seven reverse fungal ITS2 primers to rust fungi were tested. Validation of primers was performed by metabarcoding of rust communities from air and rain samples, evaluating the accuracy of taxonomic assignment, by using different bioinformatic classifiers (Banchi et al., 2020a; Chen et al., 2022). The authors developed the approach 'better clustering for QIIME' (bc4q) to ensure that representative sequences were chosen according to the composition of the cluster at a different taxonomic level.

For fungi and bacteria, the databases are biased towards (easy) culturable species, as sequences were obtained from cultured isolates captured on artificial media and analysed by cultivation, as performed in many traditional aerobiological analyses (Duan et al., 2021; Wu et al., 2021). In general there are many papers dealing with bacteria and viruses (Masclaux et al., 2013; Behzad et al., 2015; Kováts et al., 2019; Mhuireach et al., 2019; Mucci et al., 2020; Ruiz-Gil et al., 2020; Sun et al., 2020; Li et al., 2021), but fewer studies have tried to examine fungal communities (Yooseph et al., 2013; Oh et al., 2014; Tong et al., 2017; Banchi et al., 2020b; Polling et al., 2022) or pollen (Bell et al., 2019; de Groot et al., 2021), or even both contemporaneously, by high throughput sequencing. A substantial limitation is the incompleteness of databases. Estimates of global biodiversity of fungi range from 0.5 to almost 20 million species, with a best estimate of 2–3 million (Niskanen et al., 2023) which is far beyond the number of about 155.000 documented species. Also, recently, the metagenome analysis of antibiotic resistance in urban air has received significant attention (Bell et al., 2019; de Groot et al., 2021) and it is expected, that in the near future due to the continuous advances in the field, metagenomic approaches could become convenient tools for evaluating and studying airborne communities.

Although most of the reviewed studies described the used database and pipelines for sequence analysis, still ten papers did not specify the analysis of the sequences.

9. Data quality

Though DNA-based technologies are used for many years in different environmental studies, no common protocol and standardized procedure are available by now. The outcome of the studies is based on the chosen sampling and preparation methodologies, the used primers, amplification and sequencing technology, and databases used for comparison. The inclusion of control samples, such as negative controls for sampling, DNA extraction and PCR, and positive controls (including mock communities, see below) improves the reliability of the scientific results by providing a means by which it is possible to assess the accuracy of the analyses (Brennan et al., 2019). Negative and positive controls inform about extraneous material, cross-contamination, and possible index variation (Carlsen et al., 2012; Esling et al., 2015). However, the use of controls is seldom mentioned in the reviewed studies.

Positive controls and mock populations can consist of artificially synthesized molecules or DNA extracts of actual species known not to occur in the experimental system (Song et al., 2015). For air samples metabarcoding studies are very heterogeneous, thus they still suffer from the lack of standardized methods and from several biases that can

be generated from the sampling steps up to the data analyses, including DNA extractions and amplifications, the choice of the target DNA barcode(s), the use of sequencing platforms, and the lack of reference sequences in public databases (Banchi et al., 2020b). Many biases can be overcome by using mock communities, i.e. artificially created samples composed of an ad hoc DNA mix of known organisms, that are employed from the first steps of the metabarcoding study. They proved to be crucial in estimating the reliability of the results in terms of taxonomic identification and proportion of sequenced reads (i.e., relative abundance), preferential amplification, identification of primer biases, false-positive signals, and the presence of any contamination (Lear et al., 2018; Banchi et al., 2020b). These artificially created communities should include more than ten species with variable amplicon length and GC-content, and the quantity based on actual marker copy numbers. This number may be much larger, depending on the design of the study, e.g. as in a UK based study, where the positive control involved 52 different species of grass and a comparable number of negative controls (Brennan et al., 2019). Additionally, due to index switching issues, it is desirable to consider the species-specific composition of the mock community.

However, only about 10 % of the studies dealing with eDNA metabarcoding on airborne plants and fungi report on mock communities (Banchi et al., 2020b; Banchi et al., 2020c). Bacterial mock communities from other sample types are commercialized and in general more frequently included in the analyses (Lear et al., 2018; Joos et al., 2020), while mock communities for plants or fungi are less common and only a few have been proposed for standardization (Bakker, 2018; Egan et al., 2018; McTaggart et al., 2019). Even rarer are studies reporting on mock communities on multiple groups of organisms (Degois et al., 2017 on Eukarya). In the past few years, mock communities for fungi have increased and arbuscular mycorrhizal fungi (Egan et al., 2018), the gut fungi of animal mycobiomes (Arfken et al., 2023) and fungi in agricultural and forest soils (Pauvert et al., 2019) have been used to generate new mock communities. Mock communities for aerobiological studies have been first proposed by Banchi et al. (Banchi et al., 2020b) who assembled two mock communities for plant and fungi together. In the first mock community the different DNA samples were mixed in an even amount, in the second, the DNA samples were serially diluted. In both types of mock communities, the different concentrations of the fungal and plant taxa were proportionally calculated starting from the amount of the extracted DNA (Banchi et al., 2020b), in order to simulate environmental conditions at which organisms are present in different amounts.

Besides using positive controls and mock communities, the data analysis itself can strongly influence the results. Using the right tools and formats is crucial as exemplified by errors in gene name conversion when Excel is used to create gene lists (Ziemann et al., 2016). Another possible error is amplicon processing, where forward and reverse reads are not assigned correctly, without consideration for chimeric reads. This may lead to the finding of "new species" when working with ASVs (amplicon sequence variants) or loss of data when working with OTUs (operational taxonomic units) (Edgar et al., 2011; Callahan et al., 2016). In general, when using DNA based analysis, it is also important to consider the low amount of material in air and material losses during sample preparation. PCR based techniques and sequencing used to assess biological air quality are commonly facing issues with low number of particles in air, material loss during sampling, improper sample preparation, inefficient cell disruption and DNA extraction (Luhung et al., 2015). Summary of key factors influencing the results.

10. Summary of key factors influencing the results

Summarizing our findings, a variety of approaches have been used for sampling, sample processing, DNA extraction, and analysis in eDNA studies. However, not all studies provide detailed descriptions of each step, particularly regarding storage and transport times and conditions.

For analyses, metabarcoding and metagenomic approaches are commonly employed and are considered state-of-the-art techniques in bioaerosol studies. Again, not all studies describe all steps in sequence analysis, including the used databases and analytical pipelines.

In terms of the completeness of diversity assessments, there is a notable lack of studies targeting more than one organism group simultaneously. Although universal barcodes for viruses do not exist, metabarcoding strategies for fungi, bacteria, and plants are well-established, and their combination can provide deeper insights into the diversity within an air sample. These combined approaches could further enhance the interdisciplinarity of aerobiological studies and considerations.

From a geographical perspective, the spatial distribution of available data reveals a strong concentration of studies in specific regions. Most studies are focused on Europe, highly developed countries, and large cities, while only a few samples have been collected so far from sensitive areas such as Greenland, Antarctica, and the Arctic. However, rural, and remote locations are under-represented, highlighting the need for additional funding and collaborations to support sampling campaigns in these regions. Moreover, the development of robust protocols is essential to ensure high-quality samples, especially when long transport or storage times are necessary before processing. Molecular techniques, especially in their early years of application, were seen as the key to unlocking the total diversity of bioaerosols and offering a holistic view of environmental biodiversity. While every advance fills another piece of the puzzle, and each new detail contributes to a more complete picture, the acquisition of the complete information can be reduced at various stages along the analytical chain (see Fig. 3).

The first critical step in data acquisition is at the planning of the sampling campaign, which involves decisions about how, where, and when to collect samples and how many of these to collect, to obtain later results which reliably represent the studied environmental setting. Day-to-day, seasonal, and spatial variations make each sample a snapshot of specific environmental conditions at a given time. The choice of sampling device also plays a critical role, as different factors must be considered, including the target organism groups, environmental conditions during sampling, availability of devices, biological sampling efficiency, cost, and the volume of air sampled. Also, the sampling airflow can range from 1.5 L/m (low volume) to over 1000 L/m (high volume).

After sampling, either during storage and/or transportation any sample and/or the extracted DNA can be altered, or damaged and not be used for further analyses. Storage guidelines vary depending on the organism groups involved. For long-term storage (from a few months to extended periods), it is recommended that bioaerosol samples be kept at $-70\ °C$ to minimize genetic material loss or sample alteration due to organism growth. Initial storage at 4 °C immediately after sampling and short-term storage at $-20\ °C$ are also recommended.

Sample processing prior to molecular analysis can also impact the outcome of the analysis. The extraction of collected material may not be complete, or it may be biased towards certain taxa. Additionally, reagent formulation, DNA or RNA extraction methods, and the storage of genetic material can reduce the amount of recoverable information. For example, nucleotides may adhere to the walls of reaction tubes, diminishing the yield. For DNA extraction a multitude of different kits have been used with various alterations, reducing the possibility of harmonization, and recommending a common approach.

The lack of standardization is especially critical in metabarcoding and PCR analysis, where the choice of primers plays a key role in results obtained from a sample. The chosen primer set may enhance the amplification of specific taxa while neglecting others. Rigorous quality control of primers and testing against comprehensive databases such as NCBI, GeneBank, and SILVA are essential for primer design and validation. Testing primers against a wide range of sequences and isolates provides insights into amplification gaps or errors.

In metabarcoding or metagenomic approaches, the processing of samples, library preparation, and sequencing technologies can also influence the results. One of the primary limitations of DNA
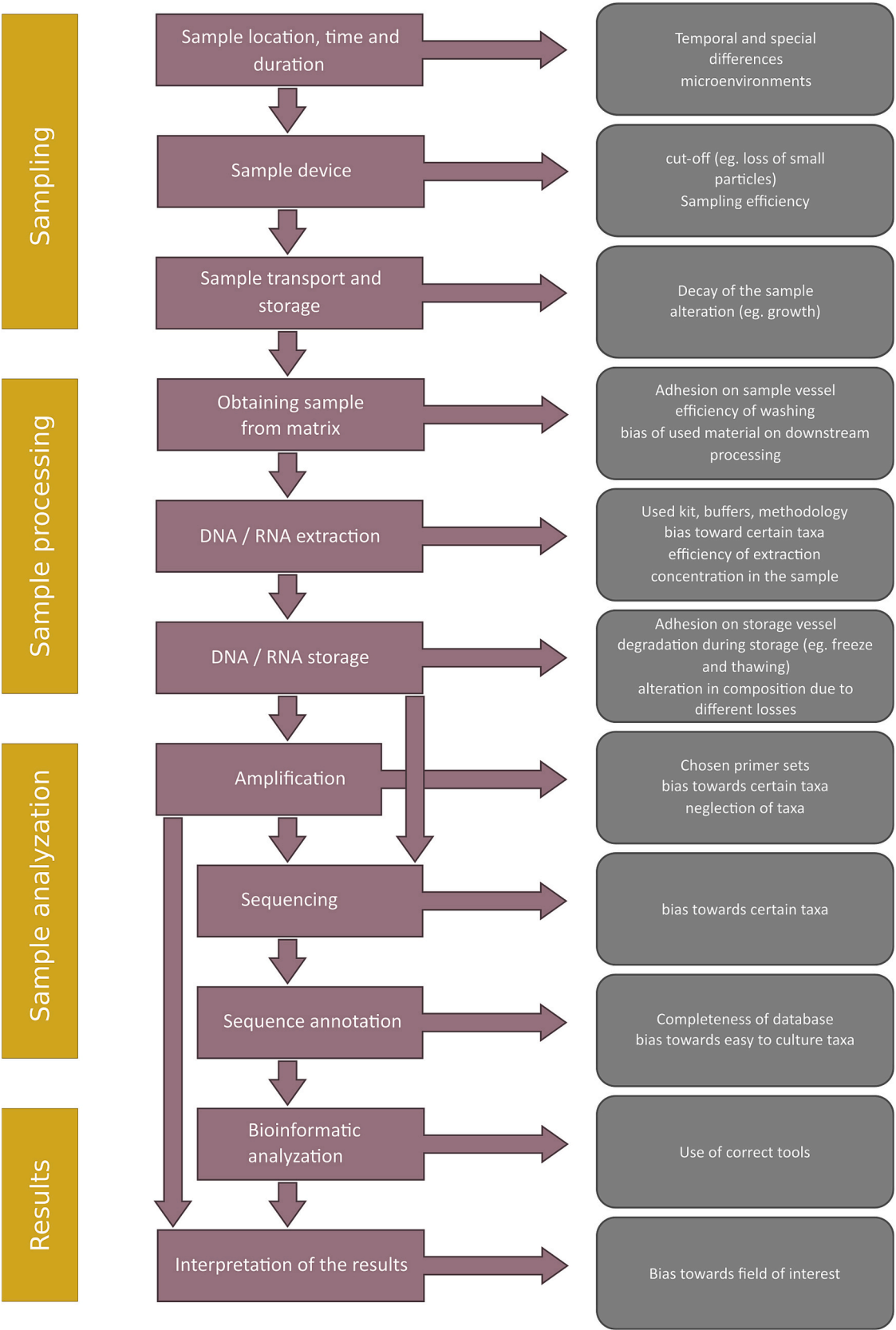
Fig. 3. flowchart of points along the analytical chain and steps where information may be lost or altered influencing the quality, depth or completeness of obtained data.

metabarcoding and metagenomic analysis lies in the reliance on existing genetic databases. Many organisms still lack published genetic sequences, meaning that without accurate annotation, the final step from sequencing data to meaningful results cannot be completed, leading to many unclassified sequences.

Lastly, appropriate controls should be included at every stage of the analytical chain. This includes validation of the procedures, negative controls, and mock community DNA samples of known composition as positive controls.

11. The future

Considering existing studies and knowledge gaps, there is an urgent need to include all information about used methodologies in published scientific articles. Especially the lack of information on used DNA or RNA extraction kits and modification, used sequence database and analytical tools is concerning. The vast variety of use extraction protocols and lack of description makes harmonization or even giving a comprehensive overview with recommendations not possible at this point.

The gaps in available data show the need for worldwide and remote sampling locations and target of more than one organism type. Even though the inclusion of viruses is in general more difficult as no barcoding primers are possible and for RNA virus a reverse transcription step is necessary. Recent European networking strategies showed the lack of interdisciplinarity and exchange between scientist from different fields (e.g. indoor, outdoor, occupational, fungi, bacteria, pollen, medicine, allergy research and exposure assessment) (Walser-Reichenbach et al., 2020).

The feasibility of widespread sampling campaigns is dependent on harmonization of acceptable sampling methods, funding and may include automated devices (like semi-automated cyclone samplers). Especially the diverse use of sampling equipment shows that there is no agreed optimal sampling solution applicable in various environments for downstream DNA analysis. Although there will be no device fitting all purposes, a couple of agreed approaches would make comparisons between sampling campaigns more feasible, especially considering that different devices have different size cut-offs, airflows and sampling times, and therefore different taxa preference in the sampling step.

Considering the advances in sequencing technology, the future may hold even more extensive data sets, which themselves need good databases and profound analytical knowledge to extract meaningful results.

The combination of harmonized sampling with established and new analytical techniques such as dPCR, metabarcoding and Oxford Nanopore Technology may be an approach for generating more complete time series of bioaerosol samples with comparable information output. To test this approach, a European wide international study is proposed by participants of the COST network ADOPT (comprising 38 European Countries) and a yearlong sampling is planned for 2024/2025.

As for the assessment of health effects, interconnecting different fields and merging existing knowledge will be one key to shed more light on dose-response relationships and the connection between biodiversity and human health (Walser-Reichenbach et al., 2020). However, to make DNA or RNA data from samples more meaningful in this regard, the collection of high-quality contextual information of the sampling site, its use, surroundings, and other influencing factors is necessary. Regarding virus sampling, collecting of this information was also stressed by a recent review (Dias et al., 2024).

This is especially true for assessing present allergen-holding taxa, as the presence of allergenic pollen or fungi alone does not equal allergen exposure. Other environmental and/or intrinsic co-factors affecting allergenicity of pollen/fungal spores need to be taken into consideration (e.g., pollutants, carbohydrates, lipids, etc.). This underlines the need for interdisciplinarity, especially in evaluating the impact of obtained data and diversity.

In assessing the results, data users must be aware that even the most comprehensive datasets will be still incomplete and cannot give the whole picture. Furthermore, to prevent the risk of losing information and be unable to generate a sufficient number of results, useful to conclude any study, we propose that the points given in Table 4 are mandatory information to be presented when publishing articles on airborne DNA analysis.

Furthermore, it is of uppermost importance that the generated data, with particular focus on molecular sequence data, are made available to the scientific community, so that evaluation and reproducibility of the results are feasible. Therefore, we propose that raw data, as is already needed for many EU funded projects under the FAIR dataset requirement, are made publicly available, providing access to a repository or even platforms for exchange of raw sequencing data (such as the one provided by the open-research-Europe, funded by the European Union, https://open-research-europe.ec.europa.eu). The increasing availability of sequence data would also contribute to the completeness of reference libraries, which are essential for the next sequence identification and annotation processes.

12. Concluding remarks

Summarizing, molecular analysis with eDNA metabarcoding and metagenomic sequencing has become essential for the study of aerobiota, but it still presents limitations and weak points. Overcoming these limitations in the near future represents a challenge for aerobiology, whose advances will also be significant for agriculture, ecology, climate change and human health. The detection of agricultural pests, invasive species, allergens, and toxins that cause diseases, as well as the establishment of taxonomic, conservation, and phylogenetic relationship studies between taxa, are among its important applications.

Metabarcoding and metagenomic sequencing not only enable the detection of novel species from indoor and outdoor environments but also provide information about metabolic processes within the communities and their interaction with the environment. Metabarcoding

Table 4
list of required information, that should be given in a publication and easy to extract for intercomparison of studies.

Required information	Details
Sampling location	Geo coordinates; Country; State; City[a]
Sampling point	Description of where the sample was taken or the sampler was mounted; indoor/outdoor, height of sampling (rooftop, cupboard, distance from exhaust or wall)
Sampling time	Date or month and year of sampling; Duration of sampling[b]
Sampling device	Device(s) used for sampling, collection surface/buffer
	Used sampling method
	Brand of devices
	Flow rate
Transport & storage	Transport and storage of initial samples as well as storing of intermediate processed samples
Number of samples	Number of samples with each sampling device and used for farther analysis
Target organisms	Which organisms are targeted with which samples
	How are processing steps optimized towards the target
Processing steps	How was the sample treated
	Details on the DNA extraction
	DNA storage till analysis
Method for analysis	Description of the method, including the type of method, target analysed
	Used devices for analyzation
	Sequencing approach
	Sequencing technology
	Region sequenced
Used database	Databases used for sequencing analysis
	Programs and packages used
Location of data publication	If FAIR datasets are produced within the described study, the location of the data should be stated

[a] If exact coordinates are not possible, due to compliance of the location, at least country and city should be stated.

[b] At least the season, year and number of sampling days.

and metagenomics need to be complemented with quantitative analyses, such as microscopical enumeration of airborne particles, automated detection methods or quantitative molecular genetics tools (qPCR, ddPCR, dPCR). Such numbers are critical for many climate change studies and mitigation strategies, e.g. in relation to human health, forestry, or agriculture.

Furthermore, some data in databases and their associated metadata are still missing but they are needed as the basis for a correct taxonomic assignment, leading to meaningful results. Much effort is needed in this area to complete the databases, thus improving the reliability of meta-barcoding studies.

Maintaining the quality control of molecular analysis based on eDNA requires the standardization of procedures, a challenge that can follow the example of standardization guidelines already established for other procedures applied to aerobiology, such as pollen and spore count or bacteria and fungal spore culture techniques.

CRediT authorship contribution statement

C.-E. Pogner: Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **C. Antunes:** Writing – review & editing, Writing – original draft. **G.P. Apangu:** Writing – original draft. **N. Bruffaerts:** Writing – review & editing, Writing – original draft. **S. Celenk:** Writing – original draft. **A. Cristofori:** Writing – review & editing, Writing – original draft. **N. González Roldán:** Formal analysis, Data curation. **A. Grinn-Gofroń:** Writing – original draft. **B. Lara:** Formal analysis, Data curation. **M. Lika:** Writing – original draft, Data curation. **D. Magyar:** Formal analysis, Data curation. **M. Martinez-Bracero:** Formal analysis, Data curation. **L. Muggia:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **B. Muyshondt:** Writing – review & editing, Writing – original draft. **D. O'Connor:** Formal analysis, Data curation. **A. Pallavicini:** Writing – review & editing, Writing – original draft. **M.A. Marchã Penha:** Writing – original draft, Formal analysis, Data curation. **R. Pérez-Badia:** Writing – review & editing, Formal analysis, Data curation. **H. Ribeiro:** Writing – review & editing, Writing – original draft. **A. Rodrigues Costa:** Writing – original draft, Formal analysis, Data curation. **Z. Tischner:** Formal analysis, Data curation. **M. Xhetani:** Writing – review & editing, Writing – original draft. **C. Ambelas Skjøth:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Merita Xhetani and Mirela Lika reports financial support was provided by Albanian-American Development Foundation (AADF and IIE). Alexandra Penha, Ana Costa, Celia M Antunes, Helena Ribeiro reports financial support was provided by Foundation for Science and Technology. Lucia Muggia and Alberto Pallavicini reports financial support was provided by Interconnected Nord-Est Innovation Ecosystem. Rosa Perez-Badia and Beatriz Lara reports financial support was provided by Government of Spain Ministry of Health. Rosa Perez-Badia and Beatriz Lara reports financial support was provided by Junta de Comunidades de Castilla-La Mancha. Agnieszka Grinn-Gofron reports financial support was provided by Ministry of Education and Science of the Republic of Poland. Zsofia Tischner reports financial support was provided by Ministry of Innovation and Technology of Hungary. Carsten Ambelas Skjoth reports financial support was provided by Novo Nordisk Foundation. Babette Muyshondt reports financial support was provided by FPS Health, Food Chain Safety and Environment, service indoor air quality. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.scitotenv.2024.177439.

Data availability

No data was used for the research described in the article.

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