Monitoring techniques for pollen allergy risk assessment

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Abstract

Understanding airborne pollen allergens trends is of great importance for the high prevalence and the socio-economic impact that pollen-related respiratory diseases have on a global scale. Pursuing this aim, aeropalynology evolved as a broad and complex field, that requires multidisciplinary knowledge covering the molecular identity of pollen allergens, the nature of allergen-bearing particles (pollen grains, pollen sub-particles, and small airborne particles), and the distributions of their sources. To estimate the health hazard that urban vegetation and atmospheric pollen concentrations pose to allergic subjects, it is pivotal to develop efficient and rapid monitoring systems and reliable allergic risk indices. Here, we review different pollen allergens monitoring approaches, classifying them into I) vegetation-based, II) pollen-based, and III) allergen-based, and underlining their advantages and limits.
Finally, we discuss the outstanding issues and directions for future research that will further clarify our understanding of pollen aeroallergens dynamics and allergen avoidance strategies.

Keywords: pollen, aeroallergens, monitoring, pollinosis, allergenicity index, air samplers

Abbreviations:
AR: Allergic Rhinitis;
PM: Particulate Matter
SAI: Specific Allergenic Index;
I$_{UGZA}$: Urban Green Zones Allergenicity Index;
AIROT: Aerobiological Index of Risk for Ornamental Trees;
CNN: Convolutional Neural Networks;
RT: real-time;
LIF: laser (or light) induced fluorescence;
qPCR: quantitative PCR;
cpDNA: chloroplast DNA;
NGS: Next Generation Sequencing;
CE: Capillary electromigration.

1 Introduction

Allergic rhinitis (AR), allergic rhino conjunctivitis and asthma affect a significant share of the global population, with a higher prevalence in developed countries. It is estimated that over 300 million people worldwide suffer from asthma, while AR occurs in about 500 million people, out of whom 200 million have asthma as a comorbidity (Ozdoganoglu and Songu, 2012; Simunovic et al., 2020).

Pollen is a common cause of these respiratory diseases, as more than 150 pollen proteins have been proven to cause allergic sensitisation (Xie et al., 2019). Allergenic plants are mostly wind-pollinated species, that have to release huge amounts of pollen in the atmosphere to reach a successful reproduction. This exposes sensitive subjects to pollen allergens for several months of the year, increasing their probability to become sensitised against one or more pollen types (D'Amato et al., 2007). Pollen sensitisation occurs when the subject inhales a quantity of allergenic pollen exceeding a certain threshold level, that variates according to the individual genetic background, the pollen type, and environmental factors. Sensitisation usually takes place in the respiratory mucosa, where humidity causes inhaled pollen to hydrate. Hydrated pollen releases some proteins (pollen allergens) that are misidentified by the immune system as possible antigens, activating an IgE-mediated hypersensitivity reaction against them (Asam et al., 2015; Mothes et al., 2004). This causes an inflammation of the upper airways symptomatic of the seasonal AR commonly known as "hay fever" or more properly "pollinosis" (D'Amato et al., 2007). According to the International Study of Asthma and Allergies in Childhood (ISAAC), the global prevalence of pollinosis at the beginning of this century was 22.1% in older children (13- to 14-yr-old) and 11.8% in younger ones (6- to 7-yr-old), with an annual increment of 0.3% in both age groups (Björkstén et al., 2008). These values however variate greatly between different geographic regions, because pollen allergy incidence is influenced by environmental and bioclimatic conditions that define allergenic plants distribution.

Pollen grains diameter ranges from 5 to 200 µm, so they can only enter the upper airways. Nonetheless, in the last decades it was proven that pollen allergens can also be carried by small particles of 2–5 µm in diameter, such as particulate matter (PM) and plant fragments. In this way, they can reach the lower, narrower airways, triggering allergic asthma. This situation often occurs during thunderstorms, when higher concentrations of pollen are resuspended in the air, and meteorological conditions promote the transfer of its allergens to other particles (Burge and Rogers, 2000; D'Amato, 2001; Harun et al., 2019). This "thunderstorm asthma" also occurs in pollinosis sufferers with no prior diagnosis of asthma. Furthermore, high pollen days during peak pollen season ($\geq$50 grains/m^3) can increase the number of emergency department presentations for asthma attacks (Erbas et al., 2018). Some authors also recorded a dose-response association between pollen exposure and asthma symptoms, suggesting threshold concentrations for different pollen types (Erbas et al., 2012; Galan et al., 2010; Tobias et al., 2004). High atmospheric levels of grass pollen, in particular, seem to be positively associated with severe asthmatic reactions in temperate climates, according to some authors (Erbas et al., 2018). However, this correlation is not always apparent, especially in subtropical areas (Ridolo et al., 2007; Simunovic et al., 2020). In European countries, also tree pollen seems to be positively related to asthma onsets (Guilbert et al., 2018; Ridolo et al., 2007).

The swelling of the upper airways caused by pollen in allergic subjects might explain asthma symptoms exacerbation. Besides, since the majority of asthmatics have rhinitis and up to 40% of patients with rhinitis have asthma, it cannot be ruled out that the two diseases sometimes exist as a continuum of inflammation, due to the functional complementarity of upper and lower airways, their interactions, and the similarity of their mucosae. This concept is known as “one airway, one disease” (Bousquet et al., 2008; Grossman, 1997)

Even if the role of pollen allergy in the global burden of asthma is yet to be comprehended, it is clear that both allergic asthma and pollinosis heavily affect the quality of life of pollen allergy sufferers, impairing their mental health, compromising their education and professional careers through presenteeism and absenteeism, and consequently lowering their productivity (Zuberbier et al., 2014).

Due to the high prevalence, the ubiquitous diffusion, and the medical and social burden of pollen allergies, their management is of global relevance. Nonetheless, to date there is still no decisive cure for this disease, hence it is important for pollen allergic subjects to be constantly aware of atmospheric pollen allergens level during the pollen season, in order to plan their movements and medications accordingly (Geller-Bernstein and Portnoy, 2019).

The regional nature of allergenic plants distribution, and the different biology fields involved in this topic, have promoted the development of a broad range of techniques and approaches to pollen allergenicity monitoring. This proliferation of methodologies for pollen allergy risk assessment calls for periodic literature reviews to summarise and classify them, and to address their optimal applications. With the present work, we propose a classification of pollen allergy monitoring methods based on their subject: (I) “vegetation-based” for ecological approaches focusing on allergenic plants; (II) “pollen-based” for aeropalynologycal approaches monitoring airborne pollen; (III) “allergen-based” for molecular approaches detecting airborne pollen allergens. We discuss their viability for risk assessment and the better context for their implementation, prospecting possible improvements and future developments.

It is however important to remark that the methods hereby presented are not exhaustive, since they are not comprehensive of the whole literature on aerobiology studies and approaches. In fact, due to the vastity of the topic, in some cases it has been necessary to limit the discussion to the most common or promising techniques.

2 Vegetation-based approach

2.1 Vegetation-based monitoring: allergenicity indices

Distribution of allergenic pollen can be the result of natural floristic patterns, or a consequence of human landscaping. Landscaping and gardening strongly influence the vegetation diversity of an area, by introducing alien species or boosting the dominance of few native species, changes that are reflected by the air biota (Burge and Rogers, 2000; Capotorti et al., 2020; Cariñanos and Casares-Porcel, 2011). While many of the ornamental native trees are highly allergenic (Thompson and Thompson, 2003), exotic pollen types are usually not problematic because the locals have never been exposed to them. Nonetheless, pollen allergens from different plants can be cross-reactive, triggering allergic symptoms in patients that have been directly sensitised only against one of them (Mothes et al., 2004). Moreover, the massive introduction of a wind-pollinated exotic plant can lead over time to the sensitisation of susceptible subjects against it, as it happened for elm trees in eastern U.S.A., Australian pine in Spain, and cypress in Italy (Cariñanos and Casares-Porcel, 2011; Mothes et al., 2004; Sposato and Scalese, 2013). To understand the allergenicity potential of the urban green areas, three main allergencity indices have been proposed in the last 20 years.

The first one published is called AI (Allergenic Index) or SAI (Specific Allergenic Index), and has been tested on urban spontaneous and anthropogenic vegetation in Italy and Serbia (Ciferri et al., 2006; Hruska, 2003; Mrđan et al., 2017). By this method, urban ecosystems are sampled along gradients to identify ecological drivers of allergophytes distribution. Then, SAI is calculated for each plant species as the sum of values attributed to life cycle,

$$SAI = \frac{\sum_{i=1}^{n} lc_i + pp_i + cr_i + a_i}{n} \quad ;$$

phenology, cross reactivity and abundance (Tab. 1). SAI for the whole green area is assumed to be the average SAI value of the individual species (Hruska, 2003), and it is calculated as follows:

with *n*= number of allergenic species, and the other parameters calculated for each species as explained in Tab.1.

This index ranges between 2 and 10, and plants or parks are considered slightly allergenic with SAI up to 3, moderately allergenic from 4 to 6, and strongly allergenic from 6 to 10. This ranking shows a positive correlation with the pathological picture of pollen-sensitized patients (Hruska, 2003). SAI has the advantage to acknowledge both cross-allergenicity and the ephemeral contribution of non-perennial species to the airborne pollen load. On the other hand, it does not refer to the actual allergenicity of plants, in terms of sensitisation incidence. Furthermore, in this index all the phenanthesis periods are considered overlapping, so it must be associated with phenograms.

Recently, Cariñanos and colleagues have proposed the Urban Green Zones Allergenicity Index (I_{UGZA}). First published in 2014, it has been fine-tuned and applied to various city parks in many European cities (Cariñanos et al., 2014, 2017, 2019; Jochner-Oette et al., 2019;

Kasprzyk et al., 2019b). The formula underwent through some changes over the years, but it can be generalized as it follows:

$$I_{UGZA} = \frac{1}{H_{max} * PAV_{max} * S_T} * \sum_{i=1}^{k} n_i * PAV_i * V_i$$

I_{UGZA} parameters, classified in biometric and biological, are explained in Tab. 2a and Tab. 2b.

The theoretical maximum value for I_{UGZA} is 1, ideally representing a surface entirely covered by the most allergenic plants at their maximum height. This assumption however is in contrast with the decision of the authors to not take into account the "exceptional values" (H=18 m, ap= 4) when calculating H_{max} and PAV_{max} (Cariñanos et al., 2016, 2014). Minimum I_{UGZA} value is 0, attainable when all the plant species are non-allergenic, or when they do not produce pollen.
Vegetation sampling strategies proposed by the authors range from a tree census of the whole park, to a selection of relevant species, to a combination of census for arboreal species and systematic sampling for herbaceous ones. Advantages of this method are the scalability (Cariñanos et al., 2017), and the evaluation of important allergenic features. There is also the possibility to simplify the method to a point that it might be applied almost directly on the floristic census of the area, on remote, without the guidance of experts.
There are anyway some downsides. First, I_{UGZA} assumes the hypothesis of a positive correlation between vegetation volume and pollen production, which to this date has not been proven. Hence, it is necessary to associate the evaluation of local airborne pollen concentrations to the allergenicity index, since I_{UGZA} alone is not always accurate in stating the real hazard posed to subjects with pollinosis by an urban green area (Kasprzyk et al., 2019a). Another improvable aspect is the standardization of sampling and calculation methods, to make the results comparable.

In 2019, Pecero-Casimiro and collaborators published the Aerobiological Index of Risk for Ornamental Trees (AIROT), merging plant biological features with non-biological factors that influence pollen production and dispersal, such as geographical features and urban landscape (Tab. 3) (Pecero-Casimiro et al., 2020, 2019). To do so, AIROT is combined with LiDAR remote sensing and Kriging interpolation, creating risk maps of pollen concentrations in urban environments, based on the distribution of allergenic ornamental trees and the presence of physical obstacles. This index can be calculated for individual plant species in different city areas, using the formula:

$$AIROT = \sum_{i=1}^{n} \frac{(PD_i \times (N_i \times \alpha_i) \times M_i \times Sh_i \times H_i)}{ST}$$

The results can be normalised on a scale from 0 (minimum risk) to 1 (maximum risk), creating a legend for risk maps and safe itineraries. However, the actual influence of its parameters on pollen concentrations is still unclear, therefore a comparison with observed airborne pollen concentrations is needed to assess this method reliability.

2.2 Vegetation-based prevention: gardening guidelines and plant control

Since urban vegetation is anthropogenic, the impact of urban greenery on public health can be reduced by adopting hypoallergenic gardening guidelines (Thompson and Thompson, 2003). The most direct way to reduce allergenic pollen availability is to avoid planting potentially allergenic species. The American Academy of Asthma and Immunology advices gardeners to only use entomophilous, non-toxic and non-invasive plants, and offers a list of recommended low-allergenic plants suitable for different regions (Green et al., 2018). Another option is to prefer sterile varieties or species with low to moderate pollen productivity. For dioecious species, mainly female individuals should be selected, whether this does not pose substantial problems of fruits and seeds littering. When utter omission of allergenic plants is impossible, it is important to not associate cross-allergenic species, to avoid potentially invasive species, and to adopt an appropriate pruning schedule to reduce or prevent the blooming (Cariñanos and Casares-Porcel, 2011; Green et al., 2018). These guidelines hardly apply to lawns, where grass allergens can also be aerosolised with mowing, even when flowers are absent. Hence, low-allergenic herbs and shrubs should preferred, along with hardscaping alternatives (Green et al., 2018). These guidelines however are difficult to apply to the existing urban vegetation.

In fact, removal of healthy trees from a landscape because of their allergenicity is not convenient from a cost-benefit point of view. Even when planning new public green areas, enforcing these guidelines in plant selection over aesthetic and practical criteria might be challenging. First, it would require a politic action (Cariñanos and Casares-Porcel, 2011), and secondly, sometimes hypoallergenic alternatives might not meet environmental sustainability criteria for urban greenery selection (Grote et al., 2016). However, hypo-allergenic gardening might not be decisive, because of allergenic pollen sources in the surrounding areas (Green et al., 2018). Moreover, it is impossible to rule out the allergenicity, and pollen from entomophilous species such as Indian bean (*Catalpa speciosa*) and horse chestnut (*Aesculus hippocastanum*) have shown cross-reactivity with common allergens (Green et al., 2018). Some countries tried to achieve allergen pollen reduction by promoting laws that forbid the planting of pollen-rich trees. This is the case of the mulberry (*Morus* sp.) in the Southwestern United States. Another approach is to control ruderal allergenic species, such as ragweed, but this is made difficult by the rapid and intense proliferation of these weeds on disturbed ground, that are typical of urban areas (Burge and Rogers, 2000).

3 Pollen-based approach

3.1 Airborne pollen sampling

3.1.1 Samplers for outdoor monitoring

Airborne pollen concentrations in a specific area are not influenced by the local vegetation alone. Pollen can be transported by wind and dispersed by turbulences within the lowest atmospheric layer, from where it can be uplifted to free atmosphere by convection and enter air masses moved by synoptic systems. This way, pollen can undergo long distance dispersal, traveling for dozens of kilometres (D'Amato et al., 2007; Green et al., 2018; Sofiev et al., 2006). Furthermore, even sedimented pollen grains can be secondarily re-suspended into the atmosphere, contributing more than once to airborne pollen load (Bastl et al., 2017). Thus, airborne pollen monitoring has always been considered the most reliable and feasible method to evaluate allergic hazard for pollinosis sufferers. To maximise the usefulness of their information, monitoring stations are generally located into urbanised, densely populated areas. They collect atmospheric pollen through air samplers placed on the rooftop, between 15 and 20 m from the ground level, raised at least 1 m off the floor, and distant from any

airflow barrier. This placement, along with a vegetation and geo-climatic evaluation of the monitored area, is important to reduce sampling biases, and should be kept similar between stations to enhance results comparability (Bucher et al., 2015; Galán et al., 2014). Since the beginning of aeropalynology, many pollen samplers have been proposed (Mullins and Emberlin, 1997). This review will explore only the ones still commonly employed for airborne pollen monitoring (Tab. 4).

Oldest and simplest pollen monitoring devices are the passive sedimentation samplers, in which pollen naturally settles on the collecting surface due to gravity. Examples of sedimentation samplers are Durham (Durham, 1946; O'Rourke, 1990), Cour Grovette (Belmonte et al., 2000; Cour, 1974), and Sigma-2-like (Miki et al., 2019; Mimić and Šikoparija, 2021; VDI 2119, 2013) samplers, that can collect daily or weekly pollen samples. For longer monitoring periods, various passive samplers collecting pollen in a jar have been developed at the end of the last century, such as Tauber (Cundill, 1991), Oldfield (Bush, 1992; Flenley, 1973), and Behling (Behling et al., 2001) traps, but they are mainly employed in environmental studies (Jantz et al., 2013; Poska, 2013). However, sedimentation samplers have been progressively abandoned in aerobiology, except for peculiar applications (Levetin, 2004).

Nowadays, airborne pollen is routinely sampled through volumetric impaction samplers that offer a steadier and controllable capture. They are based on the principle that when the air stream encounters an obstacle, it tends to drift away and bypass it, while airborne solid particles inertially collide against the surface of the obstacle (Levetin, 2004). The first instrument ever used for continuous pollen recording is in fact an impactor sampler, the volumetric Hirst-type pollen and spore trap (Mandrioli and Puppi, 1978). It is still the most widely used pollen sampler, chosen by 70% of the pollen monitoring stations worldwide (Buters et al., 2018). Hirst-type samplers are defined slit impactors because the air is aspired through a narrow inlet with a collecting surface behind. They can collect airborne pollen for either one week, on a polyester sticky tape mounted on a rotating drum, or one day, directly on a glass slide. A clockwork mechanism makes the collecting surface slide away from the inlet every hour, keeping track of the time progression. Daily samples are eventually prepared for light microscopy, usually applying glycerine jelly with basic fuchsin (Bucher et al., 2015; Levetin, 2004).
Other valid options for air quality monitoring are the rotating impaction samplers. While different models have been developed, such as Rotoslide and Rotobar (Ogden and Raynor, 1967; Solomon et al., 1968), the rotating arm impactor Rotorod® (Sampling Technologies Inc., Minnetonka (MN), 1998) is the most popular, mainly employed in U.S.A. and Canada. Particles suspended in the air around the Rotorod® impact against its two rotating rods, sticking to their collecting surface (Frenz, 1999; Heffer et al., 2005). At the end of the sampling the rods are placed in a special microscope slide, and Calberla's stain is applied (Levetin, 2004).
Hirst-type and Rotorod® provide similar results on daily pollen concentrations when compared side-by-side, and they both show an overall efficiency in pollen capture of about 80%. Nonetheless, their performance is differently affected by meteorological factors, being Hirst-type samplers more reliable with low wind speed, and Rotorod® ones with moderate wind speed (between 3 and 6 m/s). When the wind speed is higher than its air intake velocity, Hirst-type sampler might also significantly overestimate concentrations for some pollen types (Frenz, 1999; Geller-Bernstein and Portnoy, 2019; Heffer et al., 2005). For these reasons, before comparing data collected with different devices, it is important to adjust them using inter-sampler conversion factors and considering wind speed (Peel et al., 2014).

Furthermore, while Hirst-type traps can sample continuously for one week without human intervention, the Rotorod® system is prone to overloading hence its duty cycle is usually kept at 10%, and adjusted according to the sampling duration and the expected pollen concentration. Therefore, Rotorod® samplers are rarely employed for continuous multiple-day sampling (Frenz, 1999; Frenz and Boire, 1999).

3.1.2 Samplers for indoor and personal monitoring

Pollen spectra vary at different heights and locations, and between indoor and outdoor environments. Even if there is a positive correlation between pollen concentrations recorded on the roofs of monitoring stations with those recorded at ground level, airborne pollen is generally more abundant at lower heights, and shows variations in species composition at different heights (Bastl et al., 2017; de Weger et al., 2020; Kasprzyk et al., 2019a; Rojo et al., 2019). In particular, it has been demonstrated that when the sampler is placed up to 10 meters above the ground level, its height is inversely proportional to the pollen concentrations recorded. Pollen concentrations at near-ground level also show great fluctuations, due to local events of emission, deposition, or resuspension of the pollen grains, or to microscale environmental dynamics (Rojo et al., 2019). To assess pollen concentrations at human height, portable samplers can be employed (Tab. 5).

Portable Hirst-type samplers are the most dependable choice for daily continuous records at ground level, but they operate in a fixed position. To evaluate the pollen exposure of a subject throughout the day, compact and wearable pollen personal samplers have been developed. In this case, the air intake velocity of the instrument tends to mimic the human breath rate. Common examples are the sampling cassettes, available as impaction or filtration samplers. In the first case, particles impact against a glass slide, and the sample can be promptly analysed under light microscopy. Conversely, in filtration samplers, air flows through a porous membrane that captures airborne particles, with a diameter cut-off defined by the filter texture. Pollen is then recovered from the membrane using a detergent, and mounted on a light microscopy slide (Heffer et al., 2005; Levetin, 2004). Other wearable impaction samplers have been invented throughout the years, either passive like the Personal Aeroallergen Sampler (PAAS) (Yamamoto et al., 2007), or connected to a pump like the Partrap FA52 (Coppa, Biella, Italy) (Berger et al., 2014; Fiorina et al., 1999). Unfortunately, most of them are not commercially available (de Weger et al., 2020). A recently proposed, purchasable portable sampler is the Pollator (Werchan et al., 2018), an active impactor that has a capture efficiency lower than the Hirst-type trap, but a comparable sensitivity to variations and trends in airborne pollen concentrations. It can also record meteorological parameters and keep track of the position via GPS while collecting pollen. A similar device that will likely be soon in commerce is the Pollensniffer (de Weger et al., 2020). This active impactor sampler seems to have a higher collecting efficiency than the 7-days Hirst-type trap for most pollen types, and a desirable user-friendliness.

To attain a precise quantification of the pollen actually inhaled by someone, another option is the nasal air sampler (NAS), an impaction sampler worn inside the nares (Graham et al., 2000). Despite its high-declared efficiency in particle capture, this approach is limited by nasal configuration and function. In general, the reliability of personal samplers alone is disputed, but they are considered a useful complement to routine diagnostic allergy tests. Significant obstacles to their application in wide epidemiological studies however are posed by their relatively high costs, the user engagement they require, and the massive data analysis that should follow (Berger et al., 2014).

3.2 Pollen analysis

3.2.1 Manual pollen counts

Once collected with air samplers, pollen can be analysed by different means, according to the research purpose. The main aim of pollen sampling is to inform allergic patients about its atmospheric concentration, hence pollen counts are the most common analysis. While duration and frequency of air sampling are decided by each station, pollen counts are usually performed on 24 hours samples. This also means that pollen concentrations are necessarily at least 1 day old when they get published (Geller-Bernstein and Portnoy, 2019). Total number of pollen grains can be determined by optical microscopy, using a haemocytometer (Heffer et al., 2005), but it is common procedure to identify and quantify only pollen from plant species that are clinically relevant in the studied area. Allergenic pollen identification is accomplished by trained personnel in light microscopy, at 400x magnification, based on the overall grain morphology (exine, intine, cytoplasm). This method seldomly allows species identification, and airborne pollen grains are commonly identified at family or genus level, or assigned to a non-phylogenetic group called "pollen type" that includes different plant species with pollen grains morphologically indistinguishable from each other (D'Amato et al., 2007).
For timing reasons, only a subset of the sample is analysed, chosen by random fields or transect sub-sampling. According to the European Aerobiology Society, for daily samples collected with a slit impactor the subset should include at least 10% of the surface to minimise the estimation error, as explained in the European Standard EN16868 (EN16868, 2019; Galán et al., 2014). In fact, the results obtained by reading 10% of the slide surface have been proven to fall within the thresholds of Relative errors in a quality control and reproducibility analysis (Galán et al., 2014). Random field sub-sampling is not recommended because it misses the time progression, and it might be biased by swift changes in pollen concentrations. The International Association for Aerobiology poses as minimum requirements to read 3 horizontal transepts, that account for the whole day, or 12 vertical transepts, that evaluate pollen abundance in certain moments of the day (Gharbi et al., 2017). The latter appears to be the most accurate approximation of the entire slide, but short pollination peaks could be missed if they fall between transepts. Moreover, the number of sweeps needed to cover 10% of the sample surface also depends on the microscope field of view dimension, that is affected by the objective magnification and diameter. Hence, the area to be analysed remains a better reference than the number of transepts (Galán et al., 2014). Pollen counts precision also depends on sampled pollen concentration, hence estimations of daily pollen concentrations are reliable only when 50 pollen grains or more are counted in longitudinal sweeps (Gharbi et al., 2017; Levetin, 2004).
Once pollen grains in the subsample have been identified and counted, raw abundance data of each taxon are multiplied by a conversion factor to obtain average daily concentrations expressed as particles per cubic meter of air (P/m^3) (Bucher et al., 2015; Galán et al., 2014). For long-term monitoring, these concentrations can be elaborated into their integral over time, called Annual (or Seasonal) Pollen Integral (APIn or SPIn) (Galán et al., 2017). Counting pollen grains while still fresh is time-effective, but it does not provide a good image resolution. When a more accurate identification is needed, pollen can be acetolysed to better visualise exine diagnostic details. Acetolysis consists of a dehydration by glacial acetic acid (CH_3COOH), followed by an acetylation with acetic anhydride ($C_4H_6O_3$) and sulfuric acid (H_2SO_4) in a 9:1 ratio, at 100°C for 10 minutes. Samples are then washed, dehydrated in ethanol, and resuspended in glycerine, so they can be mounted on microscope slides. This treatment removes non-sporopollenic and non-chitinous organic components from the sample, including intine and cytoplasm of the pollen grains, and it gives an amber shade to

the exine, enhancing its features (Erdtman, 1969, 1943; Hesse et al., 2009). During acetolysis, known amounts of a marker are usually added to the sample. These markers can be exotic fern spores (e.g. *Lycopodium*) (Stockmarr, 1971), plastic microbeads (Ogden III, 1986), or black ceramic spheres (Kitaba and Nakagawa, 2017). In this approach, pollen grains are usually counted up to 150, and their concentrations in the sample are expressed as relative frequencies, based on the number of marker units counted (Erdtman, 1943). Being a slow and demanding process, acetolysis should be applied only to small aerobiological samples, sporadically collected for brief studies. Examples of aerobiological samples fit for this treatment are those gathered by filtration (e.g. sampling cassettes and air filters), sedimentation (e.g. Cour sampler, Tauber, Oldfield, and Behling traps), or other sampling methods lacking time progression. For instance, to evaluate personal exposure to airborne pollen, hair can be used as a sampling surface, making the volunteers wash their hair weekly and collect the rinsing water. Pollen is then concentrated, acetolysed and mounted on a glass slide (Charpin et al., 2010; Penel et al., 2017). Another non-conventional approach is to collect the dust sedimented on surfaces by vacuum, and then to concentrate and acetolyse the pollen present in the sample (Bastl et al., 2017; Gore et al., 2006).

3.2.2 Automatic pollen counts

Pollen counting is a labour-intensive and time-consuming process, that requires a specific expertise gained by technicians over a long training period. Another downside of manual counts is the subjective component of pollen identification, that tends to lower the between-analysts reproducibility (Galán et al., 2014). From the beginning of this century several attempts have been made to create a reliable automatic system for pollen recognition (Holt and Bennett, 2014). The integration of microscopy with pollen identification software allowed to automatically simulate the whole counting process, limiting the human intervention to labelling the libraries used to train the recognition algorithms, and checking their results. The first instrument produced for this purpose, to our knowledge, is the Classifynder (Holt et al., 2011). It combines robotics, image processing and neural networks to find pollen on the slide, capture, analyse and store its image, and identify its type. On fresh pollen slides with few different types, its results are consistent with those of palynologists, even if its pollen counts are lower. It also takes a slightly longer time than humans to read a slide, but compensate with a considerably higher accuracy, especially because it cannot read the same grain twice (Holt et al., 2011).
With digital microscopes becoming more common, and machine learning technologies making significant improvements, there has been an increase in efficiency of automatic pollen recognition, that is now based on more sophisticated feature extractors and supervised learning techniques (also called classifiers). For example, in 2016 Gonçalves and collaborators proposed a computer vision system to identify acetolysed pollen images, using Color, Shape and Texture (CST) and Bag of Visual Words (BOW) feature extraction algorithms, and the C-Support Vector Classification (C-SVC) classifier. They obtained an overall pollen classification accuracy greater than 64%, close to non-expert human performance, over 23 different pollen types (Gonçalves et al., 2016). However, progresses in this field have always been obstructed by the complexity of exine structure, the subtle differences between some pollen types, and the variability within type caused by grain orientation, pollen clumps, or wall rupture. Moreover, discriminating features are decided *a priori* by the researchers, a process that is time-consuming, requires informatics knowledge, and can bias the identification. In fact, human discrimination limits are reflected by the algorithm results, especially with high numbers of pollen types types (Gonçalves et al., 2016; Holt et al., 2011). Deep learning algorithms can solve this problem, being able to autonomously identify and learn discriminating features of images from different classes. In

particular, pre-trained convolutional neural networks (CNN) such as Alexnet can be employed for automatic feature extraction over large pollen images databases, a quicker approach than training a CNN from scratch. A setup where Alexnet extracts features from a pollen database, it is then retrained by transfer learning, and the features extracted thereafter are categorised using a linear discriminant classifier, allowed to correctly classify 97% of acetolysed pollen images from the same database of 23 pollen types used by Gonçalves and collaborators. It also showed a high processing speed, producing 170 predictions per second (Sevillano and Aznarte, 2018). A similar setup, associated with image pre-processing and data augmentation, led to an even better performance, exactly classifying 98% of the acetolysed pollen images tested, over a dataset produced by the Classifynder classification system containing 46 pollen classes (Sevillano et al., 2020).

Besides, it is not clear how these algorithms would perform with fresh pollen slides containing mixtures of unknown pollen types. Another limit is that automatic counts are still performed after pollen is sampled and mounted on the slide. To remove the delay between the air sampling and the publication of pollen bulletins, it is necessary to implement a real-time (RT) sampling and recognition system. This approach is already well established in Asia (Buters et al., 2018), while Europe is currently working towards an international RT pollen monitoring network with the EUMETNET AutoPollen programme (Clot et al., 2020).

Although different technologies have been applied to automatic aerosol analysis, image recognition and laser (or light) induced fluorescence (LIF) have proven to be the most effective (Tab. 6). Image recognition technology was the first to be applied to RT pollen analysis (Bennett, 1990; Šauliene et al., 2019). Japan pioneered this approach, building the first national automated pollen monitoring and forecasting network, Hanakosan, mostly based on the KH-3000 sensor (Yamatronics, Yokosuka, Japan). This sensor uses laser beam scattering to reconstruct and recognise the pollen grain morphology, but it is unable to distinguish between pollen types with the same scattering profile. Hence, while these devices optimally identify the main Asian allergenic pollen (*Cryptomeria japonica*), they still do not perform as well with more complex and diversified pollen spectra (Buters et al., 2018; Huffman et al., 2019; Kawashima et al., 2017). In other countries, where allergenic pollen grains are more variable, other instruments are preferred. For instance, in the USA a compact image-based RT pollen sensor, APS by PollenSense™ (Lucas et al., 2016), has been recently launched and commercialised (https://www.pollensense.com/). In Europe, the German ePIN automatic pollen monitoring network employs image-based BAA500 detectors (Hund-Wetzlar, Wetzlar, Germany), that use light microscopy mimicking the workflow of classic pollen counts. Their recognition algorithm can even be manually trained by experts (Huffman et al., 2019; Oteros et al., 2019; Šauliene et al., 2019).

However, in Europe LIF techniques have been historically preferred for aerosol monitoring. LIF-based sensors expose airborne particles to monochromatic light, inducing and detecting the autofluorescence typical of some organic molecules. This way, they can distinguish between bioaerosol and inorganic air pollutants (Huffman et al., 2019). Since related organisms may have similar LIF spectra, a precise identification of pollen types by fluorescence alone is challenging, and LIF-based instruments like the Wideband Integrated Bioaerosol Spectrometer or WIBS (Droplet Measurement Technologies, Longmont, Colorado) can only assess whether a biological particle could be pollen, based on its size and autofluorescent molecules combination. Other limits to this approach are the possible interference of inorganic particles containing aromatic hydrocarbons, and a change in biomolecules fluorescence properties due to growth conditions, agglomeration, or physical and chemical modifications (Calvo et al., 2018).

Nonetheless, the method can be significantly improved using multiple excitation wavelengths, and coupling LIF with other technologies (Huffman et al., 2019). It is the case

of PA-300 last model, Rapid-E (Plair SA, Geneva, Switzerland), a LIF-based sensor that provides RT airborne pollen concentrations by combining a detailed autofluorescence analysis with light scattering. It is considered to date the most effective RT device, and can distinguish between four macro-groups: (I) Grass pollen, (II) *Alnus*, *Corylus*, *Betula* and *Quercus*; (III) *Salix* and *Populus*; (IV) *Festuca*, *Artemisia* and *Juniperus*. This technology has the potential for a better resolution, but more research efforts are needed (Šauliene et al., 2019).

Another peculiar combination of LIF and image-based technology is the Poleno (Swisens AG, Horw, Switzerland). In this case, pollen identification strongly relies on the light scattering, thanks to convolutional neural networks trained with palinological databases. This device is also the first one capable of reproducing a holographic image of the pollen grain, allowing the user to verify the results or to train the algorithm by manual labelling. On one hand, an evolving algorithm can constantly improve the identification skills of the instrument, but on the other hand it reduces the reproducibility of results between individual stations. This technology is however limited by the risk of overloading at high atmospheric pollen concentrations, and by a size-dependent particle loss (Huffman et al., 2019; Sauvageat et al., 2020).
Results provided by RT automatic sensors have been compared to the traditional monitoring approach (Tab. 6), showing variable correlation with manual pollen count results, depending on the instrument, the pollen type, and the time interval considered. Although these correlations are not always directly comparable, automatic pollen counters that best approximate the daily pollen concentrations estimated by traditional approach to date appear to be BA500 and Rapid-E (Crouzy et al., 2016; Oteros et al., 2020; Tešendić et al., 2020). New information is expected in the near future as these sensors are being improved, calibrated and compared (Tummon et al., 2020).

Other RT approaches have been applied to pollen recognition, but they are not implemented in automatic sensors yet. It is the case of Raman and Fourier transform infrared spectroscopies, that use a photon beam to collect chemical information from a given sample. To distinguish different pollen spectra, it is necessary to use infrared light, avoiding the interference of pollen autofluorescence. Microspetroscopy methods in particular can provide pollen identification with high taxonomic resolution. In lab tests, near-infrared Raman microspectroscopy identified 13 plant species from pollen mixtures with 96% precision. It is however unclear if this performance could be maintained with real airborne samples (Huffman et al., 2019; Rittenour et al., 2012).

3.2.3 Molecular pollen analysis

Another precise and objective method to identify pollen taxa is using DNA markers. In the last decade there have been a few attempts to combine genomics with aeropalynology, and even if this field is still at an early stage, its near future prospective is promising. One of the first genomic approaches applied to airborne pollen monitoring is the real-time quantitative PCR (qPCR), that provides pollen spectra just 2.5 hours after the sampling. To apply this method, it is necessary to select DNA sequences suitable as species-specific markers for every allergenic plant studied, and to establish qPCR standard curves for each one of them. Airborne pollen samples compatible with this analysis can be collected with different instruments (Tab. 7), DNA is then extracted from the sample and added to the qPCR reagents mixture. This mixture contains species-specific or genus-specific primer/probe sets. Thus, qPCR is run with a standard program and pollen loads of each sample are eventually

calculated inserting cycle threshold values into the standard curve equations (Longhi et al., 2009; Rittenour et al., 2012).

Qualitative and quantitative results obtained by qPCR with TaqMan technology showed a positive and highly significant correlation with manual pollen counts of the same samples, with the advantage of a higher taxonomic resolution. Using single- or low-copy nuclear genes as markers allowed to distinguish all the 18 species present in the sample, including pollen types that can be only identified to the Family level in light microscopy, like Poaceae or Cupressaceae (Longhi et al., 2009). Highly preserved markers like nuclear genes grant precise species recognition and reliable pollen quantification (Longhi et al., 2009; Müller-Germann et al., 2015), but they require a minimum pollen quantity that is almost tenfold the one needed for visual counts (Rittenour et al., 2012).

Sensitivity can be improved targeting DNA multi-copy sequences such as the ribosomal ITS region of the nuclear genome (Müller-Germann et al., 2015; Rittenour et al., 2012), or chloroplast DNA (cpDNA) regions like maturase K (*matK*) genes (Mohanty et al., 2017a). ITS markers however might flaw quantitative results, due to differences in ploidy, or due to their copy number variability between species, within species, and even within individuals (Bell et al., 2016). Applications of cpDNA markers in palynology are also problematic, because plastids are inherited maternally and they are mostly degraded in the male gametophyte (Bell et al., 2016; Núñez et al., 2016). While it is possible to detect and analyse cpDNA in pollen grains (Galimberti et al., 2014), its copy number is likely to vary between species due to different plastid inheritance strategies. These dynamics are yet to be comprehended and they might bias pollen detection and quantification when using cpDNA markers. This issue can be overcome by collecting pollen samples from different individuals, preparing two standard curves of the cpDNA marker per species, and testing the covariance between pollen grains and cycle threshold value (Mohanty et al., 2017b).

When targeting fast-evolving sequences, DNA barcoding is another valid option for pollen recognition. This method is based on the detection of a standardised DNA region, called DNA barcode, that is preserved within species and variable among species (Bell et al., 2016; Valentini et al., 2008). Although a univocal barcode marker for plant species has not been found yet, some cpDNA sequences and the ribosomal Internal Transcribed Spacer (ITS) region are good candidates, used alone or in combination (Bell et al., 2016; Hollingsworth et al., 2009). A recent application of DNA barcoding, called DNA metabarcoding (or targeted amplicon parallel sequencing), allows to simultaneously identify and quantify biological components of small environmental samples containing mixed DNA of different species, using high-throughput sequencing techniques (Banchi et al., 2019; Bell et al., 2016; Hollingsworth et al., 2009). In 2015, Kraaijeveld and collaborators proved that DNA metabarcoding can be effectively applied to airborne pollen samples, using next-generation sequencing (NGS) (Kraaijeveld et al., 2015). Since NGS cannot integrate two distant markers simultaneously, molecular markers are analysed one at a time (Núñez et al., 2016). The sequence of interest is amplified by single or dual index PCR, using universal primers for all the plant genera present in the sample. Then, the DNA is sequenced and aligned by bioinformatic analysis, to identify the plant taxa (Sickel et al., 2015). NGS analysis of the cpDNA locus *trnL* led to unambiguous identification of various European allergenic pollen genera in airborne samples, with a greater resolution than the microscopic counts. In particular, it can confidently distinguish Poaeceae members *Holcus, Hordeum, Phleum* and *Dactilis* (Kraaijeveld et al., 2015). Ribosomal subunit ITS2 instead allowed to effectively identify 99.7% of the sampled spermatophytes on a genus level (Korpelainen and Pietiläinen,

2017), and in some cases even on the species level (Banchi et al., 2020), providing results in good concordance with morphological identification (Korpelainen and Pietiläinen, 2017; Núñez et al., 2017; Núñez and Moreno, 2020; Sánchez-Parra et al., 2021). A recent study suggested that also RuBisCO chloroplast gene (*rbcL*) could be used as barcode for NGS analysis on aeropalynologycal samples to detect short-term temporal changes in pollen spectra composition throughout the pollen season (Campbell et al., 2020). Although *trnL*, ITS2 and *rbcL* can identify pollen genera often overlooked in morphological analysis, used alone they are not fit for a thorough species-level identification and pollen quantification. For these reasons, the standard barcode for land plants requires a two-loci DNA barcode, including sequences of chloroplast markers *rbcL* and *matK* (Hollingsworth et al., 2009). *matK* has not been used for airborne pollen metagenomics yet, mainly because it is not very efficient in dealing with multiple plant families (Korpelainen and Pietiläinen, 2017). In 2019 a two-loci metabarcoding was performed on airborne grass pollen, combining ITS2 with *rbcL* (Brennan et al., 2019). This study identified to the genus level *Festuca*, *Holcus*, *Alopecurus, Lolium* and *Poa*, showing a higher resolution potential than *trnL* marker (Kraaijeveld et al., 2015). NGS studies on artificial pollen mixtures have proven that the combination of ITS2 and *rbcL* markers enables the taxonomic assignment of many pollen types to the species level, but both markers might miss or misclassify some species, especially rare ones (Bell et al., 2019; Campbell et al., 2020). However, it is not economically advantageous yet to implement metabarcoding with two markers in routine aerobiological monitoring (Campbell et al., 2020).

Overall, from published literature (Tab. 8), NGS appears a precise and detailed method to assess the pollen composition of air samples. It is more time-effective than traditional pollen counts and does not require highly trained personnel. Moreover, it can also detect pollen debris and cytoplasm, potential bearers of pollen allergens that cannot be accounted for in visual pollen counts (Bell et al., 2019; Kraaijeveld et al., 2015). On the other hand, it can lead to misrepresentation of some pollen types when airborne non-pollinic plant material is sampled (Núñez et al., 2017). Additionally, NGS is inaccurate in evaluating pollen content, due to several potential biases at isolation, preservation and amplification levels (Bell et al., 2019). In particular, the PCR amplification step might cause misrepresentation of some taxa, because it conceals the original number of the markers, and because the amplification efficiency of different polymorphisms may vary. When the relative abundance of DNA reads positively correlates with the relative abundance of pollen grains, as it is for *tnrL*, the solution might be correcting the number of DNA sequences by the total number of sampled pollen grains (Baksay et al., 2020; Kraaijeveld et al., 2015). Nevertheless, this correlation is still debated, especially for *rbcL* and ITS2 sequences. Banchi and collaborators reduced some of these uncertainties for ITS2 marker by selecting the primer combinations and PCR approaches that captured the highest plant diversity, and by creating a mock pollen community as control (Banchi et al., 2020). Nonetheless, quantitative biases cannot be ruled out, and they may also differ among markers. Thus, while some authors consider NGS a semi-quantitative method (Banchi et al., 2020), others propose to infer quantitative data by aggregation of presence-absence data from different samples instead (Bell et al., 2019).
PCR-free NGS techniques are also being developed to avoid these complications, such as shotgun metagenomics, based on shotgun sequencing (Bell et al., 2016; Kraaijeveld et al., 2015; Núñez et al., 2016). Yet, this would not entirely solve the problem, and a better understanding of quantitative biases in mixed-pollen DNA barcoding is needed (Bell et al., 2019).
Genomic analysis on airborne pollen in general would surely benefit from a method standardisation, starting from the type and duration of the pollen sampling, that are likely to

influence the outcomes. Furthermore, some authors object that the adhesive tape used for Hirst-type traps may contain PCR inhibitors, compromising sequencing outputs (Banchi et al., 2019). PCR inhibitors in air samples might be detected by adding an exogenous standard, and removed through appropriate DNA extraction methods (Rittenour et al., 2012). Another source of bias lays in the DNA isolation. In fact, differences in pollen wall resistance can make DNA extraction uneven between species, and it is also still unclear if results obtained with different extraction strategies and isolation kits are comparable (Bell et al., 2016). Moreover, DNA metabarcoding on airborne pollen needs the development of a common bioinformatic pipeline and the creation of *ad hoc* databases (Banchi et al., 2019). Another significant limit to pollen genomics is the shortage of marker sequences deposited in genomic databases for some species (Banchi et al., 2019; Bell et al., 2016). However, with the constant update of genomic libraries and the rapid evolution of sequencing and amplification technologies, these approaches will likely become more efficient and affordable in the near future, and therefore suitable for rapid airborne pollen monitoring on a large scale (Bell et al., 2016; Kraaijeveld et al., 2015; Sickel et al., 2015).

4 Allergen-based approach

4.1 Airborne pollen allergens

Conventional allergenic pollen monitoring does not thoroughly describe pollen allergenicity. In fact, allergen content might variate quantitatively and qualitatively within one pollen type (Cecchi, 2013; D'Amato et al., 2007). Because of this variability, a more operational classification for pollen allergens was suggested, based on cross-reactivity rather than botanical origin (Mothes et al., 2004). In fact, atmospheric patterns of cross-reactive allergens can be more clinically relevant than airborne pollen types combinations (Aloisi et al., 2018; Fernández-González et al., 2020).

Allergen content per pollen grain, defined pollen potency, may also vary between individuals of the same species differing in cultivar, age, or growing conditions. For example, there is evidence for tree pollen to be significantly more allergenic with warmer temperatures (Cecchi, 2013; D'Amato et al., 2007; Mothes et al., 2004). Meteorological conditions before and during the pollen season in particular seem to affect pollen potency (Buters et al., 2015). Additionally, since allergens are often involved in pollen-stigma recognition, stressed plants can compensate low pollen production with high expression of these molecules, to maximise reproduction effectiveness (Fernández-González et al., 2011, 2010). When pollen with high potency reaches regions where the same pollen type is usually less allergenic, it creates an allergic hazard undetectable by pollen counts (Galan et al., 2013). Moreover, when interacting with human respiratory mucosa, different pollen taxa may release allergens with variable intensity and speed. Allergen release mechanisms are still largely unknown, but they might attribute to each species peculiar levels of sensitization or elicitation (Hoidn et al., 2005; Mothes et al., 2004).
Pollen allergens can also be released directly into the atmosphere. In fact, allergens can be removed from pollen surface by friction or leaching, and transferred to other aerosol components such as plants fragments or air pollutants (D'Amato et al., 2007). Moreover, when exposed to rainwater, pollen can undergo germinative abortion, with emergence and subsequent rupture of the pollen tube, releasing submicronic particles that contain cytoplasmic allergens. Emission of such particles can also happen by simple osmotic rupture (Grote et al., 2003, 2000). Furthermore, fully germinated pollen can emit putative nanovesicles called pollensomes (diameter around 30-60 nm), that could act as airborne carrier of allergens (Prado et al., 2015, 2014). It is also possible that allergens are eluted

directly into the atmosphere during pollen rehydration, then diffusing in droplets (D'Amato, 2001).

Genetic analysis has attested the existence of allergenic plants debris in the finest aerosol (Müller-Germann et al., 2015), that could derive from either the ruptured pollen or its source plant. Pollen allergens however are also present in the sporophyte tissues, from which they can disperse carried by plant fragments, starch granules, or, according to some authors, Ubish bodies (D'Amato, 2001). All this implies that low airborne pollen concentrations are not necessarily proof of low allergic risk, and vice versa. In fact, substantial quantities of airborne pollen allergens have been detected outside the pollen season, and this could explain the temporal mismatch between pollen exposure and allergic symptoms often reported in epidemiological studies (Cecchi, 2013; D'Amato, 2001; D'Amato et al., 2007).

Besides the abovementioned pollen-related aspects affecting airborne allergens concentrations, also external factors might lead to a misalignment between pollen and aeroallergens peaks, such as meteorological factors (Aloisi et al., 2018; Fernández-González et al., 2020).

Overall, the correlation between airborne pollen and allergens concentrations is not always significant, therefore allergenicity cannot be deducted by pollen concentrations alone (Plaza et al., 2016).

4.2 Pollen allergens sampling

Several studies in the last decade have focused on monitoring airborne pollen allergens and comparing them with pollen spectra. Pollen allergens can be carried by particles significantly smaller than pollen grains, so they require samplers with high intake velocity and low diameter cut-off. However, they seem to be detectable only in particles over 2.5 μm diameter, possibly because particles under this threshold are often diesel particulate, that tends to absorb them (Plaza et al., 2017).

The two devices usually employed for allergens monitoring are Cyclone samplers and cascade (or sieve) impactors (Tab. 9), placed on a rooftop following similar criteria to those used for pollen traps. Cyclone sampler is a volumetric air sampler that conveys the air into a rotating stream within a cylindrical or conical tube, where airborne particles adhere to the walls due to centrifugal force. This technology allows to sort out particles based on their mass, shape and size (Lippmann and Chan, 1979). Cyclone samplers can be employed in either pollen or allergens monitoring, since their particle size selectivity can be regulated (Brennan et al., 2019). However, Cyclone air samplers are designed to collect small particles with diameter of 1 μm or less, so they are mainly employed to collect pollen debris and aeroallergens rather than whole grains. Studies on aeroallergens often adopt multi-vial Cyclone samplers that grant a customisable time resolution and a longer sampling autonomy (Plaza et al., 2017, 2016). At the end of the sampling, the vials are centrifuged, and pollen allergens are isolated from the sample with an appropriate extraction buffer (Aloisi et al., 2019; García-Sánchez et al., 2019; Plaza et al., 2017).

Cascade impactors are multi-stage impaction samplers that capture particles with different aerodynamic properties on separate collecting surfaces. Its diameter cut-off and number of stages are customisable, and the last stage is usually a filtration sampler, to ensure the capture of finest particles (May, 1945). Different versions of cascade impactors have been proposed, with variable collection media and air intake velocities (Alan et al., 2020; Choël et al., 2020; Schäppi et al., 1999), but nowadays the two most popular are Andersen-like samplers

(Andersen, 1958; Mitchell and Pilcher, 1959), and high-volume cascade impactors like the ChemVol® (Butraco, Son, The Netherlands) (Buters et al., 2015, 2008). Andersen-like samplers are the first cascade impactors developed, they have relatively low air intake velocity (28 l/min) and use glass fibre filters as impacting surface. After sampling, it is possible to dry, condition and weight these filters to evaluate the sample mass concentration (Schäppi et al., 1996). Whole filters or their sub-sections are then submerged in the desired buffer to resuspend the captured particles (De Linares et al., 2019, 2007; Schäppi et al., 1996).
ChemVol-like impactors are a more recent invention (Buters et al., 2008), they are designed to collect high air volumes (800 l/min), and they use polyurethane foam as sampling substrate. Allergens can be retrieved either by immersion in the appropriate buffer solution, or by incubating the substrate in ammonium hydrogen carbonate with bovine serum albumin, lyophilising the extract for storage and resuspending it before the analysis. Unlike extraction buffers, the latter treatment removes components that could interfere with analysis, and it also increases test sensitivity. However, extraction buffers are more effective at isolating allergens from the sample (Plaza et al., 2017).

In a side-by-side comparison, trends recorded by the two devices are similar, but Cyclone samplers tend to record higher concentrations of aeroallergens than cascade impactors, and they are more sensitive to low concentrations occurring outside the pollen season (Plaza et al., 2017). Conversely, allergen data obtained by cascade impactors tend to better correlate with pollen concentrations. This might imply that the two devices have comparable efficiency in collecting pollen grains, but Cyclone sampler performs better in capturing smaller aeroallergen carriers as well, maybe due to its wind-orienting vane. Nonetheless, both devices proved to be reliable for airborne allergens sampling (Plaza et al., 2017). Throughout the years, other devices have been proposed for aeroallergen sampling, but they never became of common use, probably because they are not practical, and because they often rely on homemade samplers that would need standardisation. It is the case of electrostatic precipitation samplers (Plaza et al., 2017), and different models of virtual impactors with low flow rate that collect particles on filters or in water (Plaza et al., 2017; Takahashi et al., 2001). Other more generic sampling methods can be found in literature, such as adaptations of Hirst-type traps for immunoblotting analysis (Razmovski et al., 2000), or dust filters from ventilation systems (Sázelova et al., 2002). Meanwhile, new aeroallergen samplers are being developed, such as the one presented by De Linares and collaborators at the 7th European Symposyum on Aerobiology, based on a High-Volume TSP Sampler that collects particles on a glass fiber filter (De Linares et al., 2020).

4.3 Pollen allergens analysis

First studies on pollen aeroallergens employed passive transfer antigen neutralization techniques or RAST-type analysis. Nowadays, those methods have been abandoned and aeroallergens are usually identified and quantified by Double-sandwich ELISA (García-Sánchez et al., 2019). In this approach, the wells of a microplate are coated with monoclonal antibodies able to specifically recognise the allergen of interest. Once aeroallergens have been captured into the wells, polyclonal primary antibodies are added to detect them. These antibodies can be biotinylated or enzyme-conjugated; in the first case, the plate is then incubated with peroxidase-conjugate streptavidine. Eventually, the plate is developed with a suitable substrate, and allergen concentrations are evaluated measuring the absorbance at substrate-specific wavelength. Calculations are calibrated by using purified allergens as standard (García-Sánchez et al., 2019; Plaza et al., 2017). Some studies also applied indirect

ELISA, coating the microplate wells directly with the sampled allergens, adding primary antibodies specific for the studied allergen and then enzyme-conjugated secondary antibodies, and developing the plate with the appropriate substrate (De Linares et al., 2019). With an estimated error of approximately 20%, ELISA assay is considered reliable and it is regarded as the standard method for aeroallergen monitoring (Buters et al., 2015). However, other approaches have been attempted, like the basophil degranulation assay. This assay evaluates air samples allergenicity based on their capability to induce mediator release from an FcεRI-humanized rat basophil cell line. The cells are passively sensitised with sera of pollen allergic subjects, and then exposed to sequential dilutions of the sampled aeroallergens extract. Levels of β-hexosaminidase are then measured as indicator for histamine release (Buters et al., 2015). Since these techniques require at least one day of processing, some authors tried to develop RT quantification methods.

One option is to evaluate aeroallergens with the BIACORE system, based on surface plasmon resonance. In this method, monoclonal antibodies against target allergens are immobilised on a sensor chip placed in the system. Then, sampled aeroallergens suspended in HEPES buffer are injected in the instrument, and their bond with the antibodies is measured in RT. It is possible to quantify sampled allergens using progressive dilutions of the purified molecules as standard. This instrument can measure up to four different allergens simultaneously, but unfortunately it needs to operate in a controlled environment, hence cannot be connected to the outdoor air sampler. Capillary electromigration (CE) instead can be performed outside, thus could be implemented in an automatic aeroallergen sensor that provides airborne allergens profiles immediately after sampling. After they are extracted from the sampled particles, aeroallergens can be separated by CE techniques such as zone electrophoresis or micellar electrokinetic chromatography, and quantified measuring the UV absorbance at 206 nm (Sázelova et al., 2002).

Another interesting approach is to associate manual pollen counts with aeroallergen quantification, as proposed by Razmovski and collaborators (Razmovski et al., 2000). They used a Hirst-type sampler with transparent, acrylic pressure-sensitive adhesive tapes, that can collect half the pollen grains but twice the smaller particles (1-20 μm) than typical adhesive-coated polyester tapes. After the sampling, the tape is adhered to a polyvinylidene difluoride or nitrocellulose membrane, creating a sandwich, to transfer eluted allergens on the membrane. Finally, the sandwich is immunostained, obtaining a more sensitive allergen detection than ELISA immunoassay. Observing the sandwich in light microscopy with the tape upwards, it is possible to visualise allergens on the membrane as coloured halos behind their carriers on the tape. Furthermore, halo intensity can be used for protein quantification by image analysis.
Regardless of the method used to calculate them, aeroallergens concentrations can eventually be expressed in picograms of allergen per cubic meter of air (pg/m^3), or elaborated into annual and seasonal indices or integrals (eg. AI or SAIn) expressed in $pg*day/m^3$. Allergy Potency (AP) is instead obtained as the ratio of allergens to pollen daily concentrations (Fernández-González et al., 2019; Plaza et al., 2017). These indices are useful to evaluate the relation between aeroallergens, airborne pollen concentrations, meteorological parameters and pollinosis outbursts. However, being retrospective, they cannot be used for allergen avoidance. Airborne allergens are also difficult to forecast because of their marked interannual variability and their unclear relationship with meteorological conditions (De Linares et al., 2019; Plaza et al., 2016). If aeroallergens were carried by pollen grains only, their concentrations could be estimated using pollen dispersal models to map pollen potency (Buters et al., 2015). Besides, in reality the estimation of their dispersal dynamics is

complicated by the dimensional variability of their carriers, that have diameters ranging from a hundred microns for pollen to dozens nanometres for nanovesicles (Plaza et al., 2016; Prado et al., 2015; Schäppi et al., 1996). In addition, to date aeroallergens emission is impossible to model since little is still known about the events underlying their expression and release (Cecchi, 2013; Plaza et al., 2017).

5 Conclusion and future perspectives

Pollen allergenicity monitoring is an old problem that requires new solutions. However, sometimes the will to innovate the field tends to overcome the necessity of standardised, comparable data. Nowadays there is a wide range of different methods available for pollen monitoring, but their relative efficiency is sometimes unclear. This divergence of approaches is partly rooted in the geographical variability of pollen allergies and plant diversity, that makes it difficult to extend local results to other regions, confining aeropalynological research on a national level (Buters et al., 2018). The rapid technological progress of the last decades also participated in this diversification, providing affordable and efficient instruments for numerous applications, and allowing quick data collection and computation (Huffman et al., 2019). However, the technological progress is also providing tools with the potential to improve the standardisation of data collection even in areas with different biogeography, such as remote sensing technologies, or automated pollen monitoring networks (Caparros-Santiago et al., 2021; Huffman et al., 2019; Pecero-Casimiro et al., 2019).

This review attempts to classify and compare all these diverse techniques, in the light of their usefulness to allergic subjects, the main aim of aeropalynology. Airborne pollen counts have always been considered the most reliable option for this purpose. They have been carried on for almost 70 years, helping to comprehend pollen spatial and temporal dynamics and to forecast its future patterns, simultaneously providing important ecological insights. Despite their long-term continuity, aeropalynologycal data collections may often result spatially and temporally incomplete, mostly because traditional pollen analysis is labour-intensive. Progresses in machine learning, in particular with deep learning technologies like CNN, might soon relieve the workload of palynologists, granting at the same time a more rapid, accurate, and precise morphological identification of pollen grains, even to the species level (Sevillano et al., 2020). The implementation of automatic pollen counting systems, in combination with the production of affordable portable pollen samplers, might also promote epidemiological studies based on individual pollen exposure, that are still not cost and time effective with current technology. To date, RT automatic pollen monitoring networks exist in Switzerland, Germany and Japan, but they still yield satisfactory results only with low allergenic species richness. The progressive training of the machine learning algorithms associated to RT pollen sensors might soon lead to the publication of precise pollen bulletins only moments after air sampling.

Thanks to computational intelligence, morphological approach seems to be the most reliable and efficient proxy for pollen identification at the moment. While DNA markers have the potential to identify pollen to the species level, they cannot be used for routine airborne pollen monitoring just yet. In fact, even if DNA metabarcoding by NGS analysis can efficiently and simultaneously discriminate almost all the plant genera even in a small aerobiological sample, it cannot evaluate their concentrations. Furthermore, metabarcoding standard for land plants requires the use of at least 2 markers in combination, complicating NGS analysis and raising its cost (Banchi et al., 2019). On the other hand, qPCR allows to precisely identify and quantify several species of airborne pollen, but its application to daily

airborne monitoring is still unlikely as well, due to its expensiveness and technical limits (Rittenour et al., 2012).
Overall, in the near future applications of LIF, spectroscopy, genomics and automated image-recognition might be the common procedure for pollen counts. While this would be a great advance for the discipline, in the perspective of allergen avoidance it might not be worth the effort. In fact, many studies reported a discrepancy between allergenic airborne pollen levels and pollen allergy manifestations (Cecchi, 2013; D'Amato et al., 2007). Clear associations between these two factors however are difficult to assess for a number of reasons. First, it is almost impossible to clinically define allergic rhinitis in large populations, and this impairs epidemiological studies since unrelated symptoms might be mistakenly attributed to the disease. Even when pollen allergy is diagnosed, reported symptom intensity after pollen exposure remains subjective, and it can also be influenced by biological or environmental factors, like air pollution levels (Ozdoganoglu and Songu, 2012). Furthermore, individual reactions to a certain pollen exposure may also vary according to allergen sensitisation, cross-reactivity and pollen potency. Hence, to objectively evaluate the allergic risk for pollinosis sufferers, IgE levels against a specific allergen of monosensitised subjects should be compared to atmospheric levels of that allergen. Airborne allergen monitoring is feasible and reliable, and unlike pollen counts it can also quantify the risk caused by aeroallergen carriers other than pollen grains (Fernández-González et al., 2011). Implementation of ELISA in routine air quality monitoring however might require some time, and it would be likely focused only on the most important aeroallergens in the region. Besides, ELISA results are produced with one or more days of delay from the actual sampling, hence cannot be used to alert the population. Moreover, they do not take into account the differences in potential allergenicity among allergen isoforms (Wolf et al., 2017). Additionally, predicting future pollen aeroallergen patterns appears even more challenging than pollen forecasting, due to the variability of their carriers and the lack of knowledge about their production and release.

Hence, it is apparent that more basic research is needed to best exploit all the available technology. Understanding how environment and genetics influence allergens production, and characterising pollen allergens release mechanisms, would lead the way to the integration of pollen and allergen quantification into a comprehensive air allergenicity monitoring and forecasting system. To do so, standardised studies comparing airborne pollen spectra, aeroallergen levels, meteorological conditions and allergic reactions are needed.
Still, this might not be enough for pollen allergy prevention. Allergen avoidance is often unpractical, because it would heavily interfere with the daily life of allergic subjects, who are more eager to take medicines than to follow such preventive measure (Ozdoganoglu and Songu, 2012). The best way to help them might be to provide a hypoallergenic urban environment, by selecting non-allergenic plants for landscaping, controlling allergenic weeds, and adopting appropriate maintenance schedules. An allergic risk assessment of existing recreational green areas should also be performed, to warn sensitive visitors against the seasonal allergenicity potential of the park, and to evaluate future interventions (Cariñanos and Casares-Porcel, 2011).

Life cycle (lc)		Phenanthesic period (pp)		Cross reactivity (cr)		Abundance (a)	
Definition	**Value**	**Duration**	**Value**	**Presence**	**Value**	**Cover %**	**Value**
Annual	1	Less than 1 month	0.5	None present	0	<1	0.5
Biennial	2	More than 1 month	2	Present	1	1-25	1
Perennial	3					25-50	2
						50-75	3
						75-100	4

6 Tables

Table 1 *SAI parameters, with their levels and related arbitrary numerical value* (Hruska, 2003)

Parameter	**Definition**		**Arbitrary values** (proposed by the authors)
$\mathbf{H_{max}}$	Maximum height reachable by pollen-producing vegetation. It can be evaluated accurately in situ, or it can arbitrarily be considered as the maximum height potentially reachable by the park vegetation.		14 m
$\mathbf{S_T}$	Total surface of the urban green area studied.		
$\mathbf{n_i}$	Number of individuals of the i-species in the green area.		
$\mathbf{V_i}$	Average volume of pollen-producing vegetation for each individual of the i-species (*i.e.* tree crowns, bushes, turfs). Foliage shape is simplified into one of the following regular geometric shapes:		
	Cylinder:	$V_i = H_i * S_i$	
	Cone:	$V_i = H_i * \frac{S_i}{3}$	

	Sphere:	$V_i = \frac{4}{3}\pi r^2$		
	Emisphere:	$V_i = \frac{2}{3}\pi r^2$		
	Cuboid:	$V_i = H_i * S_i$		
H_i	Average height of pollen-producing vegetation for each individual of the i-species. It can be evaluated accurately in situ, or it can be assumed to be the maximum height reported for the species. In the last case, arbitrary height categories are proposed.		Trees and shrubs:	2 m
				6 m
				10 m
				14 m
				18 m
			Herbs:	0.25 m
Si	Average surface covered by each individual of the i-species (crown projection for trees). It is measured in situ.			

Table 1a *Definition and values of biometric parameters for Urban Green Zones Allergenicity Index (IUGZA)* (Cariñanos et al., 2014; Kasprzyk et al., 2019b)

Parameter	**Definition**	**Arbitrary values** (proposed by the authors)	
PAV (or VPA)	Allergenicity Potential Value of each species. PAV= tp*ap*dpp	$PAV_{max} = 9$	
Tpv2:	Type of pollination. Drawn from literature.	Sterile, cleistogamous or female	0
		Entomophilous	1
		Amphiphilic	2
		Anemophilous	3
Ap	Allergenicity potential of the plant species relative to the study area. Drawn from literature and databases.	Nonallergenic	0
		Low	1
		Moderate	2

		High	3
		Major allergen in the region	4
Dpp	Duration of pollination period. Pollen grains belonging to the same pollen type are considered as a single pollination event.	1-3 weeks	1
		4-6 weeks	2
		>6 weeks	3

Table 2b *Definition and values of biologic parameters for Urban Green Zones Allergenicity Index (I_{UGZA})* (Cariñanos et al., 2014; Kasprzyk et al., 2019b)

Parameter	**Definition**	**Arbitrary values**	
PD_i	Potential Dispersability of the i-area. It is calculated by visibility analysis, using GIS and LiDAR to map all the potential obstacles to pollen dispersion.	0 - 10	
N_i	Density of the species in the i-area (trees/ha)		
α_i	pollen production according to the species and use	0.001, 0.01, 0.05, 0.1, 1	
M_i	Average maturity degree of individuals of the studied i-species in the i-area, calculated measuring trunk diameter and branch development	Young (<1 year)	1
		Adult (between 1 and 10 years)	5
		Mature (>10 years)	10
SH_i	Incidence of urban landscape on pollen dispersion in the i-area	residential/office/commercial/industrial street	1
		parkway	2
		boulevard	4
		main street	6
		wide avenue	8
		parks or public squares	10
H	Height above see level of the i-area	>1500 m.a.s.l	1
		<1500 m.a.s.l	5
ST	Total surface of the city in km^2		

Table 3 *Definition and values of Aerobiological Index of Risk for Ornamental Trees (AIROT)*

Sampler type	**Sedimentation sampler**			**Impaction sampler**		
Collection method	Passive sedimentation			Slit impaction		Rotating impaction
Sampler name	Durham sampler	Cour Grovette sampler	Sigma-2-like sampler	Hirst-type pollen and spore trap		Rotorod®
Sampling surface	Glass slide coated with grease	Cellulose garze impregnated in silicon oil	Adhesive slide or foil	Polyester tape coated with silicon oil or petroleum jelly	Adhesive-coated glass slide	Two plastic rods of 1.52 x 1.52 x 32 mm, coated with silicone grease
volumetric	No	Yes	Yes	Yes	Yes	Yes
d50*	Not specified	Not specified	2.5 µm	3.7 - 5 µm		10 µm
Air intake velocity	-	Wind speed	Wind speed	10 l/min		Calculated with Pappus's Theorem for Volumes
Pollen collection efficiency	Not evaluated	Not evaluated	Variable	80%		80%
Time progression record	None	None	None	Hourly		None
Bibliography	(Durham, 1946; O'Rourke, 1990)	(Belmonte et al., 2000; Cour, 1974)	(Hofmann et al., 2014; Miki et al., 2019; Mimić and Šikoparija, 2021; VDI 2119, 2013)	(Frenz and Boire, 1999; Hirst, 1952; Levetin, 2004; Mandrioli and Puppi, 1978)		(Frenz and Boire, 1999)

parameters (Pecero-Casimiro et al., 2019)

**** d50***: value of the particle diameter at 50% in the cumulative distribution

Table 4 *Description of air samplers currently used for outdoor continuous pollen monitoring*

Sampler type	**Slit impaction sampler**					**Filtration sampler**
Sampler name	Hirst-type pollen and spore trap	Sampling cassette	Pollator	Pollensniffer	NAS	Sampling cassette
Sampling surface	Adhesive-coated glass slide	Adhesive-coated glass	Adhesive tape on a cartridge	Vaseline-coated Melinex® strip on a glass slide	Acrylic pressure-sensitive adhesive tape	Filter membrane
Wearable	No	Yes	Yes	Yes	Yes	Yes
d50*	3.7 µm	1.7-2.8 µm	Not specified	Not specified	5 µm	Adjustable
Sampling duration	24 h	10 min	16 h	5–6 h	20 min	10 min
Optimal air intake velocity	10 l/min	15 l/m	6 l/min	7.5–9.2 l/min	25 l/min	15 l/m
Pollen collection efficiency	80-90%	95%	Not specified	Not specified	100%	65%
Time progression record	Yes	No	No	No	No	No
Bibliography	(Heffer et al., 2005; Hirst, 1952)	(Grinshpun et al., 2007; Heffer et al., 2005)	(Werchan et al., 2018)	(de Weger et al., 2020)	(Graham et al., 2000)	(Heffer et al., 2005)

**** d50***: value of the particle diameter at 50% in the cumulative distribution

Table 5 *Description of different portable and personal samplers available on the market.*

Sampler type	**Image-based**			**LIF-based**		
Sampler name	KH-3000	BAA500	APS by PollenSense™	WIBS	PA-300 Rapid-E	Poleno
Recognition principle	Forward- and side-scattering from IR-A laser beam	Optical microscopy	Image capture in a lighting environment Convolutional neural networks	Elastic scattering from red laser beam, UV laser-induced fluorescence, Machine learning	Multi-angle scattering from near-UV laser beam, Deep-UV laser-induced fluorescence, Artificial neural networks	Light scattering, Holography, UV laser-induced fluorescence, Convolutional neural networks
Air intake velocity	4.1 l/min	100 l/min	Not specified	2.4 l/min	2.8 l/min	40 l/min
Processing time	Results in real-time	Pollen counts every 3 hours	Results in near real-time	Results in real-time	Up to 4500 particles processed per minute	Results in real-time, hourly resolution
Human intervention	None	Manual labelling (Optional)	None	Data analysis (Optional)	None	Manual labelling (Optional)
Recognition accuracy	Can effectively discriminate *Cryptomeria japonica* from other pollen types	Can recognise at least 11 pollen types with over 70% accuracy	Can identify pollen groups to order and genus 92.5% of the time, with variable accuracy for different types.	Can effectively distinguish pollen in aerosol samples	Can recognise at least 5 pollen types with 80% accuracy; can identify grass pollen and 3 macro-groups of non-grass pollen	Can recognise at least 6 pollen types with over 90% accuracy
Manual check	None	Pollen micrographs from 8 focal positions	None	None	Pollen sampled on adhesive-coated slides	Holographic reconstruction of the pollen grain, Pollen samples (Optional)
Comparison with Hirst-type sampler	Pearson's correlation coefficient for total daily pollen concentrations: 0.52	Spearman's correlation coefficient for total daily pollen concentrations: 0.84	Pearson's correlation coefficient for total daily pollen concentrations: 0.5	Total pollen concentrations of the same magnitude, same average, higher maximum than Hirst-type	Correlation coefficients for total pollen on the whole sampling period: - Pearson's: 0.95 - Spearman's: 0.82 - Kendall's: 0.65	Not available
Bibliography	(Huffman et al., 2019; Kawashima et al., 2017)	(Huffman et al., 2019; Oteros et al., 2020, 2019, 2015)	(Dalan et al., 2020; Lucas et al., 2021, 2016; Lucas and Bunderson, 2019)	(Calvo et al., 2018; Huffman et al., 2019; Ruske et al., 2018)	(Crouzy et al., 2016; Huffman et al., 2019; Šauliene et al., 2019)	(Huffman et al., 2019; Sauvageat et al., 2020)

Table 6 *Real-time sensors employed for automatic recognition of airborne pollen grains.*

Application	**Evaluating qPCR efficiency**	***Betula pendula*, *Artemisia vulgaris* and *Ambrosia artemisiifolia* pollen monitoring**	**Distinguishing *Juniperus ashei*, *Juniperus pinchotii*, and *Juniperus virginiana* in air samples**
Sampler	Hand-sampled pollen from local allergenic plants	High-volume dichotomous sampler (self-constructed)	Hirst-type sampler
Sampling substrate	Polyester tape coated with silicon oil	2 glass fibre filters (diameter cut-off: >3μm and <3 μm)	Polyester tape coated with silicon oil
Sample dimension	-	7-days samples ($375\ m^3$ of air)	24-h samples ($14.4\ m^3$ of air)
Marker	Single- or low-copy nuclear genes	Nuclear DNA: BP8 and ITS for *Betula;* ITS for *Artemisia* and *Ambrosia*	cpDNA: *matK*
Probes	TaqMan probe dual labelled with a 6-carboxy-fluorescein group and Black Hole Quencher	SYBR® Green	TaqMan probe dual labelled with a 6-carboxy-fluorescein group and Black Hole Quencher
Termocycler	Light-Cycler 480 thermocycler (Roche Diagnostics)	Real-Time PCR MiniOpticon™ System (Biorad)	Step One Plus Real-Time PCR System (Applied Biosystems Inc)
Database	NCBI	NCBI, Genbank	Genbank
Taxonomic level	Species	Genus	Species
Bibliography	(Longhi et al., 2009)	(Müller-Germann et al., 2017, 2015)	(Mohanty et al., 2017b)

Table 7 *Real-time quantitative PCR approaches to airborne pollen monitoring*

Application	**Outdoor airborne pollen monitoring**						**Indoor deposited pollen monitoring**
Sampler	Hyrst-type sampler	Hyrst-type sampler	Cyclone sampler	Hyrst-type sampler	Impaction sampler (DUO SAS Super 360)	Hyrst-type sampler	Filtration sampler
Sampling substrate	Polyester tape coated with silicon oil	Melinex® tape coated with pharmaceutical sterile Vaseline	1.5 ml plastic vial	Melinex® tape coated with silicon-based adhesive	Petri dishes covered with sterile Vaseline	Melinex® tape coated with adhesive	Nylon filter
Sample dimension	1 longitudinal half of a 24-h sampling tape (7.2 m^3 of air)	1 longitudinal half of a 7-days sampling tape (50.4 m^3 of air)	24-h samples (23.7 m^3 of air)	1 longitudinal half of a 24-h sampling tape (7.2 m^3 of air)	1-h samples (10.8m^3 of air)	6 24-h samples pooled together per week (86.4 m^3 of air)	Vacuumed surfaces of 2 m^2 each
Marker	*trnL*	ITS2	*rbcL*, ITS2	*rbcL*	ITS2	ITS2	ITS2
NGS platform	Ion Torrent/Proton (Thermo Fisher Scientific)	MiSeq (Illumina)	MiSeq (Illumina)	MiSeq (Illumina)	MiSeq (Illumina)	Ion Torrent PGM (Thermo Fisher Scientific)	454 FLX pyrosequencing (Life Science, Roche), MiSeq (Illumina)
Database	Genbank	Custom database with data from Genebank and NCBI	Genbank	NCBI, Atlas of Living Australia	Custom database with data from Genebank and NCBI	PLANiTS2	Genbank
Bioinformatic pipeline	TSSV	Qiime	Python scripts	Qiime	Qiime	Qiime	Mothur
Taxonomic level	Genus	Genus	Genus	Genus	Family, genus	Genus, species	Genus
Bibliography	(Kraaijeveld et al., 2015)	(Núñez et al., 2017)	(Brennan et al., 2019)	(Campbell et al., 2020)	(Núñez and Moreno, 2020; Sánchez-Parra et al., 2021)	(Banchi et al., 2020)	(Korpelainen and Pietiläinen, 2017)

Table 8 *NGS metabarcoding approaches to airborne pollen monitoring*

Sampler type	**Cyclone sampler**	**Cascade (or sieve) impactor**	
Collection method	**Centrifugal force**	**Multi-stage impaction**	
Common models	Low-volume Burkard multi-vial Cyclone sampler	ChemVol® high-volume cascade impactor	Andersen sampler
Sampling surface	1.5 ml plastic test tubes	Toroid-shaped pieces of polyurethane foam, up to 3 stages	Glass fibre filters, up to 8 stages
Air intake velocity	16.5 l/min	800 l/min	28 l/min
Particle collection efficiency	99% up to 1.06 µm 93% in the range 0.82 - 0.75 µm	Not specified for these substrates	
Time progression record	Adjustable	No	
Bibliography	(Brennan et al., 2019; Fernández-González et al., 2019; Lippmann and Chan, 1979; Plaza et al., 2017)	(Levetin, 2004; Menetrez et al., 2001; Mitchell and Pilcher, 1959; Plaza et al., 2017, 2016; Schäppi et al., 1999)	

Table 9 *Description of most common aeroallergen samplers employed for pollen allergens monitoring*

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