1-1-2023

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10.1016/j.scitotenv.2023.163345
Frisk, C. A., Apangu, G. P., Petch, G. M., Creer, S., Hanson, M., Adams-Groom, B., & Skjøth, C. A. (2023). Microscale pollen release and dispersal patterns in flowering grass populations. Science of the Total Environment, 880, article 163345. https://doi.org/10.1016/j.scitotenv.2023.163345

Science of the Total Environment 880 (2023) 163345

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Microscale pollen release and dispersal patterns in flowering grass populations

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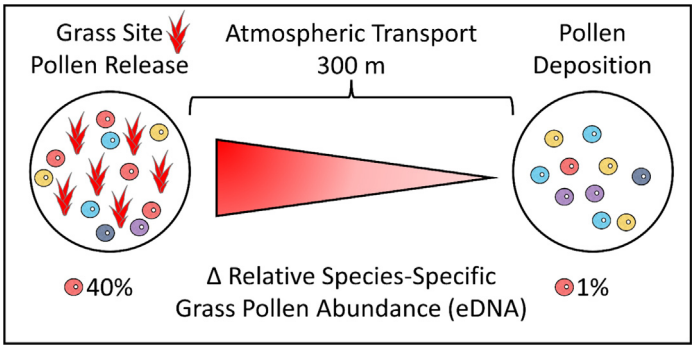
HIGHLIGHTS

- Grass pollen concentrations vary on a microscale (<2 km) geographical level.
- Most grass pollen are deposited within 300 m of the source area.
- Turbulent Kinetic Energy (TKE) is important for grass pollen emission.
- Atmospheric grass pollen biodiversity was found to vary within 300 m.

GRAPHICAL ABSTRACT



ARTICLE INFO

Guest Editor: Athanasios Damialis

Keywords:
Bioaerosol
eDNA
Festuca rubra
DNA metabarcoding
Phenology
Poaceae

ABSTRACT

Characterizing pollen release and dispersion processes is fundamental for knowledge advancement in ecological, agricultural and public health disciplines. Understanding pollen dispersion from grass communities is especially relevant due to their high species-specific allergenicity and heterogeneously distributed source areas. Here, we aimed to address questions concerning fine level heterogeneity in grass pollen release and dispersion processes, with a focus on characterizing the taxonomic composition of airborne grass pollen over the grass flowering season using eDNA and molecular ecology methods. High resolution grass pollen concentrations were compared between three microscale sites (<300 m apart) in a rural area in Worcestershire, UK. The grass pollen was modelled with local meteorology in a MANOVA (Multivariate ANOVA) approach to investigate factors relevant to pollen release and dispersion. Simultaneously, airborne pollen was sequenced using Illumina MySeq for metabarcoding, analysed against a reference database with all UK grasses using the R packages *DADA2* and *phyloseq* to calculate Shannon's Diversity Index (α-diversity). The flowering phenology of a local *Festuca rubra* population was observed. We found that grass pollen concentrations varied on a microscale level, likely attributed to local topography and the dispersion distance of pollen from flowering grasses in local source areas. Six genera (*Agrostis*, *Alopecurus*, *Arrhenatherum*, *Holcus*, *Lolium* and *Poa*) dominated the pollen season, comprising on average 77 % of the relative abundance of grass species reads. Temperature, solar radiation, relative humidity, turbulence and wind speeds were found to be relevant for grass pollen release and dispersion processes. An isolated flowering *Festuca rubra* population contributed almost 40 % of the relative pollen abundance adjacent to the nearby sampler, but only contributed 1 % to samplers situated 300 m away.

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http://dx.doi.org/10.1016/j.scitotenv.2023.163345
Received 18 January 2023; Received in revised form 17 March 2023; Accepted 3 April 2023
Available online 6 April 2023

This suggests that most emitted grass pollen has limited dispersion distance and our results show substantial variation in airborne grass species composition over short geographical scales.

1. Introduction

Pollination ecology is one of the most complicated and currently relevant biological research disciplines, with many fundamental processes remaining poorly understood (Knight et al., 2018; Mayer et al., 2011). Production and release of pollen from grasses is complex and questions remain about what drives these processes and how effective they are (Davies et al., 2015; Rech et al., 2016; Timerman and Barrett, 2021). Reproduction processes in entomophilous plants are driven by the reward seeking behaviour of the vector (pollinating insects) (Abou-Shaara, 2014; Nunez and Giurfa, 1996), while the vector in anemophilous plants (wind) has no goal-oriented behaviour, making the process random in nature with complex functional limitations (Culley et al., 2002; Friedman and Barrett, 2009; Friedman and Harder, 2004). In many anemophilous plants the source (anthers in male flowers) and the sink (most often the wider landscape environment but sometimes also the stamen of the correct species) are usually thoroughly identified (Cebrino et al., 2016; García-Mozo, 2017). However, the quantification of the processes connecting the source to the sink and the specifics of pollen release, dispersal and deposition are generally unknown for many plants due to the difficulties in studying these complex interconnected systems, primarily due to their inherent random nature. Such knowledge is important to the understanding of ecological and agricultural geneflow and for food security applications (Ronald, 2011; Serageldin, 1999; Ushiyama et al., 2009). The pollen from a subset of anemophilous plants are also allergenic to sensitised people (Akdis and Agache, 2014; Newson et al., 2014), making these processes also relevant and important to public health policies.

In some countries, pollen is identified nationally in pollen monitoring networks to warn the larger public of impending pollen seasons (Adams-Groom et al., 2020; Buters et al., 2018; Lo et al., 2019; Pecero-Casimiro et al., 2020). Most allergenic pollen can be identified to genus levels using palynological techniques and microscopy. However, grasses lack morphologically identifiable traits at genus level, making their pollen identifiable to family level only during routine monitoring (Morgado et al., 2015), although differences can be observed to some extent for cultivated grasses such as Cerealia types (Joly et al., 2007) and C3/C4 grasses (Jan et al., 2015). The lack of morphological discrimination in grasses contrasts with other genera with allergenic potential such as the Betulaceae family, where simplicity in morphological pollen classification has enabled numerous studies on reproduction, flowering and health impacts (e.g. Newnham et al., 2013; Ritenberga et al., 2018; Siljamo et al., 2013). Meanwhile for grasses, homogenous pollen morphology coupled with heterogeneous distribution ranges and largely overlapping flowering patterns has limited fundamental progress in understanding their reproductive ecology (but see Bhattacharya and Datta (1992) and Tormo et al. (2011)).

Species-specific pollen production has previously been used as proxy for the importance of certain species in the pollen record (Aboulaich et al., 2009; Prieto-Baena et al., 2003; Tormo-Molina et al., 2015), but abundance of species can be equally important. Environmental DNA (eDNA) studies from the UK suggest higher airborne pollen concentrations of *Poa pratensis* (Rowney et al., 2021) and *Holcus lanatus* (Brennan et al., 2019), when compared to *Dactylis glomerata*. This contrasts with the pollen productivity of individual grass species, where other studies have found *D. glomerata* to have a much higher pollen productivity out of these three species (Prieto-Baena et al., 2003). Species-specific differences in allergenicity enhance the importance of being able to classify pollen down to the lowest taxonomical order, with pollen from *Lolium perenne*, *Phleum pratense* and *Dactylis glomerata* identified as some of the most allergenic pollen in contrast to many other grasses (Andersson and Lidholm, 2003; Luo et al., 2016; Van Ree et al., 1998).

Studies have found that there is limited connection in grass pollen concentrations between sites >50 km apart (Kurganskiy et al., 2021), and a study from Melbourne (Thien et al., 2018) found that a sudden and short term increase in grass pollen concentrations, suggested to be associated with *L. perenne* exclusively, had a widespread and dramatic negative impact on public health. These findings suggest that grass pollen concentrations may vary considerably at short geographic scales as well as in time. Cross-reactivity between allergens of different species adds another layer of complexity to understanding the dynamics of pollen release from different species of grass and allergenic burden (Aleksić et al., 2014; Mohapatra et al., 2005). Consequently, the understanding of grass pollen at the species level in relation to release, dispersal and impact on ecological processes and human health is among the most poorly understood areas in aerobiology. Our limited knowledge of grass pollen dispersion is contrasted by the widespread distribution of grasses globally and the fact that grass pollen is considered the most important outdoor aeroallergen with the highest number of sensitizations (Burbach et al., 2009; Heinzerling et al., 2009).

Recent developments in molecular ecology and eDNA approaches have made it possible to identify species-specific pollen using molecular methods that rely on genomic markers in specific DNA regions (Creer et al., 2016; Kraaijeveld et al., 2015). Common regions used for this purpose include ITS, *rbcL*, *trnL* and *matK* (Baksay et al., 2020; Campbell et al., 2023; Galimberti et al., 2014; Hawkins et al., 2015; Milla et al., 2021). Multiple methods have been successfully utilized to identify species-specific pollen and are classified based on the quantitative nature of the method, with the most common ones being semi-quantitative metabarcoding (Bell et al., 2016; Leontidou et al., 2018) and the quantitative qPCR (Ghitarrini et al., 2018; Teng et al., 2016). Brennan et al. (2019) found using DNA metabarcoding that atmospheric species-specific grass pollen varied considerably between locations across Great Britain. Rowney et al. (2021) found using qPCR, that a small subset of specific grass pollen species can be directly attributed to asthma and respiratory conditions in human allergy sufferers.

Identifying the factors and processes involved in release and dispersal of pollen from indicative grass species will be essential in the mechanistic modelling (Kurganskiy et al., 2020), forecasting of allergenic pollen relevant for public health (Suanno et al., 2021) and the understanding of reproductive ecology in grasses (Linder et al., 2018; Van Treuren et al., 2006). To better understand these processes, we established a defined study area containing populations of grasses and observed their flowering processes over the course of a season. Simultaneously, we collected high resolution grass pollen data and pollen eDNA from multiple sites in the surrounding area and investigated how meteorology and the flowering population contributed to the distribution of grass pollen in the landscape on a microscale. We combined the flowering phenology, DNA metabarcoding and high-resolution microscale pollen monitoring and meteorology to test the following hypotheses: *There is no difference in* (i) *airborne grass pollen biodiversity and* (ii) *concentration at the microscale level* (*<2 km*) and (iii) *pollen concentration at the microscale level is governed by weather factors directly controlling the atmospheric dispersion*. Hypothesis (i) will be tested by analysing the spatial variation in species composition obtained using metabarcoding analysis and testing for significance in the alpha diversity (Shannon's Diversity Index) using a bootstrapping linear regression approach. Hypothesis (ii) will be tested for significance by comparing grass pollen concentrations from multiple locations. Hypothesis (iii) will be tested for significance by modelling high resolution pollen concentrations with the most important variables for atmospheric transport, dispersal and removal (wind speed and direction, turbulent kinetic energy (TKE) and precipitation) and the most important variables for grass pollen release (temperature, solar radiation and relative humidity). Finally, it will be

explored whether local variations in overall pollen concentrations may be associated with emission from one particular grass species and if this species can be identified in comparable samples using metabarcoding.

2. Material and methods

2.1. Experimental locations and pollen sampling

Three experimental sites located on Lakeside campus (52.254, −2.254) at the University of Worcester were used in this study. Lakeside is located about 7 km north of the city of Worcester and about 35 km southwest of the city of Birmingham in the West Midlands region of the United Kingdom. Lakeside is a flat rural landscape being mostly surrounded by arable lands and pastures, with the immediate campus area being constituted of regularly mown amenity grass areas (see Frisk et al. (2022) for a more detailed description). The three sites were closely located within the Lakeside campus (<300 m apart) and were colloquially named after their setup and referred to as Container, Tripod and Field (Fig. 1). Each site contained both a Burkard volumetric spore trap to sample bioaerosols used for pollen monitoring and a Burkard multi-vial cyclone sampler to sample bioaerosols used for eDNA approaches. The Container sampling equipment was located on a large container structure and placed 4 m above ground level (AGL), with the sampling inlets being located at 5 m AGL (42 m Above Mean Sea Level (AMSL)). The Tripod sampling equipment was located on a metal tripod structure designed as a temporary scaffolding to house sampling equipment, with the inlet of the samplers being located at 2.5 m AGL (38 m AMSL). The Field sampling equipment was located on a meteorological mast designed to house meteorological logger stations (see Section 2.2), with the inlet of the samplers being located at 2.5 m AGL (38 m AMSL).

The Field site was established as an artificial site one year prior to the study to simulate a distinct area containing natural grassland vegetation (Supplementary Fig. 1). A circle with radius of 25 m (~1964 m^2) was fenced off in the middle of a large open, mown field and was fertilized evenly using a 15:10:10 NPK (Nitrogen, Phosphorous, Potassium) standard mixture with an 80 kg N per hectare recommendation (Rollett et al., 2015) due to a concern for low productivity, caused primarily by the porous and stony soil (Mueller et al., 2010) and lack of nutrient-rich clay and silt components. A path from the outside of the circle was also created and fenced off to allow for access to the sampling equipment within while still allowing for the vegetation to establish and grow naturally without any disturbance or destruction from trampling and similar activities. A botanical survey was conducted in 2018 with an updated survey in 2019 to determine the species diversity and abundance, primarily of grasses. The updated survey was conducted to exclude the possibility of a large species turnover. The entire inner and outer part of the circle was surveyed due to the relatively small total area using standard guidelines following the UK plant taxonomic key (Stace, 2010). The updated survey found the main grass occupants

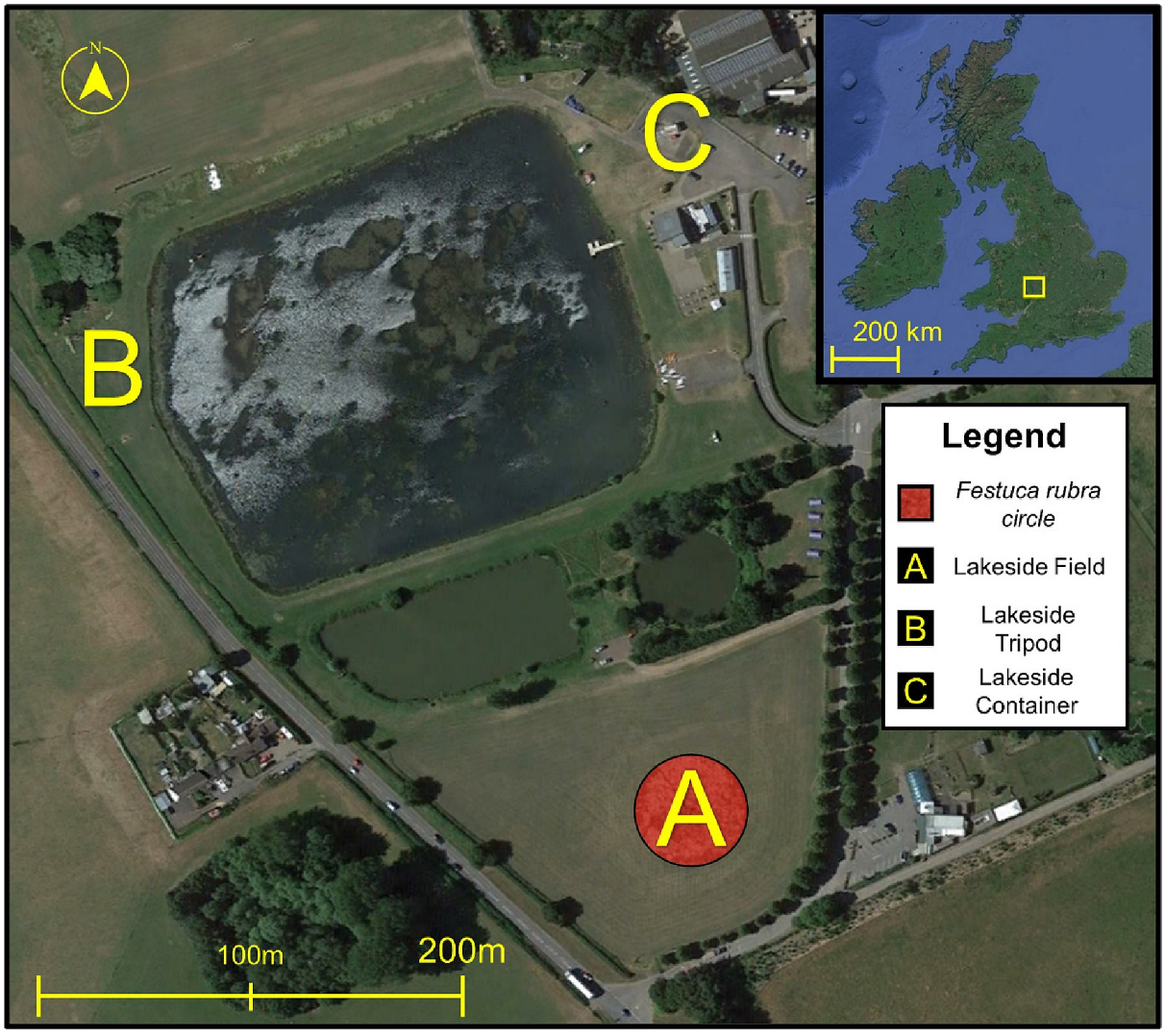

Fig. 1. Local Lakeside map. The map illustrates the location of the three Lakeside grass pollen sampling sites (Field, Tripod and Container) along with the occurrence of *Festuca rubra* in the Lakeside Field site. The sampling equipment is in the middle of the letter representing each site.

(>40 %) were *Festuca rubra* and *Lolium perenne* with minor occupancy (<1 %) of *Agrostis capillaris*, *Bromus hordeaceus*, *Poa annua*, *Poa trivialis* and *Holcus lanatus*. The vegetation outside of the circle and surrounding the other two samplers was regularly mown to prevent the growth and flowering of any other grasses. The larger Lakeside area was surveyed for other established and flowering grass plants. While *L. perenne* was frequently found no other *F. rubra* plants were found outside of the Field site. The identification of a distinct source area made it a good model species for further investigation into pollen release and dispersion dynamics.

Grass pollen was monitored at all the three sites using Burkard volumetric spore traps (Hirst, 1952) between the dates 14th of May to 6th of July 2019 (a total of 54 days) to overlap with the flowering period of the grasses within the Field location (see Section 2.3). The pollen monitoring was conducted using the standardized methodology of the United Kingdom pollen monitoring network (Adams-Groom et al., 2002; Skjøth et al., 2015) with more details in Frisk et al. (2022). The daily vertical transects (twelve per slide per location) used to construct the daily pollen concentrations were converted into bi-hourly pollen concentrations for the three locations, thereby allowing for higher resolution grass pollen data to be used in this study.

2.2. Meteorological data

The meteorological data used in this study were obtained from a Campbell Scientific meteorological logger station located in the middle of the Field site. The logger was considered adequate to broadly represent the meteorological conditions at the three sites. Four meteorological variables were collected from their respective sensors: solar radiation, ambient air temperature, relative humidity and precipitation. The sensors provide these readings in 30 min resolution. Wind speed in three directions (uX (North – South), uY (West – East) and uZ (Up – Down)) were isolated from a WindMaster 3D ultrasonic anemometer with a sampling resolution of 10 Hz (10 times a second). The measurements (uX and uY) were used to calculate the wind direction using radial geometry. The three wind measurements were also used to calculate total wind speed. Wind speed and direction in combination with pollen settling velocity can suggest likely origin of the pollen (Ciani et al., 2020; Maya-Manzano et al., 2017; Peel et al., 2013) with a higher wind speed being more likely to transport it further from source areas (Damialis et al., 2005). The variation in upwards/downwards drafts can also suggest if the underlying field acts as a source or sink (Chamecki et al., 2009). The three-dimensional wind speeds were further used to calculate turbulent kinetic energy (TKE), which has been identified as a contributory factor in how likely pollen are to be released from flowering anthers (Šikoparija et al., 2018). Temperature, solar radiation, relative humidity, wind speed and wind direction were calculated as means and resampled to match the bi-hourly pollen data. Precipitation was likewise resampled but calculated as bi-hourly sums. TKE was also resampled to match and calculated as minimum, mean and maximum values per bi-hourly datapoint.

2.3. Grass flowering phenology

The flowering phenology of the *F. rubra* population within the Field location was investigated using the 'Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie' (BBCH) scale (Meier, 2018; Meier et al., 2009). This scale is commonly used to investigate flowering progression of grasses (e.g. Cebrino et al., 2018; Ghitarrini et al., 2017; Kmenta et al., 2016; León-Ruiz et al., 2011). The flowering progression follows the protocol of Frisk et al. (2021) in which the flowering at the individual tiller level starts at anthesis (phase 1) and progresses through increased 25 % fractions of extruded anthers (phase 1 to phase 4). Full flowering (phase 4) is characterized as 76–100 % extruded anthers and senescence (phase 5) when all anthers have detached from the flowering head. Example images of *F. rubra* phase 0 (pre-flowering) and phase 4 (full flowering) are available in the supplementary material (Supplementary Figs. 2–3). The flowering observations in this study consisted of a population with 50 random tillers being observed every third day (start 25th of May) in the Field site until all observed tillers had reached senescence for multiple subsequent observation occasions. Population-based observations have previously been suggested to be the only reliable way of predicting species responses to their environment (Forrest and Miller-Rushing, 2010) and have previously been used to investigate climate-pollen interactions in grasses (García de León et al., 2015). The motivation for selecting 50 random tillers was the assumption that it would be a representative sample size and sampling methodology to determine the average flowering progression of a population in the relatively small area. The observations were conducted from the outside of the circle, along the path leading into the inside of the circle and from the inner part of the circle to avoid unnecessary disturbance of the vegetation. The optimal and healthy condition of the vegetation was essential due to the data collection related to the grasses from the other sampling equipment.

2.4. Molecular ecological approaches

2.4.1. eDNA collection and DNA extraction

To investigate the spatial distribution and relative genera and species-specific proportion of grass pollen in the atmospheric eDNA, metabarcoding was performed (see Núñez et al. (2016)). Atmospheric bioaerosols were sampled at all the three sites using automatic Burkard multi-vial cyclone samplers between the 24th of May to the 4th of July 2019 (a total of 42 days) to overlap with the flowering period of the grasses within the Field site. The bioaerosols were sampled by an active-pump system using a cyclone unit into microcentrifuge Eppendorf tubes with 24 h resolution (West and Kimber, 2015). The samples were collected from the in-field samplers weekly and were temporarily stored in −20 °C freezers until DNA extraction.

DNA was extracted from samples using the DNeasy Plant Mini kit (QIAGEN, UK) following the standard QIAGEN DNA extraction protocol outlined in Brennan et al. (2019) with the same protocol modification as utilized by Hawkins et al. (2015). Samples were initially pooled during the lysis-step of the protocol by combining three consecutive days, resulting in 14 extracted samples per site. Prepared 100 μl lysis solution (lysis buffer AP1, RNase A and Proteinase K) was added to each daily sample, vortexed, micro-centrifuged and then transferred into a new tube with additional 100 μl to create the pooling strategy (400 μl/pooled sample). The pooled samples were homogenized using a MP Biomedicals FastPrep-24™ Classic Instrument homogenizer with garnet sand of varying size-fractions to aid in the homogenization process and the manufacturer recommended setting for pollen samples of 6.0 m/s and a duration of 40 s. The rest of the process followed the standard QIAGEN DNA extraction protocol. The DNA was eluted twice using the same 60 μl elution buffer. A small fraction of each extracted sample (14 × 4 μl) for each site was then pooled to form three entire-season samples (3 × 56 μl), resulting in one sample per site to use for downstream applications. The DNA concentrations were determined using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, UK) with a blank elution buffer measured between each real measurement.

2.4.2. DNA metabarcoding and bioinformatics

The relative species-specific proportions of grass pollen were investigated using DNA metabarcoding, employing the universal (plant and fungal) spacer DNA genes ITS1 and ITS2 (Internal Transcribed Spacer) (White et al., 1990). These genes and associated molecular markers have the potential to differentiate sequence reads from different species (e.g. Op De Beeck et al., 2014; Toju et al., 2012; Yang et al., 2018), with both regions being able to distinguish grass species (e.g. Grebenstein et al., 1998; Hsiao et al., 1995; Rodionov et al., 2017). DNA amplification, PCR (Polymerase Chain Reaction) product purification and DNA sequencing were performed by Eurofins (Eurofins Genomics, Ebersberg, Germany) with the process and protocol being outlined in Apangu et al. (2022). A negative control containing DES elution buffer (DNAse-free water) and a positive control containing a pre-defined DNA mock community of different species normally found in atmospheric samples (grass, herb, tree and fungal

species) was also included (see Apangu et al. (2022)). The negative and positive controls are essential for keeping reproducibility and certainty high in genomic and molecular research (Hornung et al., 2019).

The sequence reads were processed using the software R and the R packages *DADA2* (Callahan et al., 2016) and *phyloseq* (McMurdie and Holmes, 2013). The sequence reads had been pre-trimmed and pre-merged, hence the omission of that step. The package *DADA2* was used to assign taxonomy to the sequence reads based on custom-made PLANiTS reference databases. The PLANiTS reference databases were created to be Viridiplantea (green plants) specific (Banchi et al., 2020). The original PLANiTS ITS1 and ITS2 reference databases were filtered using a UK-exclusive grass species list compiled by the Botanical Society of Britain and Ireland (BSBI) in 2009 (Cope and Gray, 2009). This was to avoid similar sequence references from species that are not present in the UK region, similar to Brennan et al. (2019). All reference sequences that matched genera, species and subspecies present within the UK grass species list were imported into the new filtered custom reference database. The taxonomical classification is based around Bayesian approach that randomly selects eight consecutive nucleotides (kmer = 8) per sequence and randomly matches them to the reference database (Wang et al., 2007). The method uses 100 bootstrap replicates, with the default cut-off for correctly assigned taxonomical reference being 50 matches.

Finally, all classified sequence reads were summed to genus level and divided by the total number of sequence reads per sample and ITS region to get the relative proportion for each genus. The sequence reads belonging to the *Festuca* genus were further classified to species level, to investigate the relative species-specific proportion in relation to the entire sample and ITS region.

2.5. Statistical analyses

To identify whether there existed any spatiotemporal divergences of atmospheric grass pollen between the three experimental sites, the grass pollen concentration data sets were first tested for non-normality using the Shapiro-Wilk test (Shapiro and Wilk, 1965), with all three pollen series showing non-normality. The grass pollen data were then analysed using Kendall's tau rank correlation (Kendall, 1938) to see their overall similarity between sites. Kendall's tau was used instead of Spearman's rho due to the more robust and efficient correlative estimation using tau (Croux and Dehon, 2010). The grass pollen was further analysed using the Mann-Whitney *U* test (Mann and Whitney, 1947) to investigate if there were any differences in the total abundance of grass pollen between the sites. These two analyses were conducted for both the bi-hourly and daily pollen resolution, to see if any differences found were primarily within or between days.

The bi-hourly grass pollen from the three sites were further modelled with meteorology to investigate which meteorological variables could potentially predict the observed patterns. The datasets were filtered to exclude observations consisting of zero pollen from the analysis, due to the greater uncertainty of using absences or zero values in the predictive modelling of pollen (Aznarte et al., 2007; Su et al., 2021). By excluding zero values the model estimates the differential in pollen abundance, rather than presence/absence, since absence is not strictly dependent on meteorology and will skew model estimates. The filtering was performed for all simultaneous observations even if only one of the three pollen concentrations were zero due to balancing the model performance. A multiple response linear regression model was created to assess the influence from the meteorological variables on the grass pollen concentrations simultaneously on all three sites. All nine meteorological variables were included in the model: TKE (min, mean, max), wind speed, wind direction, solar radiation, mean temperature, relative humidity and precipitation without any interactions. The model was subsequently analysed using Type II MANOVA (Smith et al., 1962) with Wilks' lambda test statistic (Gupta, 1971; Gupta and Perlman, 1973).

Finally, to identify differences in genus composition of the DNA metabarcoding analysis Shannon's Diversity Index (α-diversity (Whittaker, 1960)) from the R package *vegan* (Oksanen et al., 2020) was compared between the three sites using a bootstrapping linear regression approach. Bootstrapping was utilized to allow for representative distributions used in the modelling (Dixon, 1993; Efron, 1979; Freedman, 1981). Samples (n = total identified genera) used for the Shannon Index calculation were bootstrapped (m = 100) from the relative abundance of all genera identified in the metabarcoding analysis. This was calculated for all three sites and both ITS regions. The bootstrapped indices were then modelled as a linear regression and analysed using a one-way ANOVA. All statistical analyses and bioinformatics were performed using the statistical software R (ver. 4.1.3.) (R Core Team, 2022).

3. Results

3.1. Local grass pollen variation

The bi-hourly grass pollen profiles of the three sites at Lakeside showed a high overlap through the sampled season (Fig. 2). The first major peak (grass pollen concentrations > 500 grains/m^3) occurred in the evening of the 1st of June at all three sites, with the second major peak not occurring until the evening of the 15th of June. By this point more than one month of the sampling had occurred but only 20 % of the total grass pollen for the period had been captured. As the season progressed three major groups of peaks were observed: 24th of June, 27th–29th of June and 3rd–5th of July. The Kendall's correlation estimated high correlations for the bi-hourly time series between the three sites, with the tau ranging from 0.680 to 0.694 ($p < 0.001$) (Table 1). The Mann-Whitney test indicated that there was no difference between the Field and Tripod sites in terms of overall pollen abundance ($p = 0.411$) while the pollen levels were overall lower for the Container in comparison to the other two sites ($p < 0.001$). Kendall's tau increased for the daily correlations, indicating more variation within days than between them. The daily grass pollen profiles can be viewed in the supplementary material (Supplementary Fig. 4). The overall difference can also be observed in the diurnal grass pollen profiles (Supplementary Fig. 5). The diurnal Container levels were always lower overall, with the Field levels being slightly higher than the Tripod during peak hours only (18.00–20.00).

3.2. Grass pollen and weather modelling

After excluding zero-values and simultaneous observations from the other sites the MANOVA model retained 60 % of all bi-hourly data points during the sampled season. The model statistics showed that variables relevant for both pollen dispersion and removal and pollen release were important in predicting bi-hourly grass pollen concentrations for the three sites in Lakeside (Table 2). The variables initially classified as important for pollen dispersion and removal were TKE, wind speed, wind direction and precipitation. Mean TKE was found to increase grass pollen concentrations ($F_{1,367} = 2.678$, $p = 0.047$) while minimum and maximum TKE had no significant effects. Higher wind speeds had negative effects ($F_{1,367} = 4.030$, $p = 0.008$) on pollen concentrations while wind direction had a negative trend effect ($F_{1,367} = 2.547$, $p = 0.056$), as pollen concentrations usually decreased with north-western winds. Precipitation had no significant effect and was found to not be important in modelling the differences in bi-hourly grass pollen concentrations in Lakeside. All three variables initially classified as important for pollen release, i.e., temperature, solar radiation and relative humidity, were found to be important for the model prediction. Grass pollen concentrations were found to increase with temperature ($F_{1,367} = 44.362$, $p < 0.001$) while decreasing as both solar radiation ($F_{1,367} = 17.076$, $p < 0.001$) and relative humidity ($F_{1,367} = 2.772$, $p = 0.041$) increase. Temperature and precipitation values for the period can be viewed in Frisk et al. (2022) while trends for the wind speed, solar radiation, relative humidity and wind direction can be viewed in the supplementary material (Supplementary Figs. 6–7). See supplementary material for the linear model estimates (Supplementary Table 1).

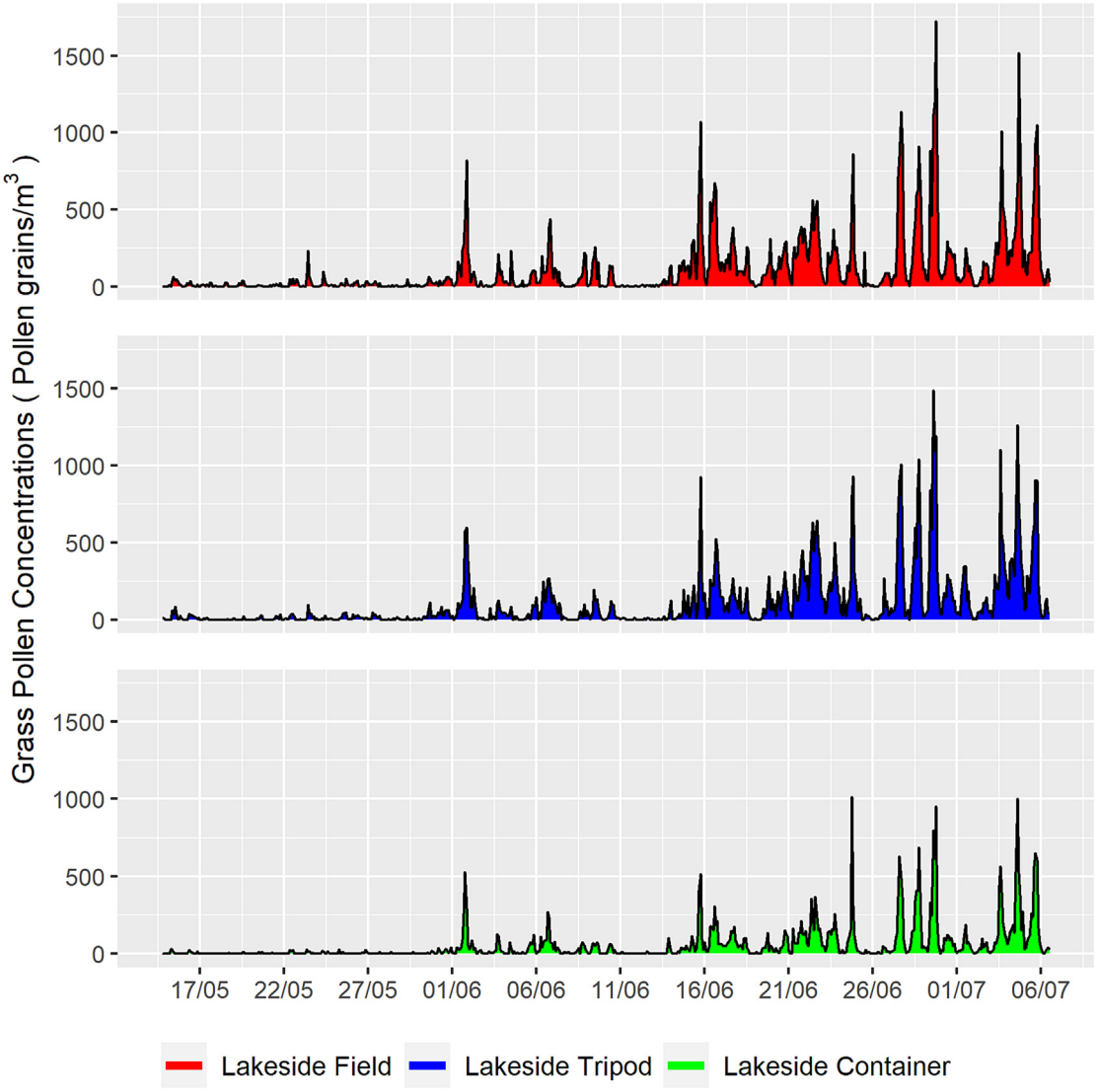

Fig. 2. Bi-hourly concentrations of airborne grass pollen from the three locations in Lakeside (Field, Tripod and Container) for the period 14th of May to 6th of July during the 2019 grass pollen season in Worcester.

3.3. Microscale atmospheric grass pollen diversity

The DNA metabarcoding analysis for the season between 24th of May and 4th of July revealed 22 different grass genera divided between the two ITS-regions (Fig. 3). Not all genera were identified in both ITS-regions, showing variability in the ability to capture atmospheric diversity. Six genera (*Agrostis*, *Alopecurus*, *Arrhenatherum*, *Holcus*, *Lolium* and *Poa*) dominated all three microscale sites and both ITS regions, consisting on average 77 % (40–92 %) of the relative abundance of the total seasonal samples. Overall, there were differences in the α-diversity between the three sites identified both in ITS1 ($F_{2,297}$ = 83.162, $p < 0.001$) and ITS2 ($F_{2,297}$ = 18.263, $p < 0.001$). Even though the samples consist on average of the same genera, substantial variation could be observed in the relative abundance, both for dominating and less frequent genera. This could primarily be observed

Table 1
Model statistics and significance levels for the comparison of bi-hourly and daily pollen concentrations from the three Lakeside samplers for the period 14th of May to 6th of July 2019.

Resolution	Comparison		Model statistics						
	Sampler 1	Sampler 2	Kendall's tau rank correlation				Mann-Whitney *U* Test		
			z	Tau (τ)	P - value	Significance	W	P - value	Significance
Bi-hourly	Tripod	Field	24.714	0.694	$<1 \times 10^{-10}$	***	195,634	0.411	ns
	Tripod	Container	23.521	0.680	$<1 \times 10^{-10}$	***	245,682	$<1 \times 10^{-10}$	***
	Field	Container	24.024	0.693	$<1 \times 10^{-10}$	***	251,579	$<1 \times 10^{-10}$	***
Daily	Tripod	Field	8.851	0.851	$<1 \times 10^{-10}$	***	1287	0.675	ns
	Tripod	Container	9.015	0.870	$<1 \times 10^{-10}$	***	1721	0.017	*
	Field	Container	9.046	0.873	$<1 \times 10^{-10}$	***	1772	0.006	**

Significance: P < 0.001 - '***', *P* < 0.01 - '**', *P* < 0.05 - '*', *P* > 0.05 - 'ns'.

Table 2
Model statistics and significance levels for the linear regression of bi-hourly pollen concentrations from the three Lakeside samplers for the period 14th of May to 6th of July 2019 in relation to turbulence, wind and meteorological variables.
Response variables: tripod, field and container. Model Statistics: Manova (Type II) using Wilks' lambda test statistic.
Abbreviations: TKE - turbulent kinetic energy.

Variable		Model statistics						
Main	Sub factor	Df	Test statistic (λ)	Approx. F	Num Df	Den Df	P - value	Significance
TKE	Min	1	0.995	0.598	3	367	0.616	ns
	Mean	1	0.979	2.678	3	367	0.047	*
	Max	1	0.992	1.030	3	367	0.379	ns
Wind speed		1	0.968	4.030	3	367	0.008	**
Wind direction		1	0.980	2.547	3	367	0.056	·
Solar radiation		1	0.878	17.076	3	367	2.110×10^{-10}	***
Temperature		1	0.734	44.362	3	367	$<1 \times 10^{-10}$	***
Relative humidity		1	0.978	2.772	3	367	0.041	*
Precipitation		1	0.997	0.375	3	367	0.771	ns

Significance: P < 0.001 - '***', P < 0.01 - '**', P < 0.05 - '*', *P* < 0.1 - '·', *P* > 0.1 - 'ns'.

between the two ITS regions, due to their differential ability to distinguish genera. However, the main difference between the samples and ITS-regions was identified for the genus *Festuca*. For ITS1, the relative abundance of *Festuca* in the Field site was 48 % while only ~1 % for the other two sites. For ITS2, it was about 2.7 % for the Field site and ~1 % for the other two sites. The analysis revealed two distinct profiles belonging to the *Festuca* genus: most of the *Festuca* pollen captured was identified as *F. rubra*, with only small portions being classified to genus level as *Festuca* sp., not being identified to a species-specific profile (Supplementary Fig. 8).

3.4. *Festuca rubra* flowering phenology

The density of *F. rubra* tillers within the Field site ranged from 27 to 283 tillers/m^2 with an average density of 143 tiller/m^2 (based on ten 1 × 1 m^2

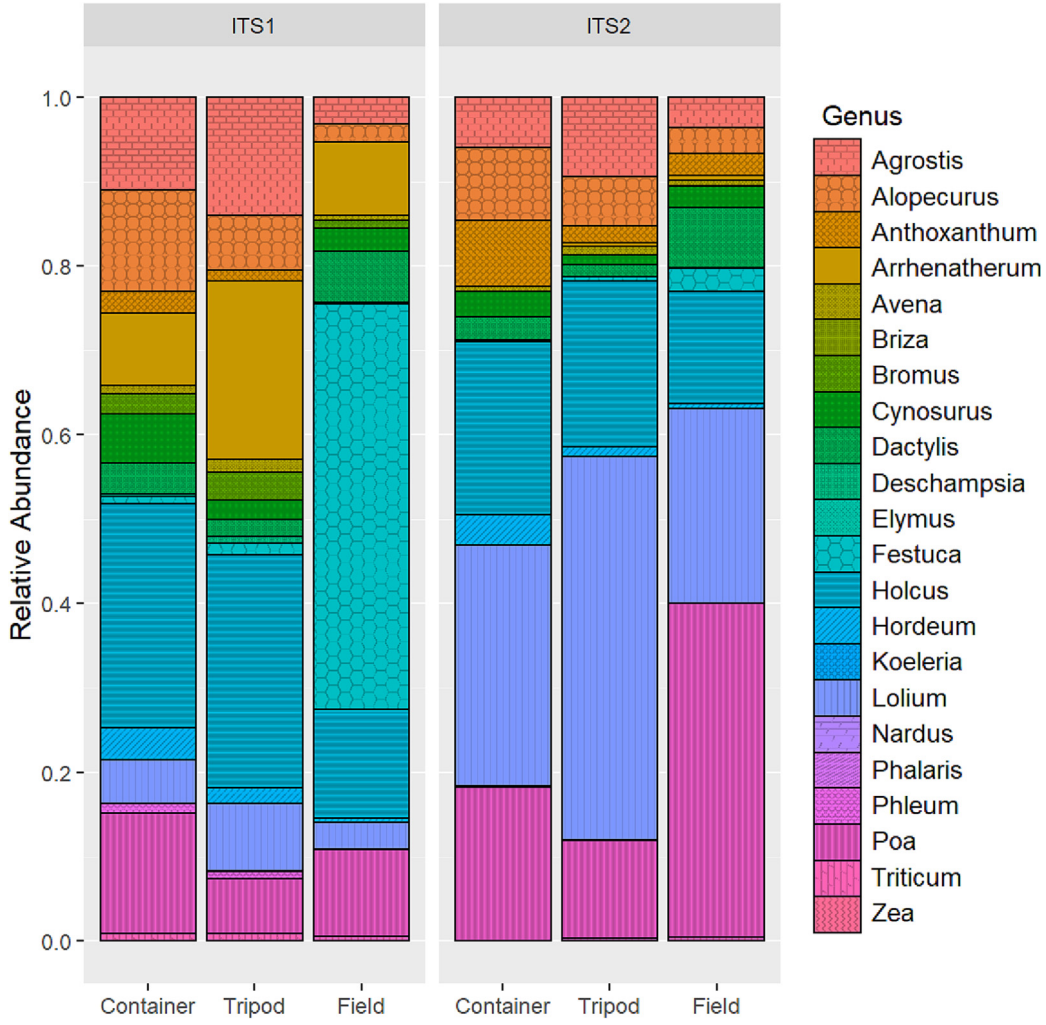

Fig. 3. Relative abundance of all airborne grass pollen genera isolated from the eDNA metabarcoding analysis of the ITS1 and ITS2-region from all three Lakeside locations. Not all genera are present in both ITS regions.

randomly scattered sampling plots). The phenological observations of *F. rubra* within the Field site started on the 25th of May and ended the 12th of July (Fig. 4). The first flowering was observed on the 31st of May, and the period until 15th of June was dominated by pre-flowering tillers, with at most 10 % of tillers showing any flowering activity. The main flowering period observed was between the 18th–30th of June, with up to 40 % of tillers in full flowering (p4) and an additional 24 % in lower flowering phases (p1–p3). By the 30th of June, no flowering tillers were observed from the random sampling. The flowering observations were stopped after four subsequent visits with no signs of flowering activity in any of the observed tillers.

4. Discussion

Our study found that atmospheric grass pollen levels vary at a micro scale (<300 m apart); this has previously never been shown on such a localized scale. One population of flowering *Festuca rubra* was found to only contribute a small proportion of pollen to sites located 300 m away. This provides strong suggestive evidence that flowering populations of grasses are likely to deposit their pollen within the immediate surroundings. Temperature, solar radiation and relative humidity, wind speeds and turbulence were found to be important meteorological factors in the grass pollen release and dispersal processes. Our findings provide important understanding that can be used to model grass pollen release and dispersal processes of other grass species likely to behave similarly.

4.1. Local grass pollen distributions

The bi-hourly grass pollen profiles from the three sites showed that there was a strong overlap in amplitude and frequency for the entire season. Although the three sites were similar, plenty of variation remains unaccounted for. These results therefore highlight substantial differences in pollen levels between sites on a microscale in rural areas. Many previous studies have been conducted to explore atmospheric grass pollen variations within local areas (mostly within cities) using multiple pollen samplers (e.g. Arobba et al., 2000; Emberlin and Norris-Hill, 1991; Fernández-Rodríguez et al., 2014a; Fornaciari et al., 1996; Katz and Batterman, 2020; Peel et al., 2014b; Puc and Puc, 2004; Ríos et al., 2016; Simoleit et al., 2017; Skjøth et al., 2013; Werchan et al., 2017) with all being conducted on a micro-alpha to meso-gamma spatial scale (1–20 km) (Orlanski, 1975). Similar to this research, these studies found high correlations but nonetheless big differences in overall profiles and grass pollen patterns. Keeping this in mind, at least two recent studies have recommended that multiple pollen samplers are needed to properly account for the localized variation in grass pollen concentrations (Katz and Batterman, 2020; Werchan et al., 2017). This confirms that grass pollen levels tend to vary on a local scale. However, our study was conducted on a micro-beta scale (<1 km), with this being the first study to have investigated variations in grass pollen concentrations using such high-resolution data and localized scale. Our results also confirmed that grass pollen levels tend to homogenize as scale decreases, as previously demonstrated by Maya Manzano et al. (2017). Multiple factors have been suggested as the cause of spatial variation within cities from the above-mentioned studies, such as differential effects of weather conditions, heterogenous distributions of source areas and other specific features of the urban landscape, such as the urban heat island effect and differential pollen transportation pathways (Peel et al., 2014b; Puc and Puc, 2004; Ríos et al., 2016; Simoleit et al., 2017). However, none of these factors are relevant for our study area since the sites were exposed to similar meteorology and grass pollen source areas. Two of the possible explanations for the local spatial variation are atmospheric dispersion eddies and transportation blockages caused by the surrounding vegetation and topography, previously suggested to be the primary causes of variations in local pollen levels (Auer et al., 2016; Boudreault et al., 2017; Emberlin and Norris-Hill, 1991; Viner et al., 2017). This has previously also been suggested to be the cause of the differences between street and roof variations in cities (Hugg et al., 2020; Peel et al., 2014a). One limitation of our study is the difference in sampling height between the pollen samplers, as this can have a role in interpreting the pollen results (Rantio-Lehtimäki et al., 1991). However, previous research has shown there to be only limited differences for grass pollen below 10 m (Fernández-Rodríguez et al., 2014b; Rojo et al., 2019), and that lower sampling heights can be justified (Hugg et al., 2020).

Another possible explanation for the local spatial variation is the dispersal distances from the surrounding grass pollen sources areas, which could explain the relative differences in the genus-specific pollen found by our study. The six genera found to dominate our aerial grass samples have previously been found to be major contributors to the grass pollen season although their relative abundance can vary widely between locations and over time (Brennan et al., 2019). The spatiotemporal heterogeneity in dominant grass pollen genera is likely due to inherent variation in seasonal phenological profiles (Cebrino et al., 2016; León-Ruiz et al., 2011), which has previously been connected to their allergenicity and to the

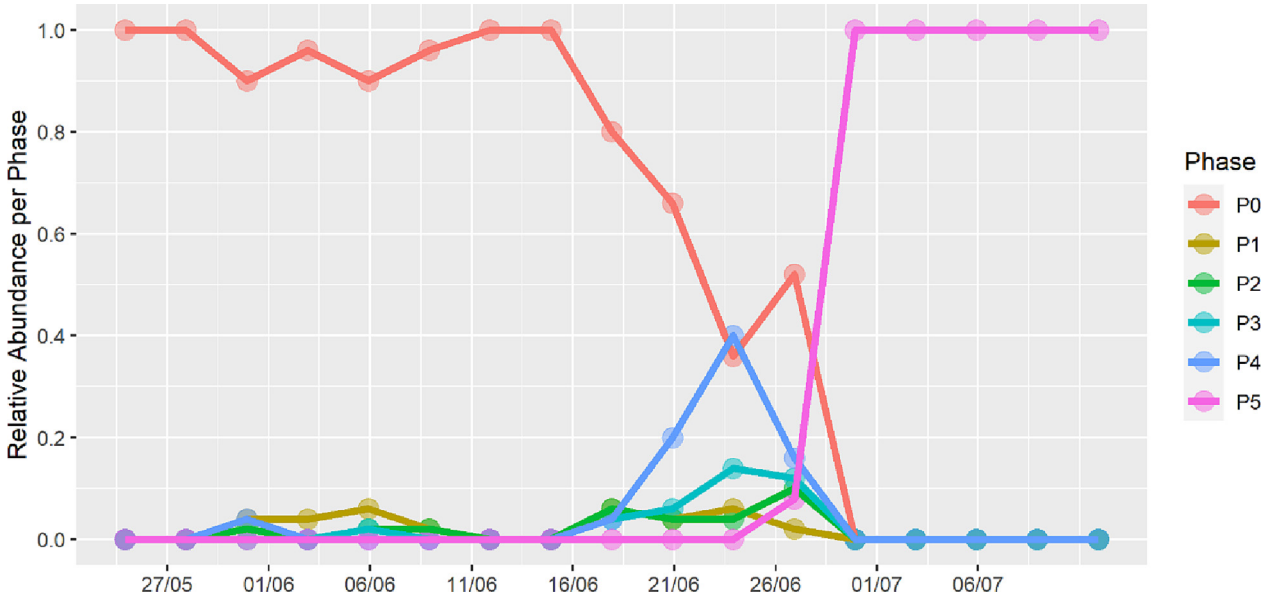

Fig. 4. Phenological flower tiller progression for the *Festuca rubra* population located in the Lakeside Field location. The included grid and x-axis corresponds to the dates in all other figures. Phase summary: P0 – pre-flowering, P1, P2, P3 – moderate flowering, P4 – full flowering, P5 – senescence.

general grass pollen profile (Ghitarrini et al., 2017). However, defined source areas are needed to definitively determine the dispersion distances of surrounding grasses. Furthermore, four of the genera found in all the air samples (*Alopecurus*, *Avena*, *Cynosurus* and *Dactylis*) were not found in the grass circle. This means that a fraction of the pollen measured over the circle can be associated to sources within the circle and another fraction can be associated to sources further away, in this case at least a few hundred meters away. This suggests that species-specific grass pollen concentrations will vary considerably at the microscale (<2 km) whenever there is a difference in species composition throughout the study area.

The use of multiple DNA markers in metabarcoding analyses has previously been recommended due to the varying degree of species discrimination depending on the DNA region (e.g. Baksay et al., 2020; Brennan et al., 2019), as observable in our results. While this can be interpreted as inconsistency, it is a natural artifact of the DNA similarity between species and the quality of barcodes and taxonomic databases (Bell et al., 2016). However, ITS1 and ITS2 have been shown to be some of the most reliable DNA barcode marker genes in properly identifying grass taxonomy (Omelchenko et al., 2022).

4.2. Grass pollen dispersal

The main flowering period of *F. rubra* within the Field site was between the 18th–30th of June. It is likely that most of the identified *F. rubra* pollen that was collected at the three sites was released during this period. This is the same period as most of the overall grass pollen collected at the three sites for the pollen monitoring. The relative abundance of *F. rubra* pollen identified at the Field site was many times higher than that identified at the other two sites, implying that only a small proportion of *F. rubra* pollen gets dispersed further than 300 m within the flat landscape. This finding agrees well with previous calculations on tree pollen dispersion (Adams-Groom et al., 2017) and grass pollen dispersion (Skjøth et al., 2013) based on the Gaussian principle. However, the observed dispersion distance provided here is a conservative estimate, due to the influence of the wind direction and vertical dispersion. During the period of study, wind directions from the south and south-east, which would be required to transport *F. rubra* pollen toward the other two sites, occurred rather infrequently. However, even if a small fraction of the wind direction (e.g., 20 %) was favouring transport of *F. rubra* pollen from the circle to one of the two sites, then this infrequent transport cannot explain the massive difference in the appearance of *F. rubra* pollen between the grass circle and the two other sites. The relative proportion of *F. rubra* pollen at the Field site corresponded to almost 40 % of the entire sampled seasonal pollen load, suggesting that the surrounding grass vegetation (within 25 m, height 2.5 m) was contributing significantly to the overall catch of the pollen sampler there. This measure does not include other grasses present in the Field site (e.g., *L. perenne*, *P. trivialis* and *A. capillaris*). The *Festuca* sp. pollen identified by metabarcoding could actually be misclassified *F. rubra* pollen, or another *Festuca* species that we had not found within the larger Lakeside area and which the bioinformatics did not recognize within the taxonomical database. This is unlikely though, since the highest proportion of the *Festuca* sp. pollen was identified from the Field site and only consisted of *F. rubra*.

Previous studies have shown that the pollen frequency (via dispersion) of *Festuca* species shows a sharp decline from around 100 m from the source area (Jones and Newell, 1946; Nurminiemi et al., 1998; Rognli et al., 2000). Although, during the right atmospheric conditions grass pollen can travel further (>1 km) from specific donor populations to specific recipient populations (Giddings, 2000). This corresponds to the key findings of our study, in which only a small proportion of *F. rubra* pollen was found to disperse further than 300 m from the source area. Parameters normally used to model general pollen dispersion distance include turbulence, wind speed, pollen settling velocity, morphological adaptations for dispersal and plant height (Adams-Groom et al., 2017; Okubo and Levin, 1989; Sjögren et al., 2015). However, for grasses, the settling velocity, morphological adaptations for dispersal and plant height are generally the same (although special cases exist for cereal pollen, see e.g. Jarosz et al. (2003) for maize pollen), leaving turbulence and wind speed as the two main parameters for comparative purposes. Pollen sampler positioning and flowering time have been shown not to be adequate in predicting grass pollen dispersion distance (Giddings et al., 1997a); neither is the addition of turbulence and wind direction (Giddings et al., 1997b). This partly agrees with our findings as wind direction did not impact the overall grass pollen levels, although turbulence did. Turbulence likely contributed to the emission process of grass pollen to the atmosphere, as has previously been shown to occur above a ragweed canopy (2.5 m) in connection to source emission processes (Šikoparija et al., 2018). However, weaker wind speeds were more likely than stronger ones to increase grass pollen levels at all sites. This suggests that the *F. rubra* pollen was able to disperse from the Field site to the other two sites during these conditions of turbulence and weaker winds, with these conditions occurring during the main flowering period. The overall effect of wind speed on grass pollen levels appears to have been mixed, with some studies suggesting that increases in wind speed do not increase grass pollen dispersal above a basic level (Adams-Groom et al., 2022; Viner et al., 2010) or atmospheric transport in grass pollen in any significant way (Damialis et al., 2005). Other studies suggest that increases in wind speed do have an effect on the dispersal capacity (van Hout et al., 2008) and on grass pollen concentrations (Emberlin and Norris-Hill, 1996; İnceoğlu et al., 1994; Puc and Puc, 2004; Waudby et al., 2022). Additionally, atmospheric transportation models of grass pollen frequently include wind speed as a relevant variable (e.g. Cresswell et al., 2010; Khwarahm et al., 2014; Voukantsis et al., 2010), which suggests its implicit importance for the dispersion and movement of grass pollen in the landscape.

4.3. Diurnal meteorological conditions and pollen release

Our findings identified temperature, solar radiation and relative humidity as important meteorological variables in determining pollen distribution and by proxy pollen release. This triad of meteorological variables is commonly used to investigate diurnal trends in grass pollen levels (e.g., Peel et al., 2014b; Toth et al., 2011; van Hout et al., 2008; Viner et al., 2010; Waudby et al., 2022) with mixed interpretation of their effect on the grass pollen release. Previous studies have identified that most grass species release pollen on warm and dry days (Spieksma and den Tonkelaar, 1986; Subba Reddi et al., 1988), which would lower relative humidity and cause anther dehiscence and subsequent pollen release (Keijzer, 1987; Keijzer et al., 1996). This agrees with our findings, as pollen levels tended to be the highest during warm temperatures and low relative humidity. Mid-morning pollen release in maize has been connected with the increase in solar radiation just after sunrise, but not to solar radiation later in the afternoon (van Hout et al., 2008). We observed that lower solar radiation contributed to higher pollen concentrations, seen during the late afternoon and early evening. This suggests that solar radiation contributes to the drying of the anthers earlier in the day, with pollen release occurring as a multistep process (Wilson et al., 2011) and airborne pollen levels increasing and peaking when sufficient number of anthers had burst during the course of the day.

Previous studies have found that *Festuca* species, including *F. rubra*, generally release pollen during the late afternoon to evening (Hyde and Williams, 1945; Jones and Newell, 1946; Liem and Groot, 1973). This has previously been linked with low humidity, but not solar radiation and temperature (Liem, 1980; Liem and Groot, 1973). Our findings indicate that all three variables, temperature, solar radiation and relative humidity, play a key role in the pollen release dynamics of *F. rubra*, due to the relatively high abundance of the species-specific pollen. One earlier study has observed that *F. rubra* pollen is liberated from the anthers in the early morning due to rising temperatures (Beddows, 1931). It is possible that there exists some divergence of pollen release in the larger *F. rubra* complex due to differing diurnal flowering responses from the varying subspecies and varieties (Cope and Gray, 2009; Heide, 1990; Hyde and Williams, 1945; Stace et al., 1992). Additionally, it is likely that the grass pollen release process in other flowering grasses responds to the same conditions as *F. rubra* due

to the predictive capacity that temperature, solar radiation and relative humidity have on the general pollen distribution patterns for all three sites with varying relative abundance of genera-specific grass pollen.

5. Conclusion

Despite showing good similarity in general season patterns, the bi-hourly concentrations of the grass pollen between the three study sites, only 300 m apart, varied significantly. We found that six grass genera dominated all three seasonal profiles. However, using the eDNA analysis, we showed that the types and relative proportion of grass species varied substantially between the three sites. A dominant population of flowering *Festuca rubra* was found to contribute only small proportions of pollen to sites located no more than 300 m away. The local vegetation was found to have a large effect on the total pollen sampler catch, with at least 40 % of the relative proportion of pollen being identified as having originated from within 25 m. Therefore, we reject the hypothesis that there is no difference in grass pollen biodiversity and pollen concentrations at the microscale level. Wind speed and turbulence were found to be important predictors for the observed pollen distribution, with the more gentle wind conditions allowing the higher transportation of *Festuca rubra* pollen from the Field site to the other two sites. Solar radiation, temperature and relative humidity were determined to be important in predicting the pollen variation and, by proxy, pollen release. This was the case for *Festuca rubra* but also for other grasses in the surrounding area, since grass pollen was found within the Field circle from grass species not present there. We demonstrated that wind conditions and vegetation have large effects on the local variation of grass pollen levels in a microscale area. This supports the hypothesis that pollen concentrations at the microscale are governed by local weather factors that directly control atmospheric dispersion and that these variables also control the emission process, which varies between grass species. Our findings can be used to model pollen release and dispersal in grasses with similar behaviour.

CRediT authorship contribution statement

Carl A. Frisk: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Godfrey P. Apangu:** Data curation, Writing – review & editing. **Geoffrey M. Petch:** Writing – review & editing. **Simon Creer:** Writing – review & editing. **Mary Hanson:** Methodology, Writing – review & editing. **Beverley Adams-Groom:** Writing – review & editing. **Carsten A. Skjøth:** Methodology, Supervision, Writing – review & editing.

Data availability

The authors do not have permission to share data.

Declaration of competing interest

The authors declare that they do not have any inappropriate financial or personal relationships that would bias or influence the research in this paper.

Acknowledgements

CAF and GPA acknowledge that the research was funded by the European Commission through a Marie Curie Career Integration Grant (Project CIG631745 and Acronym SUPREME to CAS) and by the University of Worcester. SC and CAS would also like to acknowledge NERC funding via Standard Grants NE/N003756/1 and NE/N002431/1.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.163345.

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