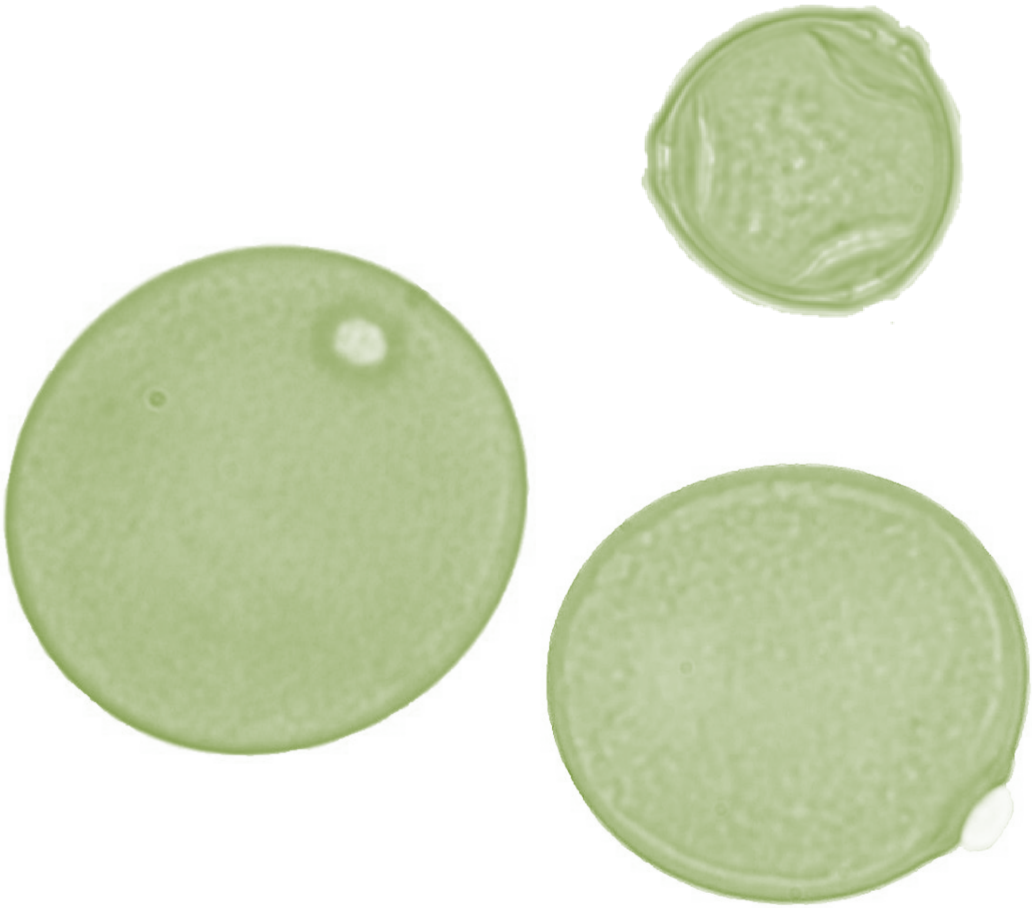
AARHUS UNIVERSITY

CROSS-DISCIPLINARY STUDIES ON POLLEN IN HEALTH AND ENVIRONMENTAL SCIENCES

PhD dissertation - Cand. Scient Pia Viuf Ørby



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DEPARTMENT OF PUBLIC HEALTH / SECTION FOR ENVIRONMENT, OCCUPATION AND HEALTH

DEPARTMENT OF ENVIRONMENTAL SCIENCE / SECTION FOR ATMOSPHERIC PROCESSES

2017

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Submitted to Allergy (September 2017)

Co-author declarations

Abbreviations

AHR - Airway hyper-responsiveness
AR – Allergic rhinitis
DEP - Diesel exhaust particles
DGAR - Danish General Agricultural Register
DOY – Day of year
EAR – Early airway response
EEC - Environmental exposure chamber
FEV_1 – Forced Expiratory Volume in the first second
HDM - House dust mite allergen
IgE - Immunoglobulin E
LAR – Late airway response
NDVI - Normalized Difference Vegetation Index
NO_2 – Nitrogen dioxide
O_3 - Ozone
PD_{20} – Dose eliciting a 20% decrease in FEV_1 compared to baseline.
PM, $PM_{2.5}$, PM_{10} –Particulate Matter (total), below 2.5 µm and below 10 µm.
SIC – Specific Inhalation Challenge
SPT – Skin prick test
SQ-U – Standardized quality units
SO_2 – Sulphur dioxide

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Preface

The work on the project was initiated under the start-up project A3- Assessment of Airborne Allergens. The study was conducted at two different research facilities under Aarhus University; Department of Public Health, Section of Environment, Occupation and Health, and Department of Environmental Science, Section of Atmospheric Processes.
The study on pollen allergen exposure was performed in the Climate Chambers, at Aarhus University.
A four month stay at Cordoba University, Spain provided the basis for the collaboration on one of the included studies, as well as a basic introduction to many additional aerobiological fields, providing additional understanding of the area.

This thesis is divided into six sections;

- An Introduction, summarizing the overall objectives and giving a brief glance at history.
- A Background section, providing insight to the included elements and previous studies in the field.
- A Methods section, accounting for the two main methods applied in data collection for the included studies; pollen sampling and a human exposure study. All the additional applied methods and data sources are described in the included manuscripts, and will not be repeated in this thesis.
- A Results and Discussions section, outlining the main results and discussion points of the six included studies.
- A section of concluding remarks and suggestions for future studies
- And finally a section comprising the Manuscripts on the six included studies.

I hope that this thesis will serve as a stepping stone towards future cross-disciplinary studies in pollen research, and inspiration for the future advice given to the many with pollen allergy.



Grasses. From left; Dactylis glomerata (Córdoba, Spain), Holcus lanatus (Vallensbæk, Denmark), Phleum pratense (Ishøj, Denmark). All photographs in this thesis is taken by the author, unless otherwise is stated.

Aknowledgements

This thesis, and the included studies, has been long in the making. Sometimes life throws you a curveball, and sometimes it throws you many. But I would like to think like Charles Dickens did - that "*I have been bend and broken, but hopefully into a better shape*".
I would like to acknowledge, that it would not have been possible to present the here included work, without the support of a great number of people. I would like to express my gratitude to all of you who helped and supported me through the years.

First and foremost, I would like to especially thank my main supervisors Vivi Schlünssen and Ole Hertel for their support and positive encouragement. I would also like to thank my co-supervisors, Jakob Bønløkke and Carsten Skjøth, and give thanks to Torben Sigsgaard, who was instrumental in initiating the chamber studies.
I am grateful for the patient introduction to the world of "R", by Bo Bibby, and for all the things I learned from Robert Peel, when we shared and office for a time - a marvelous colleague and friend.
I also greatly appreciate all the help I received from Vibeke, Tine and Peter, the amazing Climate Chamber group, and for always making me feel so very welcome. I would also like to thank all the participants in the chamber study, who voluntarily underwent challenges in the name of science.

My PhD project would neither have been a reality without the support from Astma-Allergi Danmark and their pollen crew through the years; Janne, Andreas and Karen.

I also wish to acknowledge the Faculty of Health at Aarhus University for providing me with a PhD mobility scholarship, and the Graduate School of Health and AUFF for the financial support for my stay at Córdoba University, Spain. I would also like to thank Professor Carmen Galán and her aerobiology group for my time at their department and amazing city.

My parents and my sister have been a great support in all matters; from picking catkins and grasses, to listening, and helping at home, when I was away. Most of all, I am so very grateful for my husband, Jesper, who stuck it out and even shook a whole birch tree for me, and for Naja, Osvald and Kamma, our curious and amazing children, who I believe to now be the youngest pollen experts I know.

For my husband- For always being my great support

Resumé

Pollen spiller en afgørende rolle i naturen, men af stadig ukendte årsager, forårsager de også allergiske reaktioner i nogle personer, ofte i form af allergisk rhinitis (AR), alment betegnet som høfeber. Det estimers at op mod 20 % af befolkningen i Europa er påvirket af denne allergi. AR nedsætter livskvaliteten for millioner af mennesker, og koster samfundet betydelige summer i samlede socioøkonomiske udgifter hvert år. I 2008 blev AR beskrevet i "The Lancet", som "*common, costly and neglected*", en beskrivelse som stadig passer på området i dag.

Dette tværdisciplinære Phd projekt indenfor Folkesundhed og Miljøvidenskab strækker sig over flere områder indenfor pollenforskning. Fokus er på de to allergener ansvarlige for hovedparten af AR tilfælde i Danmark; græs og birk, samt deres rumlige og tidslige variation, den allergiske reaktion og den samtidige eksponering for luftforurening. De inkluderede studier og konklusioner heraf er;

- Målinger fra tre pollenfælder i Århus by, samt kildekortlægning for græspollen, blev anvendt i analyse af variationen i pollenkoncentrationer i et byområde. Resultaterne bekræfter forekomsten af store lokale variationer i pollenlastningen i byområder, og at disse afhænger af den rumlige fordeling af kilder. På dage med høje pollental, var målinger i Århus ikke korrelerede med koncentrationer fra den operationelle pollen monitoreringsstation i Viborg.
- Det er almen praksis at opgive den gennemsnitlige døgnvariation i pollen vurderet for hele sæsonen, på trods af, at denne er meget variabel. Døgnprofilet for græspollen blev analyseret i to studier; For Århus, ved at opdele sæsonen i perioder, og for København og Córdoba, ved at anvende statistisk clustering. Resultaterne indikerer, at tidspunktet for maximale koncentrationer varierer gennem sæsonen, sandsynligvis pga. blomstring af forskellige græsser. Anvendelsen af statistisk clustering resulterede i tre profiler for hver lokalitet, hvor det hyppigst forekommende afspejlede maksimale koncentrationer i de tidlige aftentimer i København og om eftermiddagen i Córdoba. En simpel analyse af meteorologiske parametre udviste ingen signifikante kausale korrelationer for nogle af analyserne. Døgnvariation af græs er ikke tidligere vist for Danmark og ej heller er cluster-metoden anvendt på denne taxa i andre studier.
- Analyse af græs- og birke-pollenkoncentrationer og variationer i de primære komponenter af luftforurening, viste sammenfald af maximale koncentrationer af pollen og ozon, i både sæson- og døgnvariation, med en dermed potentiel risiko for, at ozoneksponering kan medføre forværrede allergiske reaktioner.
- To eksponeringsscenarier blev undersøgt i et humant eksponeringsforsøg i et klimakammer; Effekten af samtidig eksponering for ozon og allergener, og "priming"-effekten af naturlig allergeneksponering i sæsonen. Specifik bronkial inhalation med birke- og græsallergen blev udført på sensibiliserede personer med AR. Der blev udviklet en ny metode til at udtrykke dosis-respons kurver og PD_{20}-estimater (dosis der udløser et fald i FEV_1 på 20 %), ved at tilpasse en ikke-lineær regressionsmodel til målingerne. Resultaterne indikerede at hverken sæson-priming eller samtidig eksponering med ozon havde signifikant effekt på den bronkiale reaktion overfor allergener i individer med AR.

Det er min overbevisning at de her inkluderede studier har bidraget til feltet inden for pollenforskning.

Summary

Pollen has a crucial and important role to play in nature. However, for still unknown reasons it also causes allergic reactions in some humans. The condition of allergic rhinitis (AR), also known as hayfever, is a severe problem in Europe with as much as 20 % of the population being affected. AR decreases the quality of life for millions of people and induces great economic costs on society. In 2008 the area was described by the Lancet as “common, costly and neglected” (The Lancet, 2008), a fitting description that still holds today.

This cross-disciplinary project in Public Health and Environmental Science covers some of the many areas within pollen research, focus being on two of the main allergens causing AR; grass and birch, examining the spatial and diurnal pollen patterns, allergenic response and co-exposure to air pollutants.

The included studies and conclusions hereof are;

- Measurements from three pollen traps within the city of Aarhus, and grass pollen source mapping, were applied in an analysis of intra urban variation in pollen concentrations. The results confirmed intra urban variations in concentrations, and that these are linked to heterogeneity in source distributions. On peak pollen days, the pollen levels were not correlated with measurement from an operational trap 60 km´s away.
- It is common practice to indicate an average time of peak pollen concentrations although this is known to be highly variable. The diurnal profile of grass pollen was here analysed for Aarhus applying a method of separation into periods of the season, and for Copenhagen and Córdoba, applying statistical clustering. The results indicate that the time of peak may vary with the time of season, reflecting the succession of different grass species flowering. The method of statistical clustering revealed three likely profiles at each site, with the most frequent profiles showing a single peak in the early evening in Copenhagen, and in the afternoon in Cordoba. A simple analysis showed no significant explanatory correlations with meteorological parameters for any of the analyses. The diurnal variation for grass has not previously been shown for Denmark, nor has the method of clustering previously been applied for this taxa.
- Analysis of grass and birch pollen concentrations and the levels of common pollutants in Copenhagen showed both seasonal and diurnal co-variation of pollen and ozone peak levels, indicating potential relevance when considering co-exposures.
- Two topics were investigated in a human single blinded controlled study in a climate chamber, namely the effect of co-exposure to allergens and ozone, and the priming effect of the natural seasonal exposure. Specific Inhalation Challenges were performed on AR participants with grass or birch sensitization. A novel approach of non-linear model depicted a good fit dose-response curves are shown and was applied in producing dose-response curves and PD_{20} estimates, the dose eliciting a 20% reduction in lung function. Examining both the curves and PD_{20}, we did not find an effect of co-exposure to low-level ozone and grass or birch allergens on either, nor did we find an effect of seasonal priming.

Ii is my belief, that the work included in this thesis contributes to the field pollen research.

Part I – Introduction

Chapter 1

Introduction

Why focus on pollen allergy?

Pollen has a crucial and important role to play in nature, however for reasons still not fully understood, it also causes allergic reactions in some humans. Pollen allergy is a significant problem in Europe, with local prevalence's of as much as 40% being reported (Bousquet et al., 2007;D'Amato et al., 2007;Linneberg et al., 2008). Pollen allergies primarily manifest themselves as allergic rhinitis (AR), however approximately a quarter of AR patients also suffer from allergic asthma (Linneberg et al., 2002), a potentially life threatening condition. The two diseases are thought to be closely linked, as stated in the theory of "one airway one disease" (Bachert et al., 2004), which is often one of the main arguments for focusing on AR. However, the condition is a big enough issue to warrant attention on its own, as the disease decreases the quality of life for millions of people and costs society billions each year (Reed et al., 2004). In 2008 the area was described by the Lancet as "common, costly and neglected"(The Lancet, 2008), a fitting description that still holds today. In Europe for the year 2016 the costs of AR alone is estimated to be around 100 billion Euros (Schiavoni et al., 2017). Although prevention and minimization of risk factors is overall beneficial, only approximately 3% of the European health expenditure related to pollen allergy is currently being used on prevention, compared with 97% on treatment (Schiavoni et al., 2017).

Despite the fact that the socioeconomic costs of pollen allergy are great, comprising direct and in-direct costs related to doctors' visits, medication, absenteeism, decreased work productivity etc. (Pawankar et al., 2011), no European legislation exists on this area. There is no collected information system, no official threshold levels, and no requirements to measure or to take steps to prevent increases in specific allergenic plants in e.g. highly populated areas, as there is for "conventional" air pollutants.

The ultimate goal for the majority of both health professionals and researchers working in this field is to improve the quality of life for those with allergies, but there are many potential ways in which this might be achieved, and it is difficult to determine on which of these, it is most advantageous to focus. One area with huge potential is the medical option of immunotherapy, also referred to as allergy vaccination (Valenta et al., 2009). Whilst for many this can lead to the relief of their symptoms, this is neither possible nor effective for all. The use of medication such as antihistamines is another important common way to alleviate symptoms (Tripathi and Patterson, 2001). This requires either medicating on a daily basis, and thus dealing with potential associated side effects over extended periods of time, long lasting medication, or reliable predictions of when the symptoms can be expected to be increasing for targeted medication. However, one of the main pieces of advice given is still "allergen avoidance". This is difficult to achieve, with the main resource at disposal still being the daily pollen count, as it has been for decades.

There are still so many unknowns in the relationship between pollen count and the experienced symptoms that we wish to relieve. The more I learned within this field, the more apparent it has

become that we will need to come up with an array of solutions, rather look for just one master solution. As Woodcock and Custovic put it; "… *there is very unlikely to be a "one-size-fits-all" strategy…* " (Woodcock and Custovic, 2009).
The overall aim of this thesis is therefore to combine research in Health and Environmental Science, to hopefully add some pieces to the puzzle of how we best advise those with pollen allergy right now, and which of the many possible ways it would be most advantageous for us to pursue.

Objectives

It was not possible within this thesis to cover all areas within in the field of pollen research. A great number of factors will affect the magnitude of the experienced symptoms, from the multitude of aspects affecting the level of exposure, which vary substantially in both time and space (Peel et al., 2014a;Skjøth et al., 2013b;Werchan et al., 2017) to those related to the degree of response as co-exposures to air-pollutants (D´Amato et al., 2016;Emberlin, 1998). The relative importance of these factors will also vary depending on geographic location, as the relative importance of allergenic pollen species is site-specific (Bousquet et al., 2007;Burbach et al., 2009).
The studies included in this thesis focusses on the two main allergenic taxa in Northern Europe; grass and birch pollen (Bousquet et al., 2007;Rantio-Lehtimäki, 1995), with the majority of studies focusing on grass, the most widespread pollen in Europe and most frequent pollen allergy (D'Amato et al., 2007;Emberlin et al., 2000). For these taxa, the thesis addresses the resource, that underpins two of the top three advice given to those with allergies (timely and sufficient administration of medication and allergen avoidance) – I.e. the pollen count.
In many countries the distance between pollen monitoring stations tends to be in the magnitude of 100 km (Skjøth et al., 2013a). In Denmark we have two pollen monitoring stations supplying the pollen count, and it can be questioned whether this is sufficient. This is especially questionable for grass, which has been shown to have great local variation (Simoleit et al., 2017;Werchan et al., 2017). The monitoring stations are used to supply the public with one daily count, as is done in most countries. If those with allergies wish to plan their activities according to the advice of "allergen avoidance", they currently must rely on the general guidance, which in Denmark is, that "*most pollen is typically in the air around midday, however pollen concentrations can be high at all times of the day*"[1]. If potential specific diurnal pollen patterns could be determined, more specific advice could also be given.
The spatial variation in concentration of grass pollen is high in part due to the large size and low release height (Skjøth et al., 2013b), and the source-distribution within an area could be of great importance to local concentrations throughout the day. To investigate this, measurements from three pollen traps within the city of Aarhus were applied in a novel methodology for mapping potential grass pollen sources via remote sensing images and GIS analysis.
Also the daily temporal variation in grass pollen concentrations, e.g. the time of day at which grass pollen concentrations are high, may differ greatly from day to day (e.g. (Munoz Rodriguez et al., 2010;Norris-Hill, 1999). This was investigated in two separate analyses. One with focus on the

[1] http://hoefeber.astma-allergi.dk/pollengennemdoegnet

seasonality in diurnal patterns, performed on three years of grass pollen counts from three pollen traps in the area of Aarhus City. The other with focus on the methodology of statistical clustering, performed on 10 years of pollen counts from the monitoring station in Copenhagen, and four years of data from the monitoring station in Córdoba, in collaboration with Córdoba University, Spain.

To give an indication of the pollen concentration that is easily interpreted by the public, national pollen services often publish counts as being within species-specific pre-defined ranges of low, moderate or high. These levels are often also applied in forecasting. However, whether a pollen exposure is experienced as either low or high will depend on the response. One factor that may affect the response is priming - the theory that initial exposures prime the response, leading to progressively more severe symptoms following subsequent exposures of comparable magnitude (Connell, 1969). If seasonal exposure is having a general priming effect, leading to a difference in allergen tolerance between the onset and end of the pollen season, the ranges of “low”, “moderate” and “high” should perhaps not be identical at the beginning and end of the pollen season. To analyse priming effect of the natural seasonal exposure, a controlled human exposure study was performed with specific allergen challenges outside and at the end of the pollen seasons, in grass and birch atopic AR participants with no or mild asthmatic symptoms. The priming effect has previously mainly been seen in patients with allergic asthma, but according to the theory of ”the united airway”, a bronchial response should also be seen in those with AR and no or mild asthmatic symptoms.

Pollen is not the only inhalable entity to which allergic people can be exposed. There has been much focus over the past decades on the effects from the combination of air pollutants we inhale (Schiavoni et al., 2017). In this context, the co-exposure of allergens and air pollutants has also been of great interest, and whether it can exacerbate the allergic response as well as the prevalence of allergy (D´Amato et al., 2016;Emberlin, 1998).

Figure 1.1 Flowering grasses along roadsides in the center of Copenhagen, potentially leading to high local concentrations.

If peaks in exposure to pollutants potentially coincide with those for pollen grains, and are within concentration ranges seen in literature to induce exacerbated allergic reactions, future advises to allergy sufferers should include information on this. To investigate this, co-exposure allergen and ozone, a known irritant, was performed in a controlled human exposure study.

To summarize, the objectives of this thesis are;

- To investigate whether there are large intra-urban variations in grass pollen concentrations, and whether these could be linked to source distribution.

- To analyse the variability in the diurnal time of peaks in grass pollen concentration and whether this depend on the seasonal progression of different grass species flowering.
    - To further investigate the diurnal patterns grass pollen concentrations, via examining whether statistical clustering may define distinct diurnal patterns, and if so, whether these are related to meteorological factors and vary between sites.
    - A second objective in both studies was to examine whether potential differences in the profile were driven by differences in meteorological factors.

- To investigate whether the natural seasonal priming effect will result in greater bronchoconstriction at the end of the pollen season compared to outside pollen season.

- To analyse whether seasonal and diurnal patterns of pollens and pollutant peaks coincide, and exceed thresholds know to exacerbate responses.
    - To further investigate the impact of air pollutants on the allergic response, by investigating the effect of co-exposure to potentially naturally occurring levels of ozone, by specific allergen challenges.

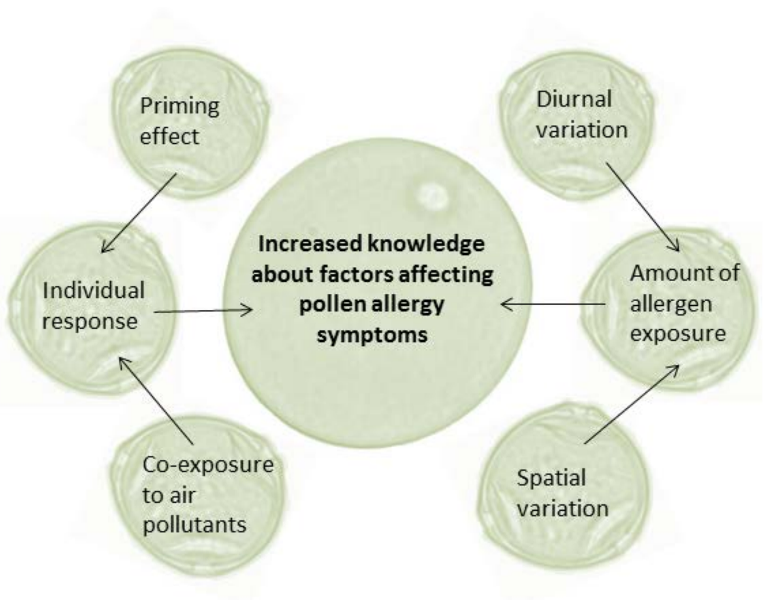
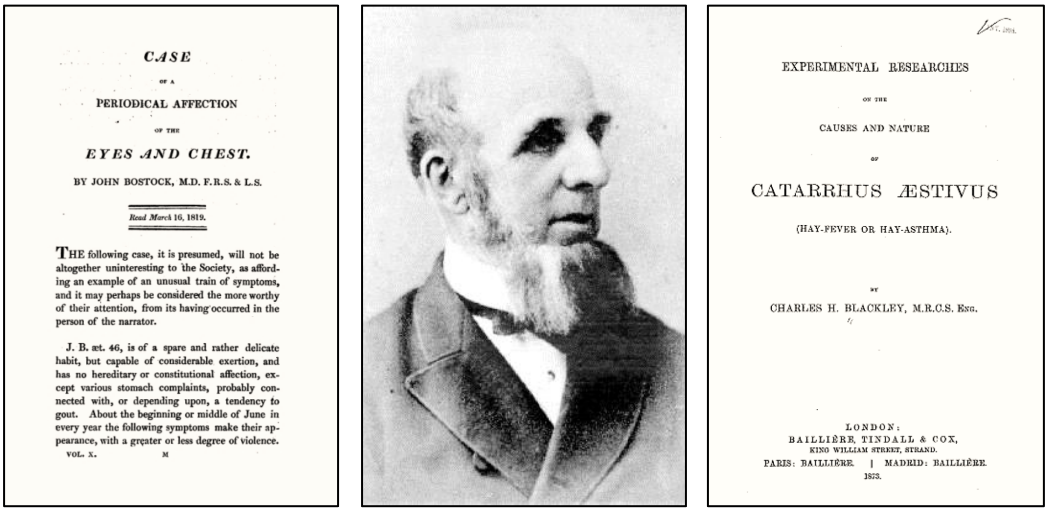


Figure 1.2 Connections between the focus areas of the studies included in this thesis.

A glance at history

Allergic rhinitis is speculated to be a modern disease, not described by many before the 19th century. Some believe there to be references to a catarrh resembling AR in texts as old as the Bible (Brostoff and Gamlin, 1996a;Waite, 1995), and a mention of a "*rose-fever*" is also seen in texts from the 15th -16th century (Waite, 1995). Yet the disease went almost unmentioned until some 200 years ago, and there is much evidence, that it was much less common until the 20th century (Finn, 1992).



CASE
OF A
PERIODICAL AFFECTION
OF THE
EYES AND CHEST.
BY JOHN BOSTOCK, M.D. F.R.S. & L.S.

Read March 16, 1819.

THE following case, it is presumed, will not be altogether uninteresting to the Society, as affording an example of an unusual train of symptoms, and it may perhaps be considered the more worthy of their attention, from its having occurred in the person of the narrator.

J. B. æt. 46, is of a spare and rather delicate habit, but capable of considerable exertion, and has no hereditary or constitutional affection, except various stomach complaints, probably connected with, or depending upon, a tendency to gout. About the beginning or middle of June in every year the following symptoms make their appearance, with a greater or less degree of violence.

VOL. X. M

EXPERIMENTAL RESEARCHES
ON THE
CAUSES AND NATURE
OF
CATARRHUS ÆSTIVUS
(HAY-FEVER OR HAY-ASTHMA).
BY
CHARLES H. BLACKLEY, M.R.C.S. Eng.

LONDON:
BAILLIÈRE, TINDALL & COX,
KING WILLIAM STREET, STRAND.
PARIS: BAILLIÈRE. | MADRID: BAILLIÈRE.
1873.

Figure 1.3 Two of the most important writings in the early years of hayfever research were the, "Periodical affection of the eyes and chest" by J. Bostock , 1819 (left), and Experimental researches on the causes and nature of catarrhus æstivus", by C. Blackley, 1873 (right), depicted on the photo in the middle.

John Bostock in credited as the first to describe pollen allergy, an affliction he himself suffered from. He portrayed it as a "*Periodical affection of the eyes, and chest*" and as an "*at length added degree of general indisposition*". He named it "*summer cold*", and noticed that confinement to his house lessened his symptoms (Bostock, 1819). In 1828 John Macculloch became the first medical professional to name the condition hay-fever, but also mentions this to be the popular name used by the public, indicating that the disease had some attention by this time (Waite, 1995).

Half a century later, Charles Blackley showed the impact of pollen on human health through several provocation tests performed on himself. He inhaled pollens and applied grains to nostrils, tongue and lips, as well as performed skin prick tests (de Weger et al., 2013;Taylor and Walker, 1973;Waite, 1995). He is also credited as the first to correlate the amount of pollen in the atmosphere to the severity of his symptoms. In one very simple experiment, he inhaled pollen during the winter, causing an immediate attack of symptoms, elegantly proving his theory (Blackley, 1873).

In the early years it was widely accepted that the affliction mainly struck those of the upper classes. It was speculated that this conclusion could be biased due to the main patient group by the investigating practitioners, being this class, but a comparison of the observations of physicians attending to the lower classes and with patients admitted to hospital, did not alter these results (Waite, 1995). Blackley also investigated the "farming class" and found no afflicted patients (Waite, 1995), a demographic group also seen to be less affected today (Elholm et al., 2013;Elholm et al., 2016), perhaps due to a newly discovered protective effect from exposure to a greater number of environmental microorganisms (Ege et al., 2011). Among the many observations made by Blackley that is still being investigated today are, his suggestion that continued exposure to pollen may have a protective affect among the lower classes (Waite, 1995).

In 1992 Ronald Finn published a paper in The Lancet, speculating upon the reason why two of the most important discoveries relating to AR, by Bostock and Blackley, were made by two physicians in north-west England, "*far removed from main academia*". He went on to speculate on whether, the industrial revolution and the accompanying air pollution, which began in this area, and the resulting damage to nasal mucosa by pollutants, may have made the public here more susceptible, inducing an increased focus on the disease (Finn, 1992). The historic facts seem to indicate that AR went from being a very uncommon disease, to one that affect around 10% of the world's population in a matter of 200 years, and the only plausible explanation for this, appears to be the coincidence with the onset of the industrialization, also overlapping with Blackleys early observations that the disease affect mainly urban citizens (Finn, 1992). Finn names it a "*disease of civilization*", a historic observation that is supported by many others (Brostoff and Gamlin, 1996a;Waite, 1995). But the link is not simple. The level of air pollution has been declining in most Eureopean cities for many decades, and yet, the rise in the prevalence and severity of AR was still increasing throughout the 1990´s and 2000´s. The explanation might be found in examining air pollutants separately, and in combination with other factors.

Blackley was not only examining the health aspects of AR, he was also one of the first to measure the amount of pollen in the atmosphere. He did so by applying a sticky substance to glass plates, and counting the grains under a microscope, much like these measurements are made today. He also created glass slides attached to kites, to examine the pollen amounts at various altitudes, and correlated pollen counts with meteorology and symptoms (Taylor and Walker, 1973;Waite, 1995). Blackley has been named "*the father of aerobiology*", although it was not until 1930 that this term was defined (Scheifinger et al., 2013).

Blackley was by all accounts a cross-disciplinary physician and scientist in the field of pollen research, covering both health and environmental science. Incidentally, he also performed experiments in which he tested whether his AR symptoms could be related to ozone, speculating that this gas had a damaging effect on the lungs (Taylor and Walker, 1973). Today it is a well-established truth that pollen is the cause of AR, and that measurements of the atmospheric load provides useful knowledge that can inform strategies for alleviating symptoms. The role of air pollutants is however still not fully uncovered.

Part II - Background

Chapter 2

Pollen

What is pollen

The main purpose of pollen is to fertilize. Pollen grains carry the male cell for plant reproduction from the anther of one plant, to the stigma of another. The majority of plants require cross pollination with other plants of the same species, and will therefore need to spread their pollen (Reece et al., 2013). Out of more than 200.000 registered plant species, only 50-100 has been reported to cause allergic symptoms (Hauser et al., 2010;Rantio-Lehtimäki, 1995), and for those, only a few of the many proteins they release are causing allergic response (Brostoff and Gamlin, 1996c). Why some proteins are induce an allergic response and others not, is still not fully understood (Traidl-Hoffmann et al., 2009). Most allergenic pollen is anemophilous, i.e. wind spread. In order to arrive at another plant of the same species, they must produce their pollen in far greater number, than the entomophilous plants, who are spreading their pollen via insects or other animals (Rantio-Lehtimäki, 1995). These pollen are generally larger, heavier and stickier, and therefore less prone to become airborne.

All pollen grains are protected by an outer wall comprising a hard outer layer, the exine, and a softer inner layer, the intine (Rantio-Lehtimäki, 1995). Within the pollen grain is a cytoplasm containing granules. The most common method for identifying pollen types are under light microscopy, by their morphology, i.e. based on size, shape, apertures, and surface features (Rantio-Lehtimäki, 1995). However, it is generally not possible to distinguish between all pollen of different genera or species using optical methods (Kraaijeveld et al., 2015). Grass pollen, for example, originate from more than 10.000 species (de Weger et al., 2011), morphological similar; near spheroidal, a single pore, granular smooth surface, 30-60 μm, with the wild species being in the smaller range, and the cereal in the larger (Joly et al., 2007;Morrow Brown and Irving, 1973;Page, 1978). Birch pollen are also very similar on species level, being three-porate and approximately 20 μm, and nearly spherical (Mäkelä, 1996).

Figure 2.1 Grass inflorescence and pollen grain in microscope, app. 40 μm (left), and birch catkins and pollen grain, app. 20 μm, as seen in microscope (right) (birch pollen grain image from Asthma Allergy Denmark).

Driessen et al. found a method of auto-fluorescence and UV- light microscopy, with which they could identify 15 of 21 examined grass species (1989b). With the usually applied microscopic visual analysis, this is not possible. However, the relative importance of different grass species to a measured pollen concentration can be estimated by evaluating the pollen production of the individual species and their abundance in an area (Smart et al. 1979), or by using genomic approaches (Kraaijeveld et al., 2015).

Pollen production in grasses

Anemophilous plants generally produce great amounts of pollen to compensate for the low efficiency of wind pollination, and trees are generally more prolific producers than herbs (Molina et al., 1996b;Subba Reddi and Reddi, 1986). This is speculated to be due to higher altitude of pollen release, and therefore wider spread of the pollen, and a need to reach individuals further away (Prieto-Baena et al., 2003).

Studies on pollen production per plant can provide information on the relative contribution of each species to the total amount of pollen, enabling estimations of the average potential pollen emission. Prieto-Baena et al (2003) found that for Córdoba (Spain), only two species appeared to be responsible of 80-85% of the total airborne pollen load. Knowledge on the pollen production may therefore aid in the understanding, of whether the flowering of succeeding species may be contributing to the variation in the pattern of grass pollen concentrations.

The pollen production in grasses have been determined by several studies (Aboulaich et al., 2009;Agnihotri and Singh, 1975;Prieto-Baena et al., 2003;Smart et al., 1979;Subba Reddi and Reddi, 1986). For some species with substantial variance between studies, e.g. has Dactylis glomerata been found to have 3475 (Prieto-Baena et al., 2003), 2133 (Aboulaich et al., 2009) and 1300 (Smart and Knox, 1979) pollen per anther. The variation in pollen production per anther may be due to the variation in the specific method applied for the different studies (Subba Reddi and Reddi, 1986).

There are several methods for determining the pollen production per anther. If the anther is expected to contain more than 2000 pollen, the anther is crushed and the pollen inside is suspended in a water-staining solution and a count is made of the pollen in a portion of the solution (Aboulaich et al., 2009;Prieto-Baena et al., 2003).

The pollen production per anther is speculated to be fixed for the individual species, however pollen production per plant can vary by the number of flowers, inflorescences or branches, where different meteorological parameters can play an important role (Prieto-Baena et al., 2003;Subba Reddi and Reddi, 1986), i.e. also for individual plants growing in different climates. Pollen production has also been related to pollen size and anther length for both grasses and trees, with the longer the anther, the more pollen produced (Agnihotri and Singh, 1975;Molina et al., 1996b;Subba Reddi and Reddi, 1986).

For this thesis, a study was initiated performing measurements of pollen production in grass species found in at least two of the three countries, Spain, UK and Denmark (*Lollium rigidum*, *Bromus Hordeaceus*, *Dactylis glomerata*, *Alopecurus pratensis*, *Phleum pratensis*). This study was however not completed for publication, as the applied method of crushing the anther was determined to be insufficient, after the observation of large amounts of pollen still remaining in the anther following crushing. Insufficient crushing would lead to uncertainty of whether the full amount of pollen is released and uniformly

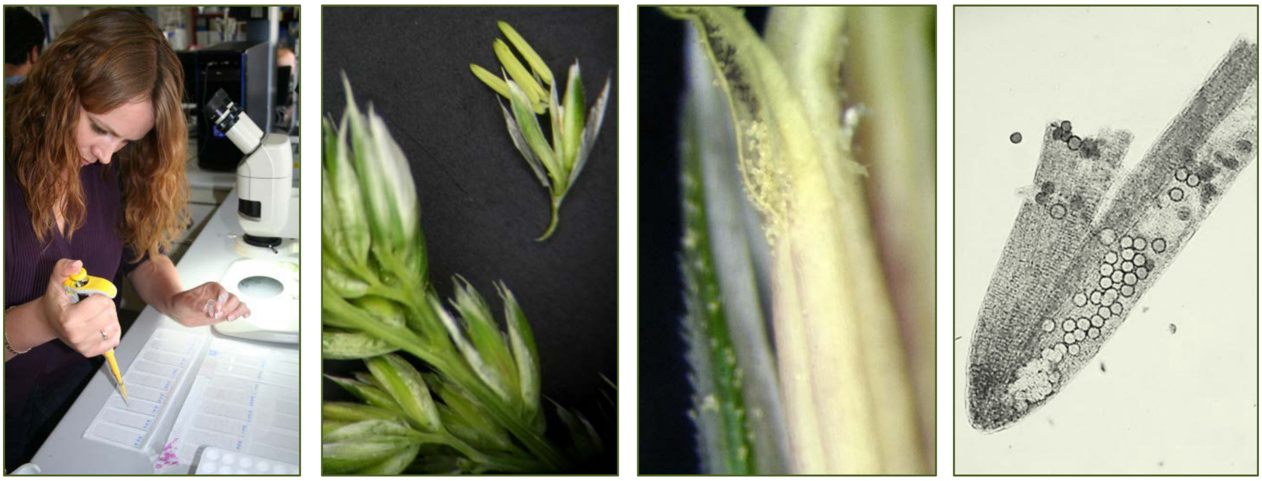


Figure 2.2 A portion of solution containing crushed anther with pollen and staining fluid is mounted and examined for examination of pollen production. Grass inflorescences were collected and anthers sampled, however, in some cases the anther was not successfully crushed and pollen remained in the anther, and did not enter the solution.

distributed in the dilution, and therefore also whether the 10-30% of the counted amount is representative (Figure 2.2). Partial results are shown in Appendix I.

Pollen allergen

Pollen allergens are water soluble proteins, where those on the surface of the pollen grain are released within seconds upon contact with a moist surface. This is ideally the stigma of a flower , but it may also be the mucosal surface of the nose, airway or eye (Knox et al., 1993;Rantio-Lehtimäki, 1995).
Allergens are named by the first three letters of the genus, first letter of species and a number specifying a grouping of the chemical structure, e.g. the main allergen in birch pollen is named Bet v 1(Rantio-Lehtimäki, 1995).

Pollen allergen is not only found in whole pollen grains, but also as smaller particles. Allergen can be found in both the exine, the granules (Emberlin and Baboonian, 1995), intine and anther lining (Rantio-Lehtimäki, 1995), and may be either released in granules from the pollen, e.g. by osmotic shock, or contained in fragments from physical degradation of the grains (Knox et al., 1997;Schäppi et al., 1997;Suphioglu et al., 1992). The smaller particles may not all contain identical allergens, although originating from the same pollen, as different allergens may be present in the granules and on the surface (Knox, 1993).
The allergenic proteins found in the pollen grains may neither be identical with those from other parts of the plant. However, for both birch and grass, identical IgE response to pollen has been found to leafy material (D'Amato et al., 1991;Fountain et al., 1992).
Recently coordinated methods for measuring allergens in airborne pollen have been developed within the HIALINE project (Buters et al., 2015;Buters et al., 2012).

Many major allergens from different plants are similar, e.g. are the main allergen of birch similar with alder and hazel (Niederberger et al., 1998;Rantio-Lehtimäki, 1995;Valenta et al., 1991), in Denmark both flowering February to April, just before the birch season, which is in April-May. Patients with cross-reactions to species who flower successively may therefore be affected for a long period (de Weger et al., 2013).

Allergenic potency of pollen may vary depending on area and year. A up to ten-fold difference in allergen content has been found for birch for day to day, for different areas, and from year to year in the same area (Buters et al., 2008;Buters et al., 2010;Buters et al., 2012). A nine-fold difference has been found for grass pollen in a study of three seasons across 10 European sites, with the main variability being locally and day to day (Buters et al., 2015). For olive a twelve-fold difference has been pollen from two sites, in Spain and Portugal, during the same year (Galan et al., 2013). The variable allergenicity has also been seen in poor correlation between allergen amount and pollen concentration for grasses in Spain (Rodríguez-Rajo et al., 2011).

Chapter 3

Spatial and temporal variations of pollen

Pollen concentrations have great spatial and temporal variation, due to a number of parameters affecting variability in production, emission, dispersion and deposition.
Focus in this thesis is primarily on the local scale, i.e. processes within the nearest kilometers of the source (Seinfeld and Pandis, 2012b). The included studies examine the diurnal variation, the influence of local source distribution and the associations with basic meteorological parameters by simple analyses.

Spatial variation of pollen

The spatial variation in pollen concentration is related to the distribution of sources (Norris-Hill, 1999) and the dispersion and deposition following emission (Skjoth et al., 2009;Skjoth et al., 2008b).
The atmospheric lifetime of pollen is predominantly within the range of hours to days rendering the range of travelled distance from meters to a few kilometers (Sofiev et al., 2006;Sofiev et al., 2013a;Sofiev and Bergmann, 2013). However, long range transport has also been seen for both trees (Skjoth et al., 2007) and weeds (Smith et al., 2008).
In an early study on pollen dispersion by Raynor, Ogden and Hayes, concentrations of ragweed pollen on an open field were seen to decrease logarithmically with distance, and only around 1% was speculated to travel more than 1 km (Raynor et al., 1970;Raynor et al., 1973).
For grass pollen, the large size, low release height, and resulting estimated short travel distances, also results in proximity to source being of great importance for the measured pollen load, and large spatial variation is to be expected. Variation between measurements may however, not only reflect variations in the actual surrounding pollen concentration, but also be a measure of the precision and placement of the measurement (Tormo Molina et al., 2013).
Birch pollen are released at a higher altitude and are smaller than grass pollen (Skjøth et al., 2013b). In Denmark, the concentrations has been found to both be affected by local emissions, not only from forest areas, but also from within the city, as this taxa is often applied in urban landscaping (Figure 3.1), and also from long distance transport originating primarily from Germany, Eastern Europe and Scandinavia (Mahura et al., 2007;Skjoth et al., 2007;Skjoth et al., 2008b).

All included studies in this thesis are based on pollen measurements in urban areas. Around 75% of the European population are living in urban areas (Eurostat, 2016), and an understanding of pollen levels and the variability here, is therefore of great relevance, however, not yet well known (Mücke et al., 2014).

Figure 2.1 There are many birch trees both outside and within Copenhagen, here seen near the center of the city, in two parks and at a cemetary.

The urban area is often characterized by a highly variable structure of street canyons, backyards, and open park spaces. Concentrations of pollen within the urban environment is therefore naturally highly variable and dependent on the distribution of sources and the dispersion patterns influenced by the physical structures (Peel et al., 2014b).

City Centre pollen dispersion has previously been examined in several studies, where several samplers have been placed within a city. Emberlin and Norris-Hill examined weekly levels of several taxa, including grass, at 14 sites in London, and found these levels to be fairly homogeneous, whereas Platanus pollen was seen to have variation of up to 646% between traps (Emberlin and Norris-Hill, 1991). In Córdoba, Spain, Alcazar et al found the spatial source distribution of Platanus to be of importance for daily allergic symptoms, in a study of pollen concentrations from four sites within the city (2004). Here, also differences in management, i.e. pruning of Plantanus trees, were seen to affect pollen concentrations. Another study in Córdoba by Velasco-Jiménz et al, examined pollen concentrations at two sites, and from two traps at the same site (2012). They found one sampler to be sufficient for estimation of seasonal peak and total loads, for a city with uniform topography and homogenous vegetation. However, they recommend more local measurements, if data is to be applied in clinical studies of effects from pollen, where the taxa is not uniformly distributed. They also found sampler efficiency not to affect inter-site differences. Mücke et al also found good correlation between daily grass pollen counts at three sites in Berlin, during a six-week measurement campaign (2014). In Badajoz, Spain, a study on two traps showed significantly different levels of grass pollen, however well correlated, also for time of day of peak concentrations (Fernandez Rodriguez et al., 2014). This indicates similar patterns within the city, however with levels being affected by local source distributions.

A recent study on the availability of urban green spaces for the urban population, showed higher availability in Northern Europe than in Southern (Kabisch et al., 2016). This indicates a higher proportion of citizens with close proximity to pollen sources in this part of Europe. The distribution of grass sources within city centers cannot be assumed to be of equal importance between northern and

southern Europe. In cities with only few sources within the city, the contribution will be almost exclusively from external sources, as found in Badajoz, Spain (Gonzalo-Garijo et al., 2006).
The previous studies indicate, that for external sources the pollen levels within the city will be lower than outside and more evenly distributed, whereas for pollen taxa found in the city, the distance to source and urban topography will have the main impact on spatial variation (Alcázar et al., 2004;Emberlin and Norris-Hill, 1991;Gonzalo-Garijo et al., 2006;Peel et al., 2014b).

In this thesis, the importance of the relative proximity to the source on the pollen measurements is this studied for grass, with focus is on horizontal variation. Vertical variation is however also relevant, especially with pollen concentrations being measured at roof level, whereas exposures occur at ground level.
Decrease in pollen concentration with height is observed where pollen sources are near the sampling site, and is dependent on emission height, with mean ratios of 1.4-2.1 (Alcázar et al., 1999a;Rantio-Lehtimäki et al., 1991), and higher wind speeds have been shown to promote vertical mixing reducing ratios (Alcázar et al., 1998). In the urban environment the background/street level ratios have also been shown to be highly affected by wind direction and the local street canyon orientation, with lower concentrations at street level (Peel et al., 2014b).

Background pollen concentrations are the primary available proxy for exposure. Not only the relationship between background and street level is of importance, also the understanding of this related to the inhaled dose needs examination (WHO, 2003b). This relationship has only rarely been examined (Peel et al., 2013). A recent study found that the amount of pollen collected on nasal filters, compared to the background concentrations, varied throughout the day, with the greatest correlation during the non-peak time where the grasses was not flowering, i.e. at the time of flowering proximity to source was the most important factor, and at this time, the background concentration was not a good proxy for exposure (Peel et al., 2013). A full understanding is not yet accomplished.

The estimation of potential sources, their distribution and location, is not only important in estimating local variations, but also of great importance for application in transport and forecasting models (Skjoth et al., 2007;Skjoth et al., 2008a;Sofiev et al., 2006), applicable in future pollen warning and symptom forecasting systems.
These mappings have been carried out mainly for ragweed (Karrer et al., 2015;Skjøth et al., 2010;Thibaudon et al., 2014) and birch (Pauling et al., 2012;Skjøth et al., 2009;Sofiev et al., 2013b) and typically for regional scale applications; 5-50km resolution (Zink et al., 2017), while local scale mapping (e.g. 10-20m resolution) has rarely been achieved.

Temporal variation of pollen

The pollen season is defined as "*the period where pollen is in the air*" (Dahl et al., 2013), e.g. including 95% of the yearly measured pollen count (Goldberg et al., 1988) or the cumulative sum method, starting when e.g. a sum of 100 (grass) of daily average count is reached (Driessen et al., 1989a). This method is more applicable in forecasting, as is does is not dependent on retrospective data (Khwarahm et al., 2014). The time of the pollen season for each species may vary from year to year, and site to site (Dahl et al., 2013). The 30-year average seasonal distribution of the main allergenic pollen in Denmark is seen in Figure 3.2.

Day to day variations may be great, and highly dependent on meteorology, with relative humidity and rainfall suppressing counts (Galán et al., 1995). High pollen levels have even been seen to be frequent at nighttime (Bogawski and Smith, 2016).

Pollen concentrations are limited indoors, and the induvial exposure is therefore very dependent on the time spend outdoors (Mitakakis et al., 2000). From this, the time of diurnal peak concentrations may be of equal importance as the daily pollen count when estimating exposure risk. Although it is known that the diurnal pollen concentration profile can vary greatly from day to day, it is common practice to provide an average profile based on the entire season; However, different profiles for grass pollen concentrations have been found at different sites. Single evening peaks have been seen in London (Norris-Hill and Emberlin, 1991), Cardiff, South Wales, whereas an afternoon were seen close by, at Cleppa Park (Mullins et al., 1986), and also in Mar del Palta, Argentina (Gassmann et al., 2002) and Porto, Portugal (Ribeiro et al., 2008). A single evening peak was also seen in Melbourne, in 1991-1992, however more pronounced in the second month of the season (Ong et al., 1995). Single morning peaks have been seen in Turku (Finland) (Käpylä, 1981), Cordoba (Spain) (Alcázar et al., 1999b;Galan et al., 1991) and Malága (Trigo et al., 1997), and two peak profiles with nighttime peaks in Washington D.C. (Kosisky et al., 2010), and in Melbourne (Smart et al., 1979) and in morning and afternoon in Jyväskylä (Finland)(Käpylä, 1981).

A few studies have examined the profile in portions of the season. Norris-Hill found a weak diurnal pattern with late afternoon peak in Wales, when averaged over the entire season, but found a clearer peak of shifting timing, when examining the season in four quarters (Norris-Hill, 1999). Smith and Emberlin separated the season in three periods defined by seasonal peak, as pre-, peak, and post-peak

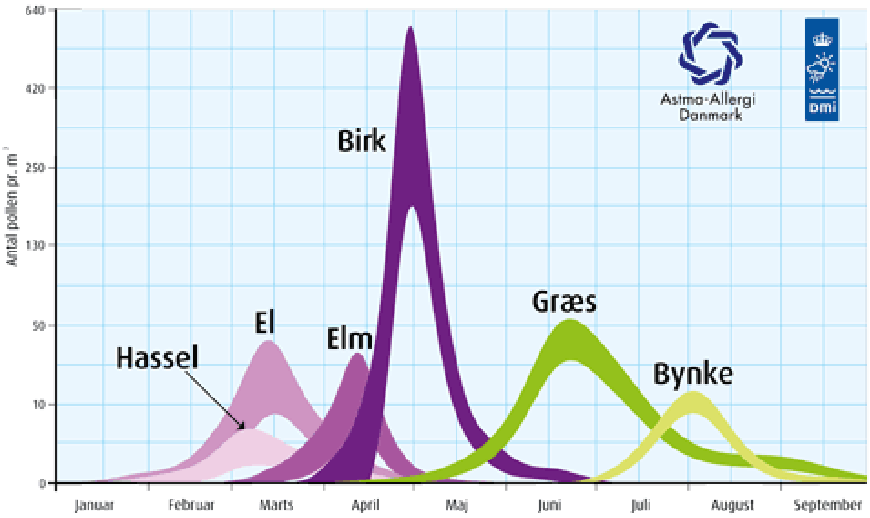


Figure 3.2 Main allergenic pollen species and their 30-year (1985-2009) average yearly distribution in Denmark. The calendar was produced by Astma-Allergi Danmark and DMI.

periods, allowing for differences in parameterization when forecasting daily pollen counts, also indicating seasonal variability in parameters affecting the pollen count (Smith and Emberlin, 2005). Munoz-Rodriguez examined the grass pollen profile for the four main months of the season in Badajoz (Spain) and found variable peak time (Munoz Rodriguez et al., 2010).
It has also been found that separating the season into pre-peak and post-peak periods, allowed for different correlation with meteorological parameters (Khwarahm et al., 2014;Mesa et al., 2003).

Some have related the diurnal pattern to magnitude of daily count. Mullins et al investigated days with pollen counts above 100 and above 50 and found no difference in the pattern. They also neither found a difference in diurnal pattern between June and July (Mullins et al., 1986). Norris-Hill neither found a difference in pattern between days with counts below and above 50 grains m^{-3} (Norris-Hill, 1999).

The highly variable diurnal pattern of grass pollen concentrations have been explained as differences in flowering species (Munoz Rodriguez et al., 2010), or as variance in dispersion, re-suspension and deposition, where convection may be responsible for later peaks when it ceases in the evening (Norris-Hill, 1999).

To the authors knowledge, the studies included in this thesis are the only reflecting diurnal grass and birch pollen patterns in Denmark.

Impact of meteorology on the variation in diurnal pattern of grass pollen

If the succession of flowering species are responsible for the variation in the diurnal pattern, variation in emission pattern will be of great importance. The pollen release in plants is controlled by anther burst and dehiscense, which both depend on factors related to meteorology, e.g. affecting dehydration, and factors regulated by the plant, as plant specific reabsorption (Dahl et al., 2013;Sofiev et al., 2013a). Meteorological parameters as the vapor pressure deficit, temperature, humidity and solar radiation may therefore directly affect emissions.
For grasses distinct species specific patterns have often been observed, however with variable degree of influence of meteorology, depending on species and conditions (Subba Reddi et al., 1988). The variability in the time of anthesis between species, as well as the synchronicity within a specie, has been suggested to be a mechanism preventing unwanted competition between species (Dahl et al., 2013).

Both airborne whole pollen, and to some degree allergen fragments, are effectively scavenged by rain, (McDonald, 1962;Sofiev et al., 2013a), and days with rain are therefore often removed from analyses of diurnal patterns (Peel et al., 2014a).
Previous studies have mainly focused on relating the impact of meteorological variables on the magnitude on the daily pollen count, and not on the intra-diurnal pattern and the variation in this. An earlier study of pollen patterns in London, differentiated for maximum daily temperature and pre-dominant wind direction, and found similar peak times in all groups for both birch and grass pollen (Norris-Hill and Emberlin, 1991). They did however find an impact on the size of the peak, with higher

peaks occurring on warmer days. Higher peaks on warmer days were also found by Fernandez Rodriguez et al, who saw statistically significant positive correlation with solar radiation, mean temperature and wind speed, and a negative correlation between hourly grass pollen concentrations and relative humidity (2014).

Khwarahm et al. also examined the relationship between the daily birch and grass pollen counts and meteorology for 10 years of counts from 9 monitoring stations in UK (Khwarahm et al., 2014). They found the correlations to shift between time of season. Temperature was seen to have a significant positive impact on pollen counts in pre-season period, affecting flowering, however in the post-peak season, the impact is not as clear, and in some cases negative. They emphasize that, since post-peak periods are often longer in duration than pre-peak, examining the season as a whole, could conceal important findings as this. The results generally showed rain and relative humidity to have a negative impact on the daily pollen counts.

Wind speed and direction are the main determinant of the local path the pollen will travel. The local wind direction can be applied as a proxy for determining the direction from which the measured pollen are originating, however, for longer distances and travel time, trajectories should be applied.

Chapter 4

Pollen allergy

Pollen sensitization and allergic rhinitis

Pollen allergy is an affliction covering an array of differential diagnoses. These overlap areas of IgE-mediated hypersensitive reactions resulting in nasal, ocular and/or bronchial symptoms following exposure to pollen allergens (Figure 4.1). In this thesis focus is on AR.

AR is diagnosed be a combination of either skin prick test (SPT) or allergen-specific IgE in a blood sample and symptom history. Degree of sensitization may not be directly correlated with clinical relevance, i.e. severity of symptoms (Graif et al., 2006). Clinical relevance has however been shown to be present in above 60% of the positive SPT for all allergens, with the highest rate for grass, of 88% (Burbach et al., 2009). A cross sectional European survey estimated 23% to have clinically relevant prevalence of AR with the affliction being frequently undiagnosed (Bauchau and Durham, 2004).

The focus in this thesis is on birch and grass allergy. The prevalence of grass sensitization is generally the highest among pollen types throughout most parts of the world, with rates of 5-29% reported from a European Health Survey (Bousquet et al., 2007). For birch the highest rates were seen in Northern Europe, where Sweden had rates of average 17% and Norway 11% (Bousquet et al., 2007). For Denmark, a random sample revealed rates of 18% with grass sensitization and 12% with birch sensitization (Thomsen et al., 2015).

Each individual may experience differences in the severity of symptoms based on the time of the season or co-exposures to other irritants, e.g. air pollutants. As stated, the amount of allergen in pollen may also differ from grain to grain, and free allergens may be present in the air. Some symptoms may also be related to other elements of the pollen than the allergen protein (de Weger et al., 2013). Therefore there is no straight forward relationship between pollen levels and symptom severity. However, the levels is still indicative of the proportion of people with allergies reacting and the severity of the symptoms (de Weger et al., 2013).

Allergic asthma - Airways

- Wheezing, coughing, breathlessness and tightness of the chest.

Seasonal allergic conjunctivitis - Eyes

- Itching, burning, watering, redness, sensitivity to light.

Seasonal Allergic Rhinitis - Nose

- Blocking, watering, itching, sneezing.
- Can also affect throat, mouth, fullness feeling in head and ears, general fatigue

Figure 4.1 The diagnoses related to pollen allergy, defined by the primarily affected organ.

Several studies indicate that the severity of symptoms correlates with pollen concentrations up to a threshold. Caillaud et al found that the degree of nasal and ocular symptoms in grass allergic patients were linear up to a threshold of 80-90 grains m^{-3} (2012). They found no threshold for bronchial symptoms, and that these symptoms show less increase with increasing pollen concentrations.
An increase in symptoms up to a threshold of 30 grains m^{-3} has been shown for hospital admissions for asthma attacks (Erbas et al., 2007;Tobias et al., 2004). For birch, 90% patients has been shown to have symptoms when airborne concentrations exceeded 80 m^{-3} (Viander and Koivikko, 1978).
A study of patients experienced symptoms, showed nasal symptoms was considered to be most severe, followed by ocular and then airway symptoms (Karatzas et al., 2014).

Some patients have been seen to have a natural ability to downregulate the allergic response without intervention, whereas it in others, a strong response appears to be able to induce this mechanism (de Bruin-Weller et al., 1999). Various types of allergen immunotherapy and allergy vaccines, either subcutaneous or sublingual immunotherapy have also been proven to significantly reduce symptoms (Burks et al., 2013).
Although medication in general has been proven to effectively improve quality of life (Tripathi and Patterson, 2001), AR still effects both psychical and social functioning. Social functioning has even been seen to be more affected than in asthma patients (Nathan, 2007). However, either medication or vaccination is without side effects, and often not experienced as inducing satisfactory relief, and as loosing effect over time (Nathan, 2007).

Allergic rhinitis and asthma

AR is considered to be a well-known risk factor for the development of asthma with most asthma patients also suffering from AR (Cruz et al., 2007;Pawankar et al., 2011;Shaaban et al., 2008). In Denmark, approximately one quarter of all AR patients were seen to also suffer from allergic asthma (Linneberg et al., 2002).
One of the theories regarding the co-morbidity of the two afflictions is the hypothesis of "one airway, one disease" and the "united airway", where the inflammation in either upper or lower airways are thought to affect the other through a systemic response (Feng et al., 2012;Grossman, 1997;Rimmer and Ruhno, 2006).

Exposure to pollen and allergen

Pollen may enter through the nose, or mouth affecting either or both the upper or lower airways. The proportion of pollen inhaled and deposited in the airways is, as for other particles, related to the size and the rate and pattern of inhalation (Heyder, 2004). Only few studies have focused on the inhaled dose of pollen, finding highly variable doses from person to person in the same environment (Mitakakis et al., 2000;O'Meara et al., 2004).
The main depository site for pollen grains is the upper airways, due to the size of the pollen grain, and the effective filtering mechanism (D'Amato et al., 1998;DS - Dask Standard, 1994;Scadding et al.,

2011). The lower airways may also be affected by micronic particles below 3-5 μm containing pollen allergens, as starch granules from rye-grass or fragments, that are within the respirable fraction (Knox et al., 1993). Rye grass pollen has been shown to rupture by osmotic shock, increasing the amount of micronic allergen particles following rain and being the likely trigger of increased number of asthma attacks (Bellomo et al., 1992;Knox, 1993;Knox et al., 1993;Suphioglu et al., 1992).

Allergic mechanism

Each specific allergen has a distinctive chemical site, known as an epitope, which defines how they are recognized by antibodies. If epitopes of an allergen are similar, an antibody produced for one, may bind to the other, creating a cross-reaction. In the allergic mechanism, the responding antibody is of the isotype immunoglobulin E (IgE). IgE favors binding to two specific immune-cells; mast cells, primarily found in tissue, and basophils, mainly found in blood serum. Mast cells contain granules of mediators as e.g. histamine and leukotriene. The release of these lead to an increase in the size of the capillaries, resulting in more immune cells leaching in, causing inflammation and swelling. Histamines are mainly responsible for the typical rhinitis symptoms, whereas leukotrienes mainly affect the airways causing the allergic asthmatic symptoms.

Mediators can also result in muscle contracting, e.g. in the bronchi. Combined with swelling membranes and mucus, an asthma attack can result.

At the first encounter with an allergen, the IgE antibodies for this specific allergen will be produced and bind to mast cells. This is called sensitization. At the next encounter the allergen will bind to the IgE, inducing the release of histamine and other inflammatory cells. Once sensitized the levels of IgE to a specific antigen will be higher, and the subject is said to be atopic.

Atopic subjects have an increased risk of developing a group of conditions known as atopic diseases, including AR, asthma and atopic dermatitis. However, atopy does not equal allergy. An allergy requires the presence of symptoms, which may not always occur in sensitized subjects (as discussed in the previous section).

The allergic mechanism is a complicated network of interconnected responses, and there does not appear to be one universal explanation. In some, the immune system may not produce IgE, whereas others have a mechanism that may prevent IgE from binding to the mast cells, by either binding to the IgE-antibody or the mast cell. There are many both known and unknown factors at play, and the full understanding of the mechanism is not yet discovered (Brostoff and Gamlin, 1996b;Janeway et al., 2005;Oettgen and Broide, 2012).

Measurements of bronchial response

A key source to increased knowledge about pollen allergies are dose-response relationships for allergen exposure and health outcome, which are scarce (WHO, 2003a).

The studies included in this thesis focus on dose-response relationships between pollen allergen exposure and the bronchial response to direct stimuli, i.e. a constrictor, as methacholine or allergen, which has a direct effect on the smooth muscles in the airways (Joos and O'Connor, 2003). In this study we performed non-specific challenges with methacholine and specific challenges with birch and grass allergen.

Bronchial hyper-responsiveness, also referred to as airway hyper-responsiveness (AHR), is characterized by an increased narrowing of the airways after exposure, and was first seen as bronchoconstriction in asthmatics following histamine exposure (Curry, 1946;O´Byrne and Inman, 2003). Although it is not linked to the exact same mechanisms, it has been shown that AHR can be inherited along with elevated levels of IgE (Postma et al., 1995). AHR is often defined as a smaller dose needed to elicit a certain drop, or a steeper dose-response profile compared to an estimated "normal" (O´Byrne and Inman, 2003).

Although AHR is a good indicator for asthma, it is not possible to distinguish exactly between asthmatic and non-asthmatic subjects by the response profile (Cockcroft, 1985;Cockcroft, 2010;O´Byrne and Inman, 2003). Inhaled allergens will typically increase airway inflammation and enhance airway hyper-responsiveness in atopic subjects. These changes are however less in non-asthmatic subjects (O´Byrne and Inman, 2003).

The bronchial response is usually separated into the early airway response (EAR) and the late airway response (LAR), where a DUAL response is defined as both an EAR and an LAR. EAR is most commonly defined as the response within 1-60 minutes following the exposure, and measured as the dose eliciting a 15% or 20% reduction (PD_{15}/PD_{20}) in the Forced Expiratory Volume in the first second of exhalation (FEV_1), compared to baseline, estimated from linear interpolation on a log dose-response curve (Bruin–Weller et al., 1996;Cockcroft, 2010;Crimi et al., 1990a;Crimi et al., 1990b;Dente et al., 2000;Paggiaro et al., 1990). The LAR is most commonly defined as a minimum drop of 15% in FEV_1 within 2-12 hours following the exposure, and measured as the maximum % fall in FEV_1. (Bruin–Weller et al., 1996;Crimi et al., 1990a;Crimi et al., 1990b;Dente et al., 2000;Paggiaro et al., 1990). The LAR symptoms can continue for weeks after the exposure has ended.

Another measure is the evaluation of the slope of the dose-repose profile, frequently applied in the evaluation of non-specific challenges (de Meer et al., 2005;O'Connor et al., 1987).

<u>Prediction of degree of AHR</u>

Previous studies have examined the association between the degree of bronchial response to allergen and various baseline characteristics, e.g. the size of SPT. The size of the reaction to SPT will however not always reflect the allergic bronchial reaction, as the local production of specific IgE may not be the same for different organs. However with large skin prick reactions, higher correlation has been found between SPT size and level of Specific IgE (Melillo et al., 1997).

SPT, specific IgE and non-specific bronchial response have all been found to be predictive for PD_{20} for the response to house dust mite allergen (HDM) (Ravensberg et al., 2007), and for cat, HDM and grass pollen (Barnig et al., 2013). It is however not all studies who find an association between PD_{20} and non-specific responsiveness (Bruin–Weller et al., 1996;Paggiaro et al., 1990).

Cockcroft el al suggested a formula for prediction of allergen PD_{20} based on histamine PC_{20} and skin sensitivity, and found results within a ±8-fold range (1987).
Barnig et al (2013) also assumed a correlation between PD_{20} and SPT, and applied a measurement of Allergen Skin Test Endpoint in estimation of the starting concentration of the SIC as one-tenth of this, in order to both reduce risk of to high doses and of induced fatigue if starting at too low doses . They however do not find any relationship between Allergen Skin Test Endpoint and PD_{20}. This method of estimation of starting doses has also been suggested by Diamant et al (2013).
It is however not all studies who find an association between PD_{20} and non-specific responsiveness (Bruin–Weller et al., 1996;Paggiaro et al., 1990).

The priming effect

The priming effect of pollen is commonly understood as an increase in reactivity to an allergen following repeated exposures. The mechanism is not fully understood, however, it is suggested to be related to repeated low doses causing increasing levels of mast cells, and immune cells such as eosinophils, and thereby an intensified allergic reaction (Liu et al., 2003b;Scadding et al., 2011).
The first study to name the priming effect was by Connell (1968), who demonstrated that repeated challenges with ragweed resulted in a decreasing threshold of nasal rhinitis symptoms. He called this "the priming of the end organ". He found that this challenge-induced priming effect on the nasal symptoms was reversible within days however the seasonal priming effect took weeks to wear off. A later split-nose experiment by Connell (1969) resulted in symptoms increasing only in the exposed nostril, indicating that the effect was in the local tissue rather than systemic. A following exposure of a different allergen resulted in severe symptoms in only the primed nostril, supporting the theory that an early response to priming is non-specific. This has later also been shown for other irritants than allergen, e.g. has ozone (Peden et al., 1995) been shown to prime the airways affecting succeeding allergen responses.
Rosenthal et al were the first to examine the priming effect in the lungs. They however, did not find any effect on PD_{35} in ragweed patients, after four successive days of exposure. Neither did they find an effect of seasonal exposure (1975).

<u>Measurements of priming effect</u>

The effect of priming can be described as a change in response to repeated administered allergen doses out of the pollen season (Bruin–Weller et al., 1996;de Bruin-Weller et al., 1999;Ihre and Zetterstrom, 1993;Jacobs et al., 2012) or as changes in response to the natural seasonal exposure (e.g. (Crimi et al., 1990a;Crimi et al., 1990b;Dente et al., 2000;Madonini et al., 1987;Paggiaro et al., 1990), or as a combination of the two (Jacobs et al., 2012). The administered doses may be of a specific allergen or a non-specific agent, i.e. methacholine or histamine, following the theory of priming being non-specific. The response can be described by a number of different measures in addition to bronchial hyperreactivity, e.g. symptom scores (de Weger et al., 2011;Ellis et al., 2010), nasal microvascular blood flow (Juliusson and Bende, 1988), or IgE (Møller and Elsayed, 1990), depending on the main objective of the individual study. When studying the bronchial response, the pattern of EAR and LAR may be of importance (Boulet et al., 2015;Bruin–Weller et al., 1996;Crimi et al., 1990a;Dente et al., 2000;Diamant et al., 2013;Grainge and Howarth, 2011;Paggiaro et al., 1990).

The priming effect appears to only affect to a certain plateau or induced tolerance. Liu et al compared one large single dose to four doses of 25%, administered one day apart, and found that the bronchial reaction reached a plateau or even decreased after 75% of the dose, when administering smaller doses (2003b). De Bruin-Weller also sees no additional decrease in PD_{20} after a second allergen challenge (1996), and Ellis et al suggests to prime patients before studies, in order to reach this level, to eliminate the priming effect as confounder in other studies (2010).

Priming appears to affect individuals with allergy independent of sensitivity. Viander et al found that the first symptoms at counts below 30 m^{-3} in birch allergic subjects were dependent on nasal sensitivity and concentration of Specific IgE in the early season, but that at late season, 80% of all subjects, independent of sensitivity, had symptoms below 30 m^{-3} (1978).
It is important to note, that diary-data for the most part is *experienced* symptoms. Two contradicting issues could affect the experience of symptoms throughout the season. Patients could either be feeling accustomed to a degree of discomfort, and therefore not registering symptoms as severe as they did the first experienced symptoms. In contrast to the previous, exhaustion could also occur, thereby experiencing symptoms as more severe. Graif et al (2006) also suggests the importance of psychological factors, as somatic awareness, when analyzing self-reported symptoms. It should also be considered whether there is a change in behavior throughout the season, affecting the amount and timing of outdoor activities.

Pre-priming

Symptom scores have been shown to be higher for those suffering from perennial allergies or allergies to other pollens preceding the investigated species, indicating that these are pre-primed by other antigens, following the theory of non-specific pre-priming. (Caillaud et al., 2012;Jacobs et al., 2014). This has also been shown for experimental exposures (Ellis et al., 2010).
Lower pre- and post-seasonal exposures may also result in more severe symptoms due to priming by homologous pollen types. E.g. the major allergen in olive, Ole e 1 is also present in ash tree pollen. In Spain some ash species flower prior to the olive season, potentially priming many atopic subjects (Vara et al., 2016). Both cross- and co-sensitizations may therefore affect pre-priming.

Priming effect on non-specific and specific challenges

Increases in non-specific bronchial responsiveness by priming is widely accepted (Dente et al., 2000), and often applied as measure of priming following both seasonal and repeated administered allergen doses. De Bruin-Weller (1996) found that, increased responsiveness to histamine following allergen exposure was inversely correlated to baseline PC_{20}, and primarily seen in those with the highest non-

specific response before allergen priming. Several studies only find priming effects in sub-groups of their participants, e.g. only for those with highest IgE levels (Ihre and Zetterstrom, 1993) or for those with a response pattern of LAR during outside season challenges (Dente et al., 2000).
These studies were performed on asthmatic subjects, indicating a higher degree of bronchial responsiveness. Some studies did however find increased non-specific bronchial responsiveness in groups of not solely asthmatics, and in their study population as a whole, following natural seasonal exposure (Crimi et al., 1990b;Walker et al., 2001).
The degree of effect on the hyper-responsiveness may also vary between perennial and seasonal allergy, reflecting the duration on impact. A study by Riccioni et al found a higher proportion of participants with AHR in a group with HDM allergy than in those with grass or parietaria allergy challenged during the season, and the lowest proportion in a group challenged outside pollen season (2002).

Overall, studies on the priming effect on specific bronchial response show inconsistent results. The response pattern may be of importance, as some studies find a significant decrease in grass allergen PD_{20} during season compared to outside season, in only for those with dual response pattern (Dente et al., 2000), and only in those shifting in response pattern from dual outside to EAR in season (Paggiaro et al., 1990). However, not all find the pattern to be of importance, as e.g. Grainge and Howarth who found no difference in either EAR or LAR following four consecutive days of high dose HDM allergen challenge in 16 sensitized asthmatic subjects (Grainge and Howarth, 2011).
A review by de Bruin-Weller et al (1999) on reactions from repeated allergen challenges, also indicated that a priming effect is not always seen. They also suggest that the effect may not be seen in all measures, and that e.g. for nasal observations, it is primarily evident in mediator levels and inflammatory cells, but that these may not be correlated with experiences symptoms. This could also be the case for bronchial response. E.g. did Crimi et al not find a priming effect when examining PD_{20}, but did find an effect in the number of participants experiencing LAR (Crimi et al., 1990a).

The magnitude of the repeating dose administered also appears to have importance. In Connells experiments, decreasing doses primed the nose, but in slowly increasing doses, as in immunotherapy, a down-regulating effect seems to appear.
It appears that repeated exposures do not always lead to a priming effect, and that differences in individual response patterns are very dominating. It should also be considered that as in other allergic reactions, there can be both early and late phase reactions to priming. Although some factors affecting the degree of priming are known, as pre-priming, the whole picture does not seem to be fully understood yet.

Chapter 5

Pollen, allergy and air pollution

For decades, air pollution has been linked to inducing an increased response to allergens as well as increased prevalence of allergic respiratory diseases (D´Amato et al., 2016;Emberlin, 1998). The impact of air pollution affects a multitude of processes from the pollen production in the plant (Rezanejad, 2007), to the reactivity of the allergic individuals (D'Amato et al., 2007). Historic observations often link the increase in AR with industrialization/urbanization, and name air pollution as the main cause (Brostoff and Gamlin, 1996a;Finn, 1992). Even so, the complexity of the connection is indisputable, as seen in e.g. the continued increase in prevalence of allergic disease in the western world, even after the general levels of air pollution declined (Brostoff and Gamlin, 1996a).

The impact of air pollution on pollen

The impact of air pollution on pollen has been seen as e.g. impact on the plant, inducing production of more deformed and fragile grains (Rezanejad, 2007). Impact on the pollen grain it-self has been seen, both *in vivo*, from pollens collected in areas with high pollution levels (Rezanejad, 2007;Shahali et al., 2009), and in *in vitro* studies, of pollen exposed to various pollutants (Motta et al., 2006;Santra et al., 1991). Pollen grains have been seen to have exine damage, being more prone to release cytoplasmic granules, and allergen content to be either reduced, heightened or altered (Ghiani et al., 2012;Motta et al., 2006;Rezanejad, 2007;Santra et al., 1991;Shahali et al., 2009).

The released granules have been shown to result in a higher cellular response than whole pollen (Motta et al., 2004), and due to their small size, they can reach the lower airways, and are not as effectively washed out of the atmosphere during rain. An increase therefore poses an increased risk of especially allergic asthma attacks.

The most amble air pollutants in the urban atmosphere are NO_2, PM and O_3 (D´Amato et al., 2016), and studies herof have either focused on single or combined exposures.

Ozone has been shown to increase allergenicity of birch pollen in a polluted area (Beck et al 2013), and to alter and increase grass pollen allergen content in a study on *in vivo* exposure (Eckl-Dorna et al., 2010). Another study found a degradation of allergen recognition following *in vivo* exposure to ozone, NO_2 and SO_2, with more degradation occurring after combined exposures (Rogerieux et al., 2007).

The impact of air pollutants on pollen has been speculated to be both species- and dose dependent (Frank and Ernst, 2016), but to overall induce increased risk of allergenic potential (Schiavoni et al., 2017). However, *in vivo* studies appear to show more resistant pollen than *in vitro studies*, and transferrable conclusions are therefore complicated (Senechal et al., 2015).

The impact of air pollution on allergic disease

The impact of air pollution on the allergic response has been examined in several studies applying a wide range of methodologies and different focus areas (Schiavoni et al., 2017). The overall mechanism is speculated to be related to increased permeability in the epithelial mucosal lining, and due to oxidative damage, inducing various inflammatory responses (D'Amato et al., 2007;Schiavoni et al., 2017). The increased inflammation will allow for exacerbated responses following allergen exposures, as well as previous inflammation caused by allergens could induce aggravated responses following pollution exposures.

Exacerbated allergic responses has been shown for particulate matter (PM) as e.g. increased hospitalization rates for asthmatics with AR (Tecer et al., 2008). PM can affect both the pollen grain by contamination through attaching to the exine, degrading this and potentially altering allergen release, but also through the attachment of micronic pollen particles to other PM (Emberlin, 1998).

Especially diesel exhaust particles (DEPs) have been associated with increased allergic reactions. The mechanism is associated with the DEP increasing IgE production (Nel et al., 1998), and hereby enhancing airway inflammation (Bosson et al., 2008), and even inducing allergic response in otherwise non-reactive individuals (Diaz-Sanchez et al., 1999). Free allergens have also been shown to bind to DEP´s in the $PM_{2.5}$ size range, thereby introducing a pathway for inhalation of free allergens (Emberlin, 1998;Knox et al., 1997). No studies were found that indicate a lower limit for potential aggravated allergic responses by PM.

A synergistic effect has been seen for exposures of SO_2 and NO_2 and allergy (Devalia et al., 1996). However, the Current SO_2 levels in ambient air are far from the examined levels (Guerreiro et al., 2016).

Combined exposures to O_3 and NO_2 has also been shown to induce an increased airway response to pollen allergens (Bayram et al., 2001;Jenkins et al., 1999;Molfino et al., 1991). However, a review on the effects of NO_2, suggests that no effect is seen below 200 ppb.

Co-exposure of allergen and ozone

Ground level ozone is a common air pollutant, resulting from photochemical reactions between volatile organic compounds and NOX gasses (Seinfeld and Pandis, 2012a). In the urban environment, most direct emissions are NO, rapidly interacting with ozone to form NO_2 (Palmgren et al., 1996). This generates a depletion of ozone in the urban atmosphere, inducing lower levels in areas with high traffic emissions and NO levels, and higher ozone levels in rural areas, where long distance transported ozone is not depleted.

High ground level ozone concentrations can generally be seen in regions of general high levels of pollution, much sunlight, and during smog periods of low ventilation (Derwent and Hertel, 1998).

Ozone is less soluble in water than e.g. SO_2 and therefore not as effectively removed in upper airways, and can easily reach lower airways.

Ozone inhalation have been shown to induce increased airway inflammation and increased AHR, e.g. by enhancing epithelial damage and permeability (Hernandez et al., 2010;Holtzman et al., 1979). Holz et al (2002) investigated the effect of different ozone concentration levels, exposing both AR and allergic asthmatic participants to filtered air, 125 ppb, 250 ppb and 4 consecutive days of 125 ppb, for 3 hours of intermittent exercise. They found repeated ozone exposures of 125 ppb to have the greatest impact on both the number of EAR, LAR and inflammation, seen in sputum, with the strongest response seen in allergic asthmatics.

The impact of co-exposure to allergen and ozone may be of importance for both prior, following or simultaneous exposure. Vagaggini et al (2002) found that a preceding allergen exposure led to increased inflammatory eosinophilic response in sputum, following a later exposure to ozone. No increase in neutrophils was seen, indicating that not all response mechanisms are activated in this process. In the studies by Peden et al, they found enhanced nasal inflammatory response and increased eosinophils in bronchial lavage fluid after ozone exposure (1995;Peden et al., 1997). As eosinophils are key cells in inflammatory and asthmatic response, this may indicate that atopic individuals are at greater risk than non-atopic, and that ozone is increasing the already underlying inflammation.

High ozone concentrations often occurs in episodes, whereas elevated pollen concentrations or high exposures to indoor allergens could occur for long periods. Since it is proven that allergen affects AHR, it could be of importance to also examine ozone response after prolonged allergen exposure.

Long term exposure of ozone has been shown to increase the prevalence and severity of asthma (D´Amato et al., 2016;Malig et al., 2016). One could therefore speculate whether long term ozone exposures of lower concentrations would also induce increased effects on the allergic response. Although studies on the co-exposure and health effects are not consistent, and do not appear to show clear effects of low ozone levels, it has been suggested that, theoretically ,there should be no lower limit of the adverse effects of ozone exposure (Van Bree et al., 1995).

<u>Studies on the bronchial response to co-exposure of ozone and allergen</u>

The majority of studies on co-exposure to ozone and allergens were conducted 10-20 years ago and almost none in the recent years. This despite the fact that most studies conclude that there is still substantial gaps in the knowledge on the mechanisms of the effect of ozone on the allergic response.

The studies predominantly examine participants with no or only mild asthma. This is mainly for safety reasons, but it can be argued that the more severely affected asthmatics are the group that will be most affected in case of high pollutant and pollen episodes (Chen et al., 2004;Kehrl et al., 1999;Peden et al., 1997).

The lowest level of exposure was reported by Molfino et al. (1991) in a study on seven atopic patients with mild asthma. Following one hour of exposure to 120 ppb, only half the allergen of ragweed or grass was needed to induce a reduction of 15% in FEV_1 (PD_{15}), compared to after exposure to clean air. A similar study by Ball et al (1996), applying same exposure level and duration found no significant differences between exposures. Hannania et al (1998) likewise exposed subjects to 120 ppb, and

challenged them the following day, assuming a lasting priming effect of the ozone, but found no impact on PD_{15}. It has later been suggested that the findings by Molfino et al. may have been affected by carry-over effect induced by as little as one week between challenges, and by having 6 of the 7 subjects exposed to ozone second, perhaps rendering the priming effect of repeated allergen exposures a relevant factor, and not only the ozone as priming agent (Ball et al., 1996;Hanania et al., 1998).
Jenkins et al (1999) suggested that it was the exposure level rather than the exposure time, that was the essential factor to increasing the allergic response. They measured no increase in the airway response to allergen following 6 h exposure to 100 ppb O_3 and 3 hours of 200 ppb NO_2, either combined or separate. However, they did find a significant effect on PD_{20} following 3 h of exposure to 200 ppb O_3 and 400 NO_2 as well as following separate exposures. Following this they suggest that the threshold of ozone is between 100 and 200 ppb. They also suggest that inhaled concentrations rather than dose is essential, since double exposure duration did not yield the same response as double concentration.
Kerl et al examined the effect of 7.6 hours of exposure to 160 ppb during light exercise, in HDM allergic mild asthmatics. They found that allergen challenge the following day, induced a 9% larger decrement in FEV_1 following ozone exposure than filtered air ($p<0.01$), and that 7 of 9 subjects only required significantly less allergen to elicit a 20% reduction in FEV_1.
Chen et al (2004) found no effect on PD_{15} in 14 mildly asthmatic HDM atopic subjects exposed to 1 h of 200ppb during exercise. They did however, find that a subgroup of their participants, most reactive in lung function to the ozone exposure, was also more responding to the subsequent allergen challenge, and suggest that the effect may only affect a subgroup of the asthmatic participants.
Jörres et al (1996) found significant effect on both allergen PD_{20}, methacholine PC_{20}, and percent reduction in FEV_1 in mild asthmatic atopic participants, following 3 hours of intermittent exercise during exposure to 250 ppb. However, for AR participants they only found a lower percent reduction in FEV_1, no difference was seen in PD_{20} or PC_{20} bronchoconstrictor response in mild asthmatics.

Overall, previous studies indicate that ozone has a priming effect and could aggravate the allergic response. However, this effect appears to be particular dependent on concentration of exposure, and inflammatory state of the participants, suggesting that ozone co-exposure may only pose a risk to a subgroup of those with pollen allergy. Also, as the effect of ozone may primarily be via inflammation, rather than on a direct lung function effect, the lag of the exposure may be of importance (Kehrl et al., 1999).

Part III - Main applied methodologies and evaluation of these

Chapter 6

Pollen sampling

Charles Blakely measured pollen by collection in a sticky substance on glass slides, and subsequent examination and counting by microscopy. This is still the essence of the majority of pollen monitoring activities across the Europe, where the pollen concentration of the air, is still the only routinely measured variable.

From an allergological perspective, the relevant measure is the received allergen dose. However; the pollen concentration is currently the primary readily available proxy on which information to the public can be based. The relationship between allergen content and pollen count is not uniform, as discussed in Chapter 2.

In Europe, national monitoring networks have been operating in most countries since the 1970´s (Scheifinger et al., 2013) and The European Aeroallergen Network (EAN) has collected and stored data since 1980´s (Galán et al., 2014). The monitoring is however, not performed by the same type of institution across Europe. The task is undertaken by e.g. universities, government or private organizations.

Standardization of the method is of great importance for potential comparison between sites, which is a useful application of data in research of e.g. spatial variations (Maya Manzano et al., 2017), the effect of local relief, climate, or production of models. A comprehensive standard-guideline was therefore developed by Galán et al for producing a set of minimum requirements for the set-up of future pollen monitoring stations and future Quality Control (QC) of pollen counts (2014), as an update to the previous standard by The European representatives of the International Association of Aerobiology (Jäger, 1995). The requirements specify e.g. placement of the trap at a flat roof, not near the edge, flowrate of 10 l min^{-1} and weekly control, type of adhesive, mounting media, staining fluid, counting method, training and quality control.

This chapter is focused on the method of pollen concentrations measurement applied in the included studies, and the potential errors introduced by this method.

Measurements of pollen concentrations

There are today many methods for measuring pollen concentrations in ambient air, some more applied in certain regions of the world.

The most widespread method for pollen sampling in Europe is the volumetric spore trap. The design was first developed by Hirst in 1952 (1952), and it is still the standard method applied in pollen monitoring across Europe (Cecchi, 2013). The majority of pollen monitoring is currently performed with traps from the producers Lanzoni, Burkard Scientific Ltd. or Burkard Manufacturing Ltd (Oteros et al., 2017;Scheifinger et al., 2013). Figure 6.1 shows images of the Hirst and Burkard traps. Air is sucked through a 2 x 14 mm orifice at a rate of 10 l/min and onto a drum rotating past the inlet at 2 mm/h. The drum is covered with transparent plastic tape coated with an adhesive. The rate of 10 l/min was chosen as it was the more optimal setting compared to higher speeds, where fine dust would more easily adhere, and efficiency was shown to go down (Hirst, 1952). The trap can be operated up to 7 days. The tape is subsequently cut according to 24h recordings, and mounted on glass slides, where a staining fluid is added for improved visualization of pollen characteristics. Single glass slides can also be directly mounted in the trap, for daily counting, applying an alternative slide assembly. The pollen are consequently identified and counted applying light microscopy, where magnification and counting method can vary slightly between countries. However the minimum requirements recommend at least 10% of the slide being counted at 400 x magnification (Galán et al., 2014). The count will always be a proxy for the true amount of pollen on the slide, and counting the whole slide is considered too time consuming.

The traps for continuous monitoring are placed at roof level, at approximately 10-20 m above ground. A wind vane assures that the orifice is continuously facing the wind.

Pollen concentrations are typically reported as bi-hourly or daily counts, and concentrations often categorized according to species specific intervals of "many", "moderate" and "few". For grass, "few" is typically less than 30 grains m^{-3} and "many" is more than 100 grains m^{-3}. For birch, "few" is less than 10 grains m^{-3}, and "many" is more than 50 grains m^{-3}. In the studies included in this thesis, the level of "many" is applied in the definition of "peak pollen days".

<u>Continuous pollen sampling in Denmark</u>

In Denmark, the continuous pollen monitoring has been performed by the consumer organization "Astma-Allergi Danmark" since 1977. There are two monitoring stations, one in Copenhagen at the roof of the Danish Meteorology Office, and one in Viborg, at the roof of the regional hospital (see *Manuscript I* and *II* for description of placements). Both are Hirst-type Burkard spore traps.

Pollen is identified and counted at 640 magnification on 12 transverse strips for every two hours, according to the method described by Käpyla and Penttinen (1981). A higher temporal resolution would introduce errors due to the rotation speed, the width of the orifice, and the airflow through the

Figure 6.1 The original Hirst spore trap (Hirst, 1952) (far left), and a Hirst type spore trap from Burkard; orifice and drum (center and right), currently applied for research purposes at Aarhus University.

inlet. The bi-hourly value is representative for the period of ½ hour before and after the timestamps for the count. E.g. the value registered at 01 is representative for the time 00:30 to 01:30.

Daily average pollen concentrations are expressed as grains m^{-3}.

The total area counted is 65.52 mm^2, which is 9.75% of the total area of the slide, equivalent to the number of pollen grains in 1.44m^3 of air. This is slightly less than the recommended 10% (Galan Soldevilla et al., 2007).

For the studies on Danish pollen counts included it this thesis, pollen counts have all been performed by Astma-Allergy Denmark and supplied as bi-hourly measurements.

Pollen sampling campaign in Aarhus 2009-2011

A pollen monitoring campaign was performed in the City of Aarhus, Denmark in 2009-2011 comprising of three traps within the city. The traps were placed at the roof of the school Rundhøjskolen, in the southern part of the city, in the city Centre at the Aarhus University´s

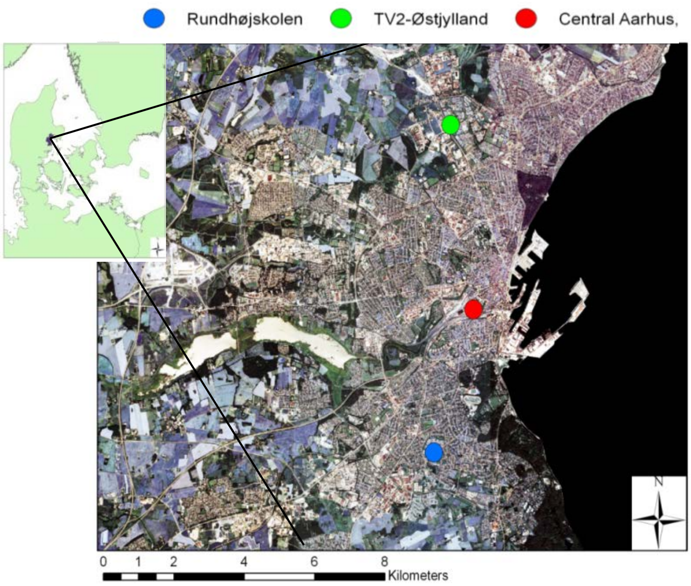


Figure 6.2 The placement of the three pollen traps in Aarhus city in 2010-2012.

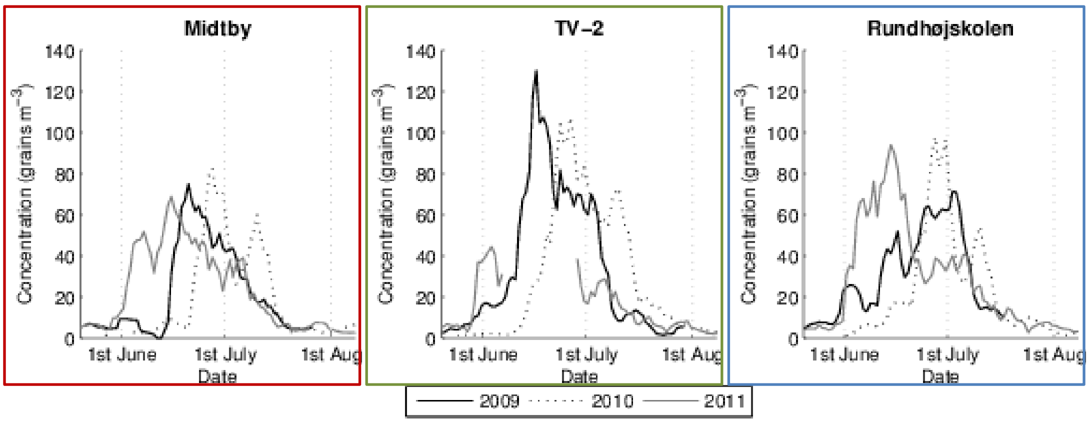


Figure 6.3 Daily grass pollen counts from three traps in Aarhus during a monitoring campaign from 2009-2011.

(Department of Environmental Science) urban background air quality monitoring station (Ellermann et al., 2007), and in the northern part of the city at the top of the TV2-Øst station building. Counts are shown in Figure 6.3. All three traps are within the urban area, however with different surroundings in close proximity (see *Manuscript I* and Figure 6.2). Due to technical problems, data is missing from 9th-24th June 2011 at the TV2 station.

Pollen monitoring in Cordoba

Cordoba city is located in the southern province of Andalucia, Spain. The city is located in the Guadalquivir valley, where the vegetation is primarily cereal and olive. The valley is surrounded by the Sierra Morena mountains to the north, and to the south the Sierra Subbeticas mountains (Hernandez-Ceballos et al., 2011).

The aerobiological studies in Cordoba University began in the 1980's. Since 1992 the aerobiology group in Córdoba has been coordinating the work in the "Andalusia Aerobiology Network" (Red Andalucía de Aerobiología - RAA) and the "Spanish Aerobiology Network" (Red Española de Aerobiología - REA). The group is also responsible for developing a standardized methodology for aerobiological sampling (Galan Soldevilla et al., 2007) and the Spanish participation the European Aeroallergen Network (EAN). There are currently a total of 47 Aerobiological monitoring units in Spain. Since the majority of the monitoring stations are in urban areas, a wide array of studies on ornamental plants are performed (Alcázar et al., 2004). The REA network also acts as a national pollen allergy prevention service, performing the pollen counts and the forecasts announced to the public.

The pollen monitoring in Córdoba is performed with a Lanzoni Hirst-type spore trap, placed north-west of the city at the Rabanales Campus of Cordoba University, where the aerobiology unit is located.

The Spanish Aerobiology network applies a method of four continuous horizontal sweeps across the slide with a microscope of 400 times magnification, resulting in a count of 12-13% of the slide (Galan Soldevilla et al., 2007). This results in an estimation of the daily pollen count. For producing hourly data the slide is divided into twenty-four 2mm sections, and a count recorded for every hour. The hourly

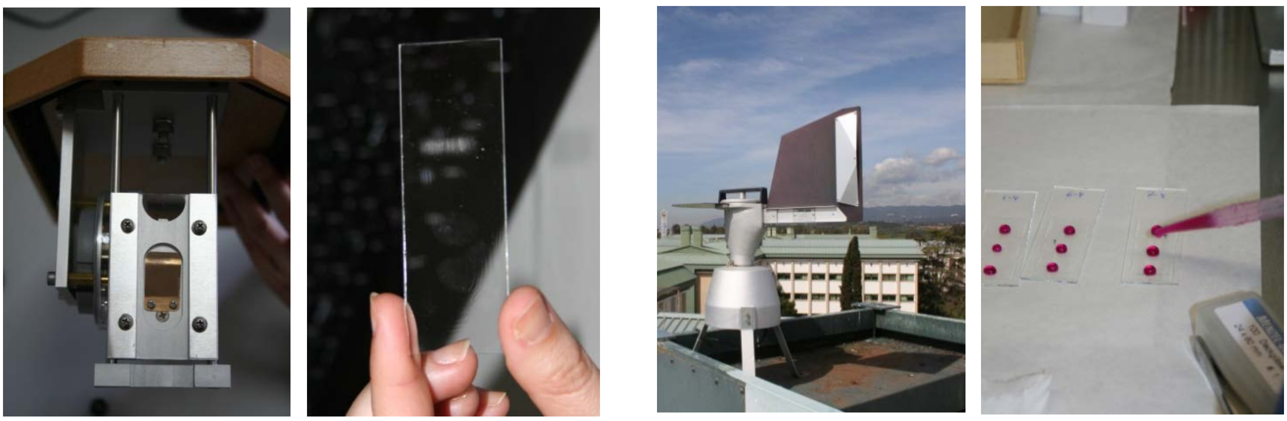


Figure 6.4 Pollen sampling in Córdoba- Left: Pollen sampling on slides applied in a two-hour sampling campaing on orange-pollen in Córdoba, one of the studies performed here on ornamental plants.
Right: The continous pollen monitoring station at the roof of Córdoba University where preparation wiht fuchsina staining and following counting is performed on site.

pollen counts are recorded on paper sheets, and daily pollen counts are digitized. For the study included in this thesis, analysis of the diurnal pattern therefore required digitization of hourly counts. The data entry program Epidata Entry[2] was applied for digitization of grass pollen counts from 2008-2011. Potential inaccuracy could have been induced by the higher resolution than bi-hourly counts, as described above. Data for the analysis included in this thesis, was re-calculated to 2-hour values. Quality control checks and re-calculation of data was performed in Matlab.

Potential errors and bias in pollen sampling and counting

As the method applying Hirst-type spore traps and light microscopy has been applied for nearly eighty years, several issues regarding factors affecting the precision have been examined, such as capture efficiency, monitoring conditions (placement, surroundings, altitude (Alcázar et al., 1999b)), counting method (and person counting), sampler errors and quality control methods (Galán et al., 2014;Oteros et al., 2013b;Oteros et al., 2017).

Recently, a comprehensive European technical standardization of the pollen monitoring applying the Hirst-type trap has also been developed for purchase (CSN P CEN/TS 16868; Ambient air - Sampling and analysis of airborne pollen grains and fungal spores for allergy networks - Volumetric Hirst method).

A recent study by Oteros et al identified a potential bias in the measurements, in relation to the calibration of the flowrate by use of a flowmeter, that potentially add resistance to the flow, to an extend where the flowrate is affected (Oteros et al., 2017).

In a test of 19 different traps, applying the supplied flowmeters from the three producers (Lanzoni, Burkard Scientific Ltd. or Burkard Manufacturing Ltd), the calibrated flow was compared to the flow during monitoring, measured by an electronic hot wire anemometer with negligible resistance. The mean differences were between 5% and 72% indicating a higher flowrate during monitoring than

Figure 6.5 Flowmeters applied in the calibration of the spore traps. Two of the applied flowmeters in Còrdoba, a Lazoni (left) applied at the operational monitoring station and a Burkard Manufacturing (right) applied in a campaign on olive pollen monitoring.

[2] http://www.epidata.dk/

during calibration, with the error depending on the individual trap and rotameter. No consistency in errors depending on manufacturer or rotameter was found, and therefore no uniform solution or correction factor can be applied. This study also only supplied the results on a single measurement, and emphasize that the error may also change over time. Future calibration of pollen traps should be continuously performed with a devise not introducing resistance as for measurements of PM and other air pollutants, per EU requirements (CEN, 2014).

The counting method applied in Córdoba of examination of four longitudinal traverses has been shown to give consistently lower estimates from the outer lines. This could otherwise be considered the most appropriate method, as it does not miss potential peaks at times between horizontal transverses. A study on the count from 718 days in Badajoz (Spain) revealed statistical significant difference between the amounts found in the outer and inner lines, with the outer lines containing on average 7% less grains for grass pollen (Molina et al., 1996a). Another study compared the count of the whole slide to estimated constructed by different counting methods, and found 16% less grass pollen by the method of four longitudinal lines, and 14% for the method of counting 12 transverse lines (Comtois et al., 1999), as applied by Astma-Allergy Danmark.
An analysis of the four years of grass pollen concentrations examined from Córdoba in this study, showed the two outer lines to be 3-13% lower than the inner lines, with line 1 consistently being the lowest (average 12%). The highest difference was found in the season with the lowest total load, and the lowest in the season with the highest total load. This is consistent with previous findings (Comtois et al., 1999;Molina et al., 1996a), indicating that at lower concentrations more traverses may need to be counted to achieve same accuracy as in slides with more grains.

The procedure of manual pollen counting is highly time-consuming, introduces a delay and is expensive due to the high labor intensity. Automated counting is therefore a great focus area, with methods being developed applying e.g. multifocal microscope images and pattern recognition software to traditionally sampled slides, fully integrated systems with microscopy, laser light particle counters applying scattering and fluorescence measurements (Scheifinger et al., 2013). However none of the methods have yet become a widespread replacement for the current Hirst-type sampling and manual pollen counting.

As the samplers themselves have been shown to introduce variability, several studies have focused on comparing samplers located at the same site, e.g. Tormo Molina et al. (2013), who compared four traps at ground level and found both daily and hourly concentrations to be significantly correlated, however with a lag between peak of several hours between samplers. Velasco-Jiménez also found no significant differences between two samplers at the same site, when comparing daily pollen counts(Velasco-Jiménez et al., 2012). They did however, not examine the hourly counts. Several other studies of multiple samplers at the same site, have also come the conclusion, that the samplers measure similar daily counts (Irdi et al., 2002;Pedersen and Moseholm, 1993).

In future studies, measurements of pollen should also be accompanied by measures of allergen amount. In Europe the HIALINE (Health Impacts of Airborne Allergen Information Network) has performed an intensive work on allergen measurements from 2009-2012 (Buters et al., 2010;Buters et al., 2015;Buters et al., 2012;Galan et al., 2013), where the aerobiology group in Cordoba also participated . Measurements of allergen concentrations in the ambient air were performed with a Chemvol® high-volume cascade impactor, where air was sampled at 800 l min^{-1}, and aspirated through a filter for PM > 10 µm, and PM 2.5- 10 µm (Figure 6.6).

Although the here described method of pollen measurement is associated with a wide range of potential errors and bias, the method has the strong advantage of being mechanically simple and robust, and therefore reliable. It has also been applied in 30-40 years at many sites, and been applied in producing data for long time series.
Pollen counts applied in the studies included in this thesis, have all been measured fulfilling the minimum requirements (except the 9.75% counted in Denmark not being above the recommended 10%). However different samplers, flowmeters and counting methods could have induced variance between the concentrations, not related to true differences.

Future studies of the data could include examination of the bias introduced by the flow calibration for each trap and applied flowmeter. For counts from Córdoba a complete analysis of the variance between the four counted lines could be performed to examine the potential effect of counting four transverse lines, and for Denmark additional area could be counted, especially for days with lower counts as the precision will be less here.

Figure 6.6 Collection of samples for measurement of allergen content in Córdoba for the Hialine project.

Chapter 7

The exposure study

The exposure study described in this thesis was initially set up as a pilot study for a subsequent larger study to be conducted on co-exposure to pollen grains and ozone. The study was performed under the A3-Start-up-initiative at Aarhus University – “Assessment of Airborne Allergens”.

The study described in this thesis is therefore a pilot project, with the objective of assessing not only outcomes, but also the methodological set-up for future studies.

The study and all applied methods and materials were approved by the Regional Scientific Ethics Committee for Central Denmark (M-20090215). The trial was performed in accordance with the Helsinki Declaration.

Each participant signed a written consent form and received a monetary compensation of 750 DKR per study day.

We measured a large number of variables during the study. However, only the bronchial response has been analysed. In this chapter the methods of the overall set-up, SIC´s and assessment of bronchial response will be critically evaluated.

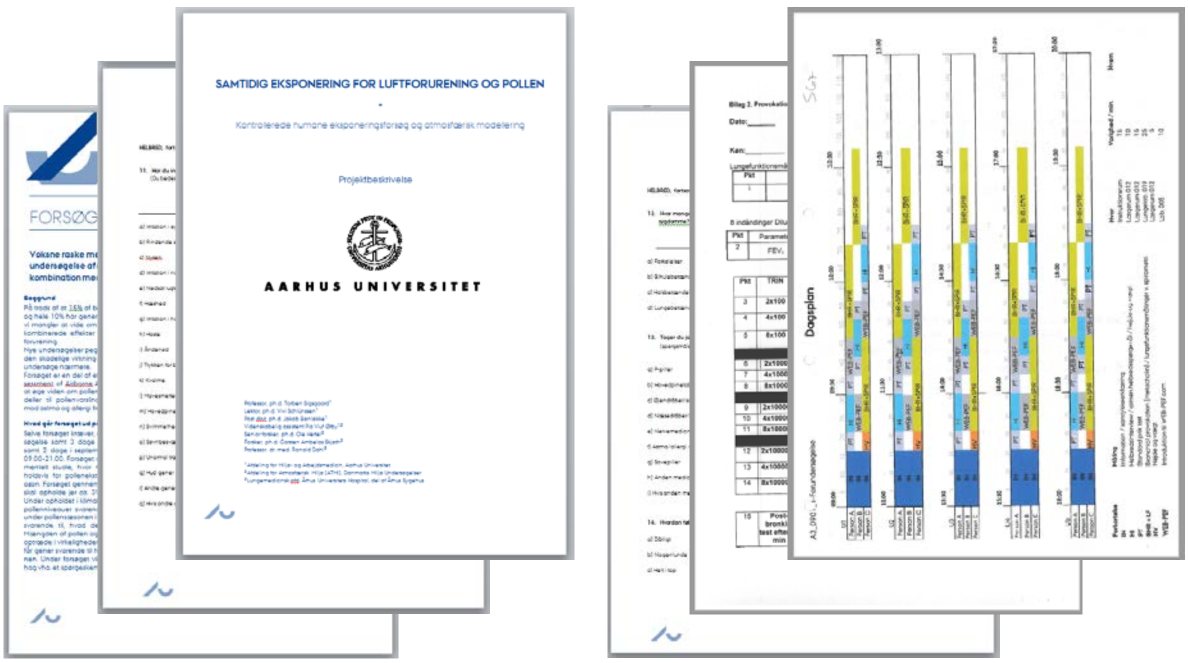


Figure 7.1 Examples of the documents applied in the clinical study; Application for the Ethics Committee, participants-bulletin, health questionnaires, spirometry scheme and challenge day-plan.

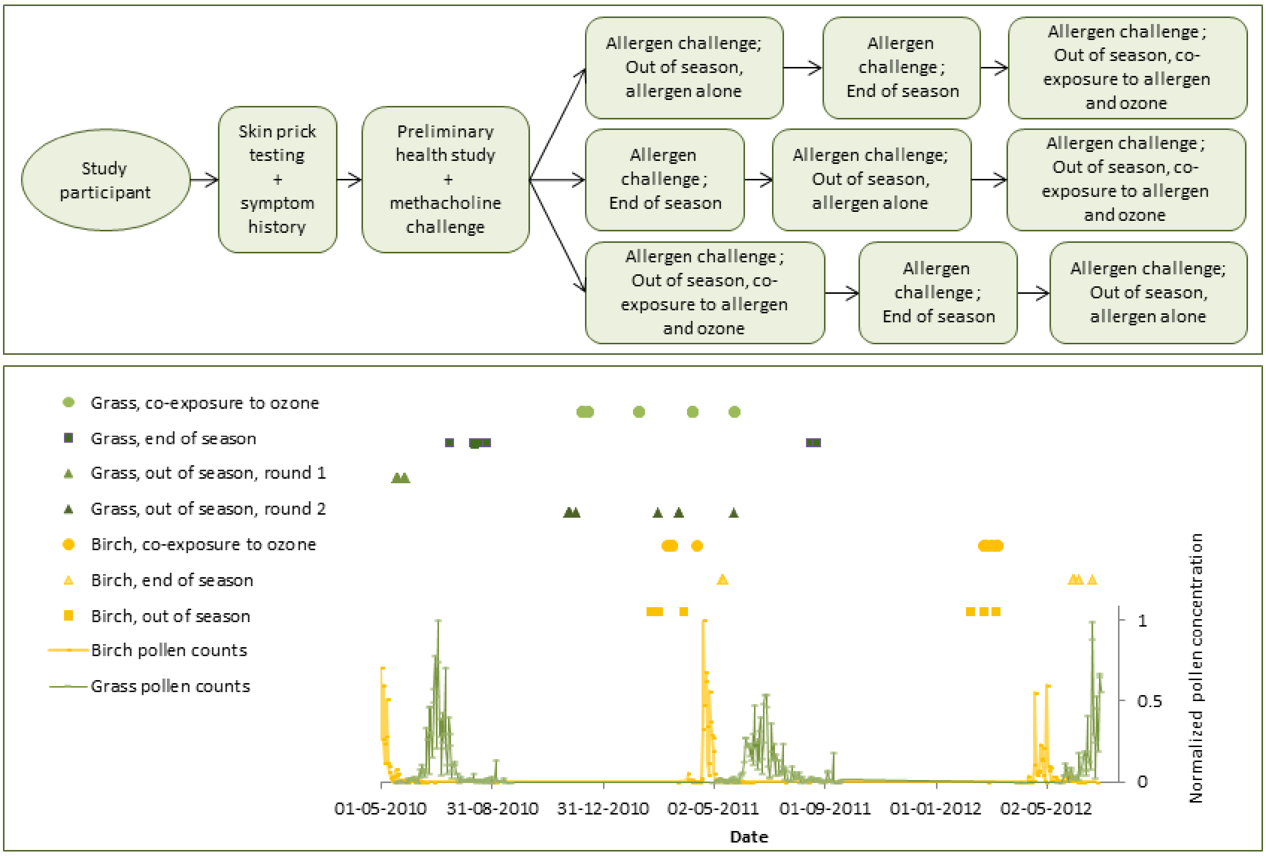


Figure 7.2 Study design (above), and timeline indicating the timing of the specific inhalation challenges, as well as the normalized birch and grass pollen concentrations during the study, measured at the operational trap in Viborg (below).

Study design

The study was set up to investigate two different objectives; Co-exposure to ozone and seasonal priming effect. Challenges were performed with and without ozone, outside the pollen season, and without ozone at the end of the pollen season. Figure 7.2 shows the study design and timing of the SIC´s and the pollen season during the study period.

The study participants were recruited among students at Aarhus University, and allergy was confirmed by SPT and symptom history. Interested eligible participants were hereafter invited to an information meeting, including preliminary examination and non-specific inhalation challenges.

The initial study round (1) enrolled 13 grass allergic participants, who were challenged outside the pollen season. A second enrollment round recruited additional 7 grass participants and 17 birch participants.

The study was designed as a case-cross-over study, where participants were their own controls. The order of challenges were determined by when the participants were available to attend, and therefore not in a specific order, however neither perfectly randomized. The challenges were planned to be performed a minimum of two weeks apart to exclude carry-over effect. However, due to illness, the “out-of-season” challenges for two participants were moved, resulting in challenges with co-exposure to ozone being performed only one week after the previous challenge (ID 105 and 133).

For the study of co-exposure to ozone, the birch challenges are on average 18 (sd.7) days apart and the grass on average 169 (109) days apart. The high interval is due to the time between "out-of-season-" round 1 and 2. If only round 2 is considered, the average interval is 36 (37) days. For the study of the priming effect, the birch challenges are on average 90 (28) days apart and the grass challenges are 196 (57) days apart. Again, if only the nearest "out-of-season"-challenge is considered, the intervals is 103 (35) days.

The majority of study days comprised of a morning- and an afternoon-session, with 1-4 participants at each session.

<u>The environmental exposure chamber</u>

The study was performed in an environmental exposure chamber (EEC) at Aarhus University, where a controlled ozone exposure was administered and all environmental conditions were kept uniform throughout the study.

The chamber system comprises of a main chamber of 5.4 * 5.4 * 2.7 m (length, width, height), and a smaller chamber of 2.92 * 4.15 * 2.70 m connected by a hallway, also connecting to toilet facilities. The chamber has been applied in e.g. studies of wood smoke (Riddervold et al., 2011) and later in an experimental set-up with whole pollen (Kenney et al., 2016). The chambers are made of stainless steel with rounded corners. In the center of the room a table and chairs were placed for the participants to sit and work by during the time in the EEC.

Participants and examiners wore shoe covers and disposable coveralls while in the EEC to avoid contamination. Following the challenges, participants were seated in adjoining facilities for three hours awaiting concluding measurements and performing hourly FEV_1-measurements.

The initial 13 "out-of-season" grass SIC´s, were performed in the smaller chamber, with participants seated in the large chamber between challenges.

The chambers were provided with filtered air, and airflow, temperature, humidity and CO_2 were measured every 60 sec. The temperature during all study days were 22.5°C (std. 0.5°C) and the humidity 42.0 % (std. 2.6%).

<u>Ozone exposure</u>

The ozone was produced with pure oxygen using an ozone generator from BMT Messtechnik model BMT802. The generated ozone entered the chambers through the ventilation system. The ozone output of the generator was continuously adjusted according to the readings made by the detection system. The ozone concentration detecting was performed by an API (Advanced Pollution Instrumentation, Inc , ser.no 1058) UV-absorption instrument (254nm), reading the concentrations in a sampling loop from four different locations in the chamber. The average ozone level throughout the study was 120.9 ppb (std. dev. 1.8 ppb).

Pollen seasons during the study

The grass pollen seasons of 2010-2011 and birch pollen seasons of 2011-2012, during the period of the EEC study, was compared to the 30-year average seasons (Sommer and Rasmussen, 2010;Sommer and Rasmussen, 2011;Sommer and Rasmussen, 2012). In 2010, the weather at the time of average grass flowering was colder than normal, resulting in a week later onset of the season. The season was shorter than normal, however more intense, and slightly higher. In 2011, the onset of the pollen season was delayed by a cold and long winter. However, the spring was warmer than normal, and the birch season started 3 days early. The season was slightly below, but near normal, short and intensive. These lower levels could be due to westerly winds, crossing an area with few birch trees around the time of season peak. Due to the sunny spring, grass season began 4 days early. However, above average rain during the summer resulted in slightly lower than normal season. In 2012 a warm, sunny and dry march resulted in an abrupt onset of the birch pollen season. Due to an error at the monitoring station, there was a gap in measurements from Viborg around the season peak, however overall seasonal progress was similar to Copenhagen, indicating a season slightly above normal, but ending early due to cold wet weather in early May. For visualization of the seasonal progression, normalised pollen levels are shown in Figure 7.2. One "end-of-season" study-day was three weeks after the end of the pollen season due to unfortunate planning issues, and the abrupt ending of the season.

The yearly norm is listed by the Asthma Allergy Association for Copenhagen only. However, similar grass pollen counts are usually measured at the two sites, and it is therefore comparable. The current norm is 2200 pollen. For birch the norm from Copenhagen is 6037 pollen, however, this is not comparable to the site in Viborg, since a birch forest south-east of Copenhagen is producing high pollen concentrations in the city. The yearly total for Viborg was 3585 birch pollen in 2011, and not provided for 2012 due to the gap in measurements. For grass it was 2278 pollen in 2010 and 2097 in 2011.

Participants

A total of 92 potential participants were interviewed on symptom history and had SPT performed during the recruitment rounds. SPT included grass, birch, artemisia, horse, dog, cat, house dust mites (HDM; Dermatophagoides pteronyssinus and Dermatophagoides farina) and fungal spores (Cladosporium herbarum and Alternaria alternaria). Inclusion criteria was a weal size of minimum 3 mm for either or both birch and grass allergen, and a history of AR symptoms during these pollen seasons.

None of the participants had been exposed to tobacco smoke, had recent infections, used asthma medication, or had used antihistamines within 72 hours prior to the SIC. Baseline FEV_1 was calculated before each SIC, and required to be higher than 70% of predicted. Mean % of predicted Baseline FEV_1 for each participant was calculated with the "GLI-2012 Desktop Software for Individual Calculations" (Philip H.Quanjer et al., 2014) based on age, sex and height. See Appendix II. Following all preliminary examinations and information meetings, a total of 36 participants were enrolled in the study.

	Grass	*Birch*
Participants (n)	*19*	*17*
Gender	*F 10* *M 9*	*F 10* *M 7*
Weight	*F 68 (13), M 77 (8)*	*F 70 (14), M 86 (16)*
Height	*F 169 (5), M 183 (8)*	*F 169 (6), M 179 (6)*
Age	*24.2 (2.7)*	*24.4 (2.3)*
*Wheal size , SPT mm**	*9.4 (3.2)*	*6.5 (2.5)*
Number of positive SPT (min. / max.)	*3.1 (1/6)*	*5.9 (4/8)*
% Predicted, Baseline FEV$_1$	*F 99 (14), M 86 (12)*	*F 97 (12), M 96 (12)*

Table 7.1 Study participant characteristics, mean (std. dev.). F= female, M=male. * Wheal size of SPT for the allergen applied in the SIC (grass or birch).

Based on previous studies in the climate chamber and literature on the number of participants needed to detect a change in $PD_{20}FEV_1$ after exposure, above 10 participants should be sufficient power (Devalia et al., 1994;Jenkins et al., 1999).

All included study participants underwent a clinical examination before and on study days to insure that they had no other relevant health conditions.

Participants allergic to grass and with a weal size of minimum twice the weal for birch were included in the study to be challenged with grass allergen. Participants with a weal size for birch above 3 mm and larger than 0.5 times that for grass, were included to be challenged with birch allergen. No potential participants were mono-sensitized towards birch. Focus was to minimize the risk of pre-priming grass-participants by birch before the grass season, therefore grass allergy in birch participants was not considered an issue. Even so, two included grass participants also had a positive SPT to birch.

Average characteristics of the participants are listed in Table 7.1 and a complete list of individual characteristics and dates of challenges are shown in Appendix II.

No significant difference between excluded (birch, n: 23, grass, n: 32) and included SPT tested participants was found for the weal size of the skin prick test (birch, p =0.74 / grass, p = 0.57).

SPT results indicated multiple allergies, including sensitizations towards perennial allergens HDM in the 14 of the participants included in the birch-study and 7 of the participants included in the grass-study, inducing a potential issue of perennial pre-priming.

Initial interview information on self-reported asthma and airway symptoms was registered. Twenty of the 36 included participants reported the presence of respiratory symptoms more frequent than a few times a year (morning cough, wheeze, or chest tightness). Of these, 10 reported mild asthma, hereof 7 with symptoms more than a few times a year.

Measured variables

SPT were acquired at the recruitment sessions. During the initial health examination, non-specific challenges and basic health examinations were performed, and a basic health questionnaire was filled in. On days of challenges, a medical examination was performed, a questionnaire was filled in, and a blood sample taken before the SIC´s. Following this, an examination program was performed, which were repeated immediately after and again approximately three hours after the SIC´s. Figure 7.3 shows photos from selected examinations during the study days. The examination program consisted of the following procedures: NO in expired air, questionnaire on overall well-being on 29 variables of symptoms, nasal lavage, and acoustic rhinometry.

All participants were also instructed to indicate general level of irritation on a potentiometer every 30 minutes during the time in the ECC. Lung function was measured every 15 minutes before, during the SIC´s, and the first hour after. Following this, the participants were instructed to measure every hour until bedtime, however, these measurements were however not consistently performed by all.

Figure 7.3. Measurements during the clinical study involved nasal lavage (a), questionnaires to be filled on laptops (b), indication of general level of irritation on a potentiometer (c), acoustic rhinometry (d) and measurements of NO in expired air (e and f).

The blood sample and nasal lavage fluids were analysed for levels of leucocytes.
The acoustic rhinometry measured the minimum cross sectional area in the nose on both sides and distance to this area and volume in three positions.
Each participant were in addition given a personal spirometer to take home, and asked to measure FEV_1 during the pollen season, a minimum of three times per day.
For the studies included in this thesis, analysis of specific and non-specific bronchial response and SPT was performed.

Non-specific inhalation challenges

Non-specific inhalation challenges were performed with a maximum cumulated dose of 4.51 mg Methacholine bromide. Undiluted fluid of 167 mg/ml of Methacholine bromide (Aarhus Hospital Pharmacy) were applied, and a NaCl 0.9% solution as dilution fluid.
FEV_1 was measured with a handheld MicroDL spirometer connected to a computer. Baseline FEV_1 was measured after inhalation of diluent as the highest of 3 consecutive measurements. If the FEV_1 was above 60% of predicted, a saline solution were administered as a control. The highest of 3 measurements hereafter was noted as baseline FEV_1, and Methacholine bromide was administered in increasing doses. FEV_1 was measured 1 minute after each dose, and if the fall was less than 20% of the baseline value, the next dose was administered. This method was followed until a drop of more than 20% in FEV_1 were reached, or the highest dose was administered. If the participant at any time felt unexpected discomfort, the test was ended and a bronchodilator (Bricanyl Turbohaler) was given. FEV_1 was tested after 15 min to confirm that the lung function was improving.

Specific inhalation challenges

SIC´s were performed with aerosolized allergen administered by a SPIRA dosimeter nebulizer, with an output of 95% of the allergen as particle drop sizes <10μm, i.e. main deposition in the thoracic region (DS - Dask Standard, 1994;Heyder, 2004). The procedure during SIC´s were as described above for non-specific challenges, however with 15 minutes between steps. The SICs were performed according to the procedure listed in Figure 7.4. Allergen solution was produced from extract from ALK containing 100.000 SQ-U/ml. The grass extract contain 20.2 μg of Phleum pratense (Phl p 5) per 100.000 SQ-U, and the birch extract contain 12.3 μg of Betula verrucosa (Bet v 1) per 100.000 SQ-U (Larenas-Linnemann and Cox, 2008). Solutions of doubling doses from 1.4 – 5600 SQ-U were applied. International recommendations are that future references of allergen be based on micrograms of the major allergen (Larenas-Linnemann and Cox, 2008).

To limit the exhalation of allergens into the chamber air, extra ventilation was installed near the dosimeter, and participants were instructed to exhale in an extraction device.

		Baseline	Level 1 Dilution 100 SQU			Level 2 Dilution 1,000 SQU			Level 3 Dilution 10,000 SQU			Level 4 Dilution 100,000 SQU			
Step	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Conc.	*	Diluent	2×100	4×100	8×100	2×1,000	4×1,000	8×1,000	2×10,000	4×10,000	8×10,000	2×100,000	4×100,000	8×100,000	Bricanyl
Dose, SQU			1.4	2.8	5.6	14	28	56	140	280	560	1400	2800	5600	

Procedure based on % drop in FEV1, based on baseline measurement after diluent (step 2)	
< 5 %	Next level – skip two steps., If < 5 % drop at level 12, no response is assumed and the provocation ended.
> 5 – 15 %	Next step.
>15 – 20 %	Repeat same step, maximum 3 times. If still < 20 %, next step is administered.
> 20 % or unacceptaple discomfort.	Disrupt provocation and proceed to administer bronchodilator (Bricanyl).

Figure 7-4 Procedure for the SICs. The administered dose was dependent on magnitude of the drop in FEV1 induced by the previous dose, resulting in an administration of either repeated dose, or an increase of either one step or one level.

The protocol was developed for clinical application of diagnosis, and allowed for individual procedures of administered doses to be followed, instead of a fixed procedure for all. This has also previously been seen in e.g. Rosenthal et al (Rosenthal et al., 1975). Different starting points have also been suggested based on the end-point concentration of the SPT and the PC_{20} histamine (Barnig et al., 2013;Cockcroft et al., 1987;Diamant et al., 2013;Melillo et al., 1997).This is a clinical consideration to account for the risk involved in administering a high dose in highly allergic participants, or too many low doses in non-responsive participants, inducing fatigue. However, it complicates later statistical evaluation of results, and it should be underlined that the administration of dosages will greatly affect many of the measured outcomes.

In this study we assume the allergen dosages administered with 15 min intervals to be cumulative, as in other studies, e.g. Kehrl et al, who administered the same dose with 10 minute intervals (1999). A cumulative effect of doses is thus implicit in the protocol, as it allows for the administration of repeated doses when the fall in FEV_1 is above 15%. Bronchial allergen challenge in asthmatics have previously been shown to result in bronchoconstriction within 10-15 minutes after exposure, reaching maximum after 15-30 min and resolving within 1-3 hours (Weersink et al., 1994). We can therefore not assume that the full effect is neither still present nor whether it is achieved after 15 minutes.
The effect of the cumulated dosages could also be expected to be different if administered in one dose (Liu et al., 2003a), and is therefore not to be considered equal to a one-dose administration. Whether or not the doses could be considered as individual administered concentrations, or as cumulated doses, is therefore not completely certain.

A total of 121 SIC´s were performed. Of these, 22 SICs performed on 11 different participants are not finalized with either 20% drop in FEV_1 or the maximum dose administered. Of these, two were ended due to severe discomfort (ID 138 and 148), all other was due to the protocol, assuming no response to allergen at a less than 5% drop at 1400 SQ-U. It should be noted that, we do not know whether this would have been the case if the maximum dose had been administered. Five of the participants only experienced a very minor response in all 16 SICs (3 grass and 2 birch), and for 6 additional SICs (5 grass and 1 birch) a minor response were seen. It is not possible to predict whether these would have dropped below 20% if the maximum dose had been administered, however it is clear that potential information was lost due to this part of the protocol.

In future studies the maximum dose should be high enough to elicit a response in all sensitized subjects, as also suggested by Mellilo et al (Melillo et al., 1991).
All SIC´s are shown in Appendix IV, as non-modelled log-dose response curves.

Analysis of bronchial response during inhalation challenges
As described in Chapter 4, the EAR is typically described as the PD_{20}. This value is consistently derived from linear interpolation between the last two allergen doses administered, and therefore solely dependent on these doses and the response at this point in the challenge. However, if the dosed had been administered with different increments, the two final points may have been different. If the entire dose-response profile is viewed, the shape is also rarely linear on this scale (see measured points and modelled curves in Appendix IV and V). In this study we therefore chose to apply a self-starting four-parameter non-linear regression model to the dose-response profiles. This model allows for all measured responses to be included, affecting the final PD_{20} estimate.
As previously noted, we did not have a measure of LAR for all our participants. We could therefore not make a systematic analysis on the response pattern including the LAR. However, for those who did record the FEV_1 for above 3 hours following the SIC´s, both participants with and without LAR was seen, indicating that our study group may consist of different endotypes of response patterns.
There may also have been an effect of the application of bronchodilator on the LAR. As the LAR was not analysed, this was not further examined.

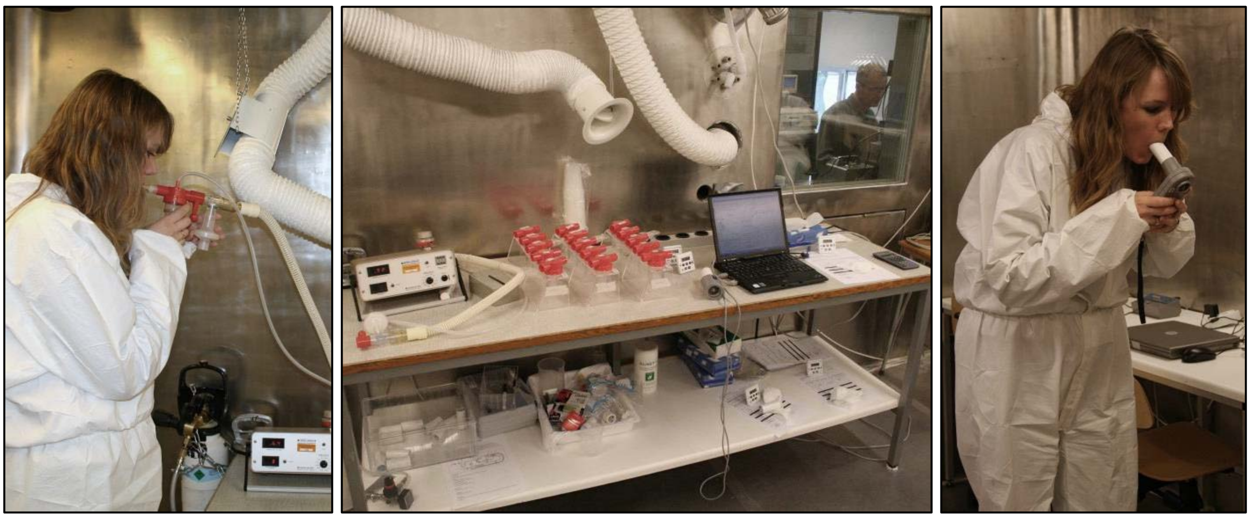


Figure 7.5 Set-up for the SIC´s. Aerosolized allergen solutions were administered with a dosimeter, and the bronchial response measured with a spirometer coupled to a computer.

Statistical analysis

All analyses were performed in the R- programming environment for Data Analysis and Graphics, Version 3.2.3 (Pinheiro and Bates, 2006). The applied non-linear regression model was programmed using the Linear and Nonlinear Mixed Effects Models, nlme package. Both methacholine and allergen dose-response curves were modelled and PD_{20} estimates extracted.

The nmle model: $$Fev1(x) = a + \frac{b-a}{1-exp^{\frac{x_{mid}-x}{scal}}}$$

Where x is the cumulative dose, and *a*, *b* and *scal* are model parameters.

- a is an estimate for the horizontal right side asymptote, ie. an estimate of FEV_1 at the lowest possible dose.
- b is an estimate for the horizontal left side asymptote, ie. an estimate of FEV_1 level at highest possible dose. We do not have data entries at this point, and the question is whether we should set b to 0, as it is the lowest FEV_1 possible. However, the question is whether it is reasonable to assume that FEV_1 will drop abruptly until 0, or wether it would fade off at some low level.
- x_{mid} c is the infliction point of the curve, ie. the value mid-way between a and b. In our study, this is logarithm to the cumulated dose eliciting a drop in FEV_1 of half of the difference between the maximum and the minimum FEV_1, where the minimum is unknown.
- d is a scaling parameter, estimating the shape of the cuve, ie. how compressed the form is. D is high if the curve has a steep shape and low if the curve is very flat. In our data, d is estimated as between 0 and 1, most often closer to 1 than 0.

All model parameters must be estimated as an entry for the model, hereafter the model will create a predefined number of iterations around these values to find the best fitting estimate. The estimates are found by investigating the output scaling parameters and their 95%-CI intervals, and by plotting data for visual estimation. If the entry point is not proximate to a possible end value, the model will not run or give a good fitting result. The dose-response curves were estimated by 1000 simulations around the mean curve for a total of 400 points.

Modelling non-specific bronchial hyperresponsiveness

Fourteen of the 36 participants had a more than 20% reduction in FEV_1 during the non-specific challenges. Applying the model calculations, an estimate of PD_{20} was calculated for all participants. The combined methacholine PD_{20} estimate based on measured response from all participants was 4.4 (95%-CI: 2.8; 6.8) mg methacholine bromide. No systematic errors in the residuals were seen. Individual PD_{20} values are shown in Appendix II and methacholine dose-response curves are shown in Appendix III.

Modelling specific bronchial hyperresponsiveness

No statistically significant difference in PD_{20} were found between the two periods of SICs with grass allergen alone outside the pollen season (p=0.66), and they were therefore combined for the purpose of

modelling log-dose response curves and extracting PD_{20} estimates for analysis of the potential priming effect and effect of co-exposure to ozone.
Individual dose-response curves are shown in Appendix IV for all study periods and modelled dose-response curves are shown in Appendix V for each of the two analysis; co-exposure to ozone and priming effect. Chapters 10 and 11 presents the results on the SIC´s

Associations between PD_{20} and baseline characteristics
As stated in Chapter 4, the response to allergen is potentially related to individual characteristics as e.g. the concentration of specific IgE, baseline lung function, the non-specific bronchial responsiveness, and the number of sensitizations. As proxies for these, the association between the size of the SPT, the baseline FEV_1, the Methacholine PD_{20} and the number of positive SPT´s were estimated. As no statistical significant difference was seen between any categories of the PD_{20} estimates (with/without ozone and season/out of season), the analysis was performed on a combined datasets for each of the analyses.
Associations are calculated as simple linear regression, and given as the beta-estimate (95%-CI) in Chapter 10 and 11; $\log(PD_{20}) = \text{alfa} + \text{beta} * \log(x)$

Evaluation of the method applied in the pilot exposure study
The planning of the study was not optimal, and two participants were therefore not challenged until three weeks after pollen season. The timing of challenges can be difficult, and allowances may have to be made. However this could have induced an error in the estimation of the priming effect. Also, the two challenges only one week after a prior challenge were a result of planning difficulties, however, a closer look on the response curves for these challenges, indicated that the following challenges did not induce an increased response.
The order of the challenges in this study was not random, which could affect results. A view on the dates of the challenges revealed, that for challenges included in the study of co-exposure to ozone, all but three of the birch challenges with co-exposure to ozone were after allergen alone. For grass the same occurred, with all co-exposure to ozone challenges after the allergen alone challenge, however three had an additional allergen-alone challenge after the co-exposure to ozone. For the challenges included in the study of the priming effect, all seasonal challenges follow after the out-of-season challenges. However, for the 6 grass participants with an additional out-of-season challenge, this is performed after the two other. Order effect was therefore not possible to investigate.
The intervals between the challenges were not identical for the study participants. This can be difficult to achieve due to other considerations. However, a large difference in intervals could induce confounding factors. Some difference is due to the differences in the duration of the season, and could not be avoided. However, the difference in the timing of the challenges included in the study of co-exposure to ozone, may have affected the results. Future studies should ideally be planned with similar intervals between exposures for all participants.

It was not possible to recruit only mono-sensitized participants in this study. All but five grass-participants were multi-sensitized. Pre-priming may therefore have occurred by either exposure to HDM or in the two grass participants sensitized to birch. Pre-priming could also have been induced by homologues pollen types, which is primarily problematic for birch allergic participants, since many of the spring flowering trees have pollen with allergens that are homologous to birch and often induce cross-reactivity (Rantio-Lehtimäki, 1995;Valenta et al., 1991). The standard SPT applied in this study, did not test for sensitization towards any of the other spring flowering trees. However, none of the participants listed allergies towards these. Although no symptoms are experienced, and therefore no allergy reported, pre-priming could still occur if sensitization is present, since even exposure to low doses of allergen, not eliciting symptoms, could cause bronchial inflammation and increase responsiveness (Ihre et al., 1988;Ihre and Zetterstrom, 1993).

The participants in this study had AR with no or mild asthmatic symptoms. The mixed asthmatic status of the participants is both a strength and a weakness of the study. The group has not previously been studied as intensively as allergic asthmatics, and constitutes a larger patient group. However, it is also a group that may be too heterogeneous in bronchial response, for differences elicited by other factors (ozone/priming) to be evident.

Non-specific bronchial inhalation challenges were initially performed as an indicator for disease severity and as control for severe hyper responsiveness. Future studies could benefit from applying this measure as a general outcome, planning the non-specific challenges to be performed both before and after SIC.

The analysis of the associations between allergen PD_{20} and baseline characteristics was performed on the modelled Methacholine PD_{20}. However, with only 44% of the challenges inducing an above 20% fall in FEV_1, this measure is not as well founded as for the SIC´s. It cannot be excluded that the application of the traditional measure of log-dose-slope therefore would have been a more appropriate method.

The design of the provocation protocol steps and allergen doses can affect the resulting outcomes greatly. Whether or not all is given identical doses, and the magnitude of these doses, affects the possibilities for analysis methods, as well as the results. Larger single doses will not induce the same response as smaller increments in multiple doses (Liu et al., 2003b). Future studies should consider administering the same protocol for all, although this implies a risk of fatigue in those tolerating high doses. Also the procedure of not administering the maximum dose for those not responding to the lower doses, introduced potential missed response in these.

The lack of LAR-measurements in all challenges induced the lack of possibility for analysis of the response pattern of EAR and LAR, and therefore excluded the option of potential differentiation based on this. Future studies should all be performed from the morning, in order for LAR measurements to be recorded for all.

A study of patients experienced symptoms, showed that a ranging put the nasal symptoms as most severe, followed by ocular and then airway symptoms (Karatzas et al., 2014).
In this thesis, the focus was on the analysis of the bronchial response. With the allergens being administered though oral inhalation, in a size fraction compatible with deposition in the thoracic region, the majority of allergen could be expected to be deposited here, causing local histamine release and bronchial muscular response. General wellbeing and nasal symptoms could however also have been affected, which may be evident in the additional data from the study. It was however not within the scope of this thesis to analyse these additional measures.
Since the bronchial symptoms generally is not considered the most frequent, nor the most severe, by AR patients, the effects of the priming may have been more apparent in other symptoms.

The study did not include any placebo-challenge, nor did we include a non-atopic control group. Both would have required a much larger study, but could have added information to the results.
As this study was considered a pilot-study, where the objectives also included evaluation of the methods for future similar studies in the ECC, methodological issues was to be expected. Most of the above mentioned issues could be improved in future studies, however the optimal set-up is rarely possible, and some elements of potential errors will inevitably be induced.

In this study we applied allergen extract, not pollen grains, and the results are difficult to transfer to the measured pollen concentrations experience in a natural setting. Pollen grains also contain other elements that may affect the response, e.g. lipid mediators (de Weger et al., 2013), further complicating the comparison between natural exposure and the current studies.

<u>Advise for a future study on the bronchial response to specific inhalation challenge</u>

Based on the preceding statements, the following should be considered in a future similar study.

- Similar time intervals between challenges for all, and random order.
- Administering the highest dose to all, unless a 20% decrement in FEV_1 is achieved. The highest dose should be estimated to elicit a 20% response in all.
- Measure LAR.
- Potentially add non-specific bronchial measurement the day before all allergen SICs, to give estimation on the current state on bronchial responsiveness. Some measures have also been seen to be better reflected in non-specific responsiveness.
- Apply the here presented non-linear model to fit the log-dose-response curve and for PD_{20} estimates. However, consider applying log-dose-response slope, if the proportion of non-responders is high.
- Attempt to recruit mono-sensitized participants, if not possible, preferably no perennial allergies. Consider testing for spring tree sensitization.
- Consider including a healthy control group or a placebo challenge.

Part IV – Main results and discussion

Chapter 8

Local scale variation in grass pollen concentrations

Grass is the most ubiquitous plant we have in Denmark, also in urban areas where flowering grasses can be found both in and around the city centers. The pollens are large (30-60 μm) and released at low height, both factors affecting the dispersion pattern, and resulting in high concentrations near the sources and greater spatial variation than seen in e.g. the smaller and higher released birch pollen.

This study therefore sought to examine the following two objectives;

- whether there are large intra-urban variations in grass pollen levels,
- and whether these could be linked to variations in the source distribution.

For this study, data from 2009 from the pollen sampling campaign in Aarhus described in Chapter 6 was applied. Additional methods included the mapping of potential grass pollen source areas applying Remote sensing and GIS, and linking these to the pollen counts. The overall method is described in this chapter, whereas all additional details on applied methods are described in *Manuscript I*.

The application of geographical information systems is determination of source distribution

The mapping of potential grass pollen sources was achieved by a series of map classifications and

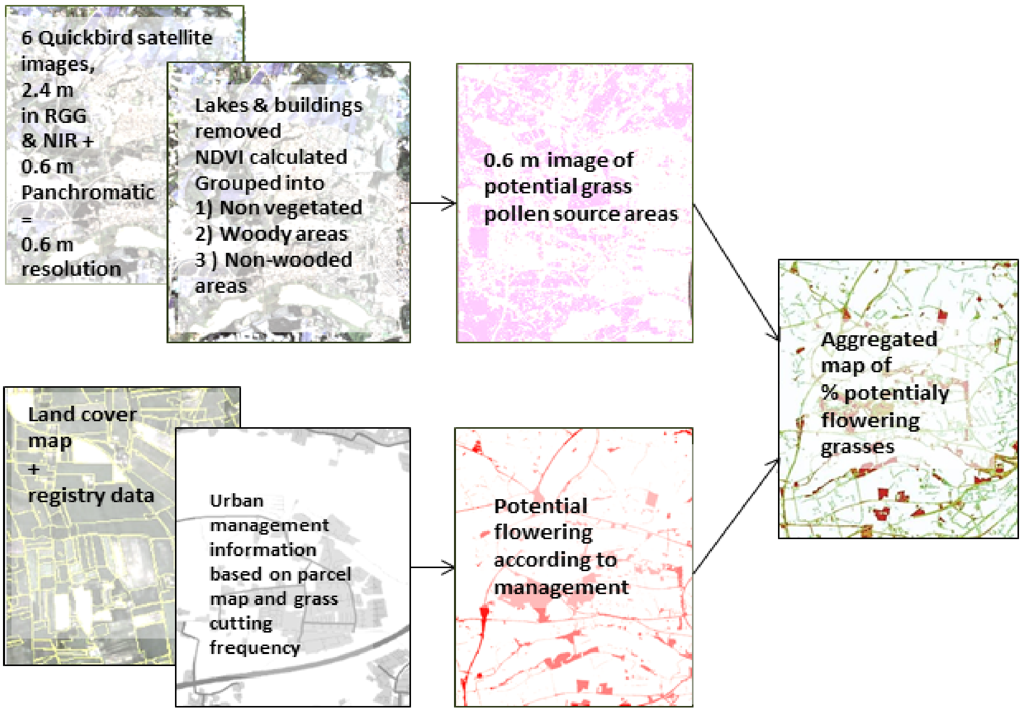


Figure 8.1 Remote sensing and GIS analysis process for the production of the map of percent risk of flowering grasses.

combinations performed in ArcGIS (Figure 8.1)

Six Quickbird satellite images, covering the area of greater Aarhus, were combined and Normalized Difference Vegetation Index (NDVI) was calculated for determination of vegetated non-wooded areas (Figure 8.2). The final map showed 84 % accuracy with the largest issue being miss-classification of trees as grass areas. The classification cannot distinguish between weeds and grasses, and an overrepresentation may therefore also occur.

Whether or not grass in an area will flower is dependent on management. If the grasses are frequently cut, or if the area is strongly grazed by animals, the grasses will not reach maturity and flower.

For the agricultural areas in Denmark, the potential grass areas can be determined via the Danish General Agricultural Register (DGAR). At DGAR information is recorded on crop types for each field and linked to maps of field parcels. The data on crop types was for this study categorized into five classes of potential grass areas and one class of other crops. For the agricultural grass areas, harvest is usually performed around the time of flowering. It is therefore likely that they will reach some degree of pollen release, and these areas are considered potential pollen sources.

<u>The risk of flowering grasses in the urbanized areas.</u>

Land parcel maps were categorized according to whether potential grasses would flower or not, and buildings, parks and cemeteries were considered regularly managed. Areas considered unmanaged comprised of major roads, railroads, construction- and unmanaged land, agricultural and areas without buildings. The cutting frequency of the managed areas was obtained from Aarhus municipality, and a frequency above 12 times per year, were considered to eliminate the potential for flowering. The image of potential grass areas were finally combined with the map of management and parcel information into a aggregated map of 14.4 m resolution with the percentage of possible flowering grasses in each grid (Figure 8.2). The central area of Aarhus was seen to have moderate potential grass pollen sources with "hot-spots" of fields near the TV2 station, and narrow areas along roads, railroads, industrial areas, and

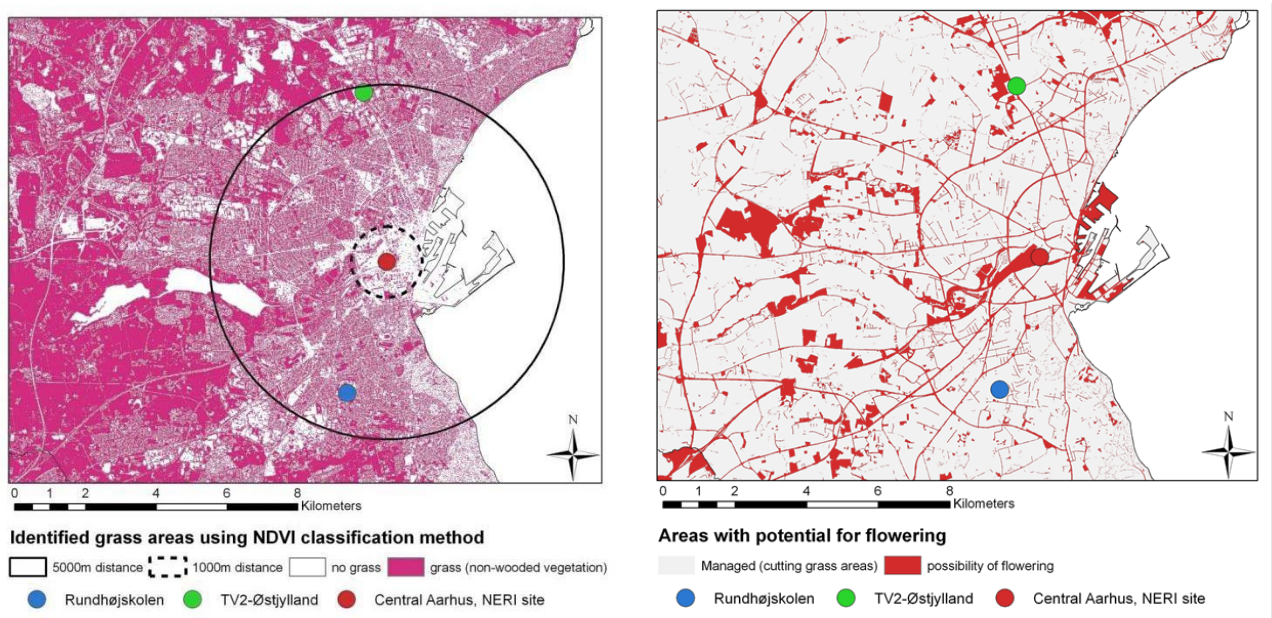


Figure 8.1 Non-wooded areas determined via NDVI-index from Quickbird satellite images (left) and areas of potential flowering according to management practice (right).

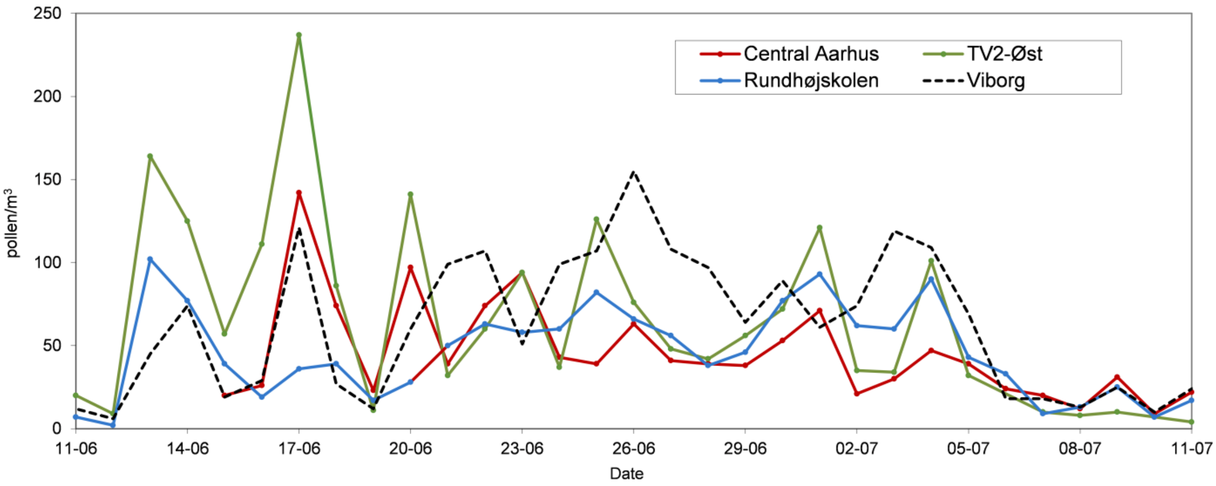


Figure 8.3 Daily pollen counts from the three traps in Aarhus and the nearest operational trap in Viborg, 60 km away, around the time of seasonal peak.

near areas of streams and wetlands.

	Rundhøjskolen	*TV2-East*	*Central Aarhus*
Correlation with trap in Viborg (all data in season)	0.76	0.61	0.70
Correlation with trap in Viborg (above 50 grains/m^3)	-0.35	0.15	0.06
Correlation with trap in Viborg (up 50 grains/m^3)	0.68	0.74	0.88
Total pollen load (Viborg; 2373 grains)	1799	2421	1461

Table 8.1 Correlation of daily pollen counts between the three traps in Aarhus and the trap in Viborg, and the total pollen load recorded at the stations.

<u>Intra-urban variations in grass pollen concentrations</u>

The daily pollen counts from the three stations in Aarhus were compared with counts from the nearest operational monitoring station in Viborg 60 km away. Table 8.1 and Figure 8.3 show results from the correlation with this trap and a timeline of the time around seasonal peak.

The greatest differences between the stations are seen in the period of the highest pollen counts, between the 14th of June and the 5th of July. The correlation between the traps in Aarhus and the one in Viborg is 0.6-0.76 for the whole season, however, a separation into days above and below 50 grains m^{-3} show that the peak days are poorly correlated. This indicates that on days with high pollen counts, the proximity to local flowering sources is very important, and pollen counts 60 km away is not a good indication of the local pollen level.

As seen in Figure 8.3, the daily pollen counts from the three traps in Aarhus were also more identical during days with low concentrations. On days with higher pollen counts not even theses traps, only 7 km apart, show similar counts. It appears that the traps at Central Aarhus and TV2 are the most similar in pattern.

Linking variations in pollen concentrations with spatial distribution of sources

The local air mass transport and dispersion of pollen was approximated from 30-minute wind directions measured at the Central Aarhus station. For each of the three stations the distribution of wind directions on peak days with pollen counts above 50 m^{-3} were calculated (Figure 8.4).

Wind directions during the pollen season were mainly from the sectors of 225-315 degrees.

On days with peak levels recorded at the TV2 station, 55% of winds arrived from the sector of 270-315 degrees. This was consistent with the source map, indicating a major "hot-spot" of grass pollen sources located in this direction. The highest total pollen load was also recorded at this station (Table 8.1). None of the other stations have clear hot-spots in the vicinity or as clear an indication of a main wind direction on peak days.

On days with peak levels recorded at the Central Aarhus station, 40 % of winds arrived from the sector of 270-315. Again this was consistent with the map, indicating the majority of sources in this direction. However, this was not as clear a "hot-spot" as the one seen near TV2, which is also indicated as the less frequent winds from this sector on peak days, compared to the wind distribution at TV2.

On days with peak levels recorded at the Rundhøj station, approximately 30 % of winds arrived from each of the sectors 45-90 and 270-315 degrees. This was the most diffuse distribution of the three stations, and were again consistent the distribution of potential sources, where only medium density sources was seen to the east and to a lesser degree in all directions.

The highest degree of similarity between pollen counts, seen in the timeline, was seen between TV2 and Central Aarhus (Figure 8.3), and could be due to the proximity to "hot-spots" at both these sites.

The analysis of the local air mass transport support the hypothesis, that intra urban variations in pollen concentrations are linked to the distribution of pollen sources, and that these sources can be efficiently located by Remote sensing images and GIS analysis of management information.

Implications of results

The potential implications of the measurement method was discussed in Chapter 6, e.g. the issue of comparing data from several traps, assuming comparable replication of actual pollen concentrations, and will not be discussed further here.

The results shown here indicate that the pollen counts measured at the operational pollen monitoring station in Viborg do not correlate with pollen counts measured in Aarhus on days with high pollen counts. This is consistent with the hypothesis of the importance of local sources, and also seen in the visual inspection of the daily pollen counts from the three traps, where differences are seen, even for traps only 7 km apart.

A recent study by Simoleit et la (2017) on the impact of local pollen sources on the measured counts also support the results found here. They find local sources to be of great importance to the pollen counts, and find high concentrations of grasses near a large road to be a significant source area in the urban environment. They report significant positive correlations between two traps also

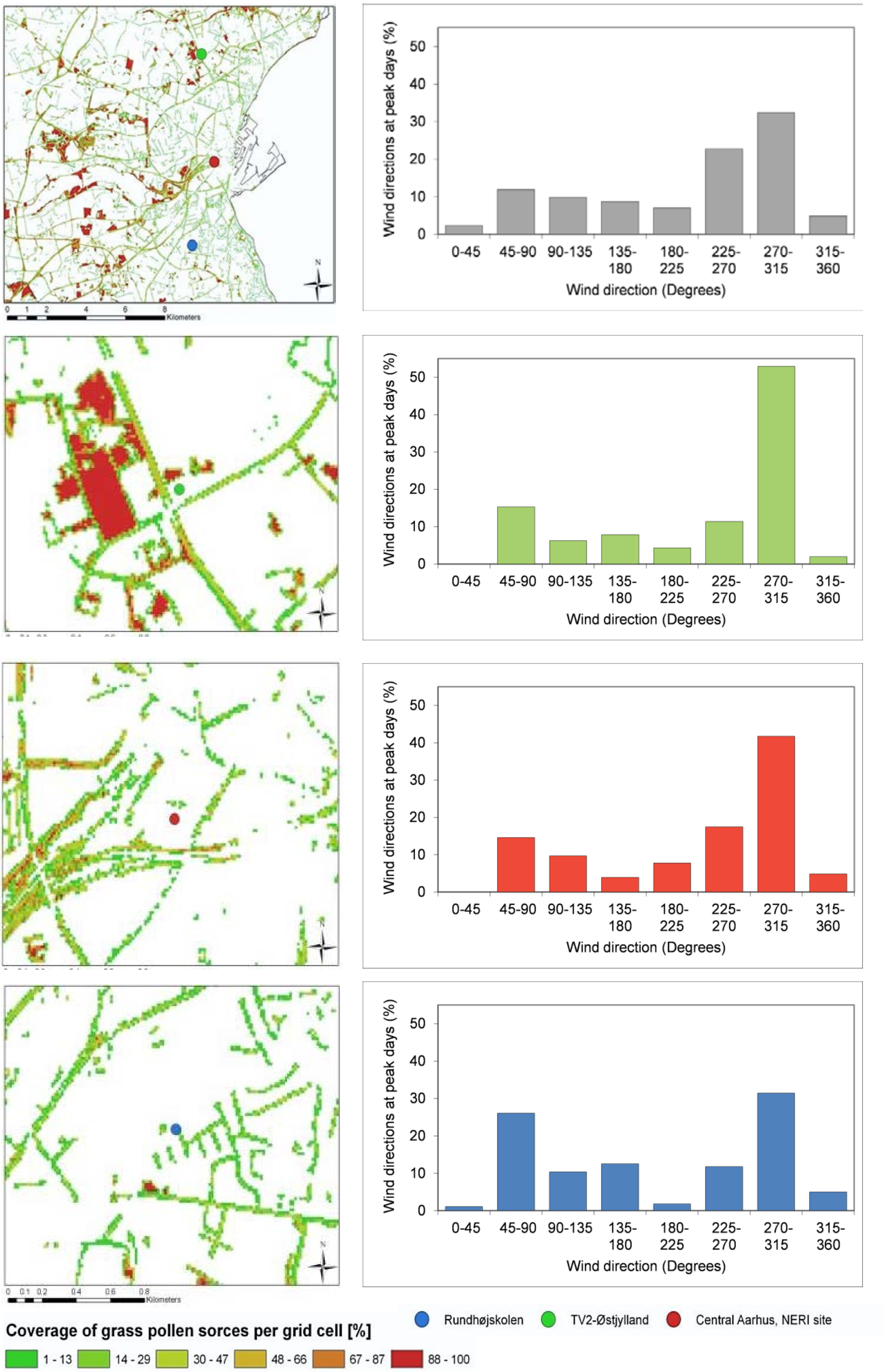


Figure 8.4 Maps of potential grass pollen source areas with percentage of coverage per 14.4 m grid cells (left) and distribution of wind-directions measured at Central Aarhus (right).
Top; Map of all of Aarhus, and wind directions for all days during the pollen season.
Below; zoom on the areas around the three stations, and distribution of wind on peak days for TV2 (green), Central Aarhus (red) and Rundhøjskolen (blue).

placed approximately 7 km apart, however, this could be due to the correlation being calculated for the entire season and not only peak days.

Large local differences in the magnitude of the pollen counts was found in another recent study on 14 traps at street level in Berlin, where up to 306% difference in total load was seen (Werchan et al., 2017). Previous studies on intra-city variation generally show good correlation between daily pollen counts (Alcázar et al., 2004;Emberlin and Norris-Hill, 1991;Fernandez Rodriguez et al., 2014;Mücke et al., 2014;Velasco-Jiménez et al., 2012) (Chapter 3). Many do however suggest that the correlation is dependent on homogenous spatial distribution of sources and that local variation may be present and clinically relevant, as well as the magnitude of concentrations may vary. None of these studies however examined peak and non-peak days separately, and the overall degree of correlation may have been affected by the higher correlation on days with low concentrations.

The pollen source map indicate that the most urbanized parts of Aarhus have few and moderate sources. In these areas local sources will not be as dominating, and in larger cities with greater areas of urban core, it could be expected that the here seen pollen will originate from sources further away, and more complex dispersion will affect the concentration levels. Smith et al (2013) found that for ragweed, a smaller pollen than grass, but also released at low height, scales from 0-2000 km may be of importance when accounting for the sources contributing to a pollen measurement. However, there is little doubt that the main impact on the measured pollen loads of a source as ubiquitous as grass is the local sources.

Overall, it appears that for sources outside the city, pollen concentrations will be lower inside than outside the city and fairly homogenous. Pollen sources located within the city, will however affect the concentrations relative to distance from source and also affected by urban topography (Alcázar et al., 2004;Emberlin and Norris-Hill, 1991;Gonzalo-Garijo et al., 2006;Peel et al., 2014b).

The pollen counts applied in this study are background measurements obtained 10-20 m above ground level. According to dispersion theory, the pollen concentrations will be higher at ground level near the source, also indicating even larger gradients in local scale concentration variation at the breathing zone. Rantio-Lethimäki et al found approximately one magnitude larger concentrations at surface level compared to rooftop (1991), however the opposite was seen in Peel et al (2014b), in an urban area with few sources . It has also been shown that the ratio of background concentration with inhaled dose can vary throughout the day (Peel et al., 2013), again indicating the importance of the proximity to the source, and even more so at the time of emission.

The pollen source map indicates that agricultural areas may be essential hot-spots for pollen sources outside the City Centre. Although two-thirds of Denmark consists of agricultural areas (Skjoth et al., 2008a), the majority are fields not producing flowering grasses. In this study less than 10% of the agricultural area around Aarhus was considered potential pollen source areas. It is therefore evident that

Figure 8.5 Many grasses grow along the railroad in Aarhus, as seen here (left, middle). To the right is seen the pollen trap and weather station at Central Aarhus.

land cover maps alone cannot estimate potential grass flowering areas, and management must be included. Also the potential of flowering of urban grass areas is very dependent on management. The information on cutting frequency applied in this study is of great value, but not often available on a larger scale. Alcázar et al. also found management of urban planting to affect pollen counts, in a study on Platunus trees, where pruning methods affected the concentrations (2004).

Models of pollen dispersion can to some extend lean on the vast experience achieved in the field of pollutant modelling (Sofiev et al., 2013b), however the great challenge is modelling of the sources. For this the here presented method could be applied, however, the data is not easily available for all regions. Previous source mapping has primarily been on larger scales, and mainly apply land cover data, assumptions on growth conditions, elevation models and forestry databases (see Chapter 3). The mapping is also mainly performed for larger areas. The here presented method is relevant in future local scale mapping and potential correlation with clinical data.

Chapter 9

Diurnal variations in grass pollen concentration profiles

Although it is known that the diurnal pollen concentration profile can vary greatly from day to day, it is common practice to provide an average profile based on the entire season. Especially concentration profiles of grass pollen have been shown to vary, and it is speculated to be related to the vast number of species in this taxa, with different seasonal and diurnal emission patterns (see Chapter 2 and 3).
The concentration will also be dependent on a complex combination of factors affecting both emissions and dispersions, making the profile difficult to predict.

Meteorological factors constitute the main impact on both emission and dispersion, both through the impact on the growth and flowering process, the pollen release and on the subsequent dispersion or re-suspension of the pollen. It can therefore be speculated, that the differences in the patterns are driven by either purely the seasonal succession of flowering species or by the meteorological factors affecting emission and dispersion, the latter having both great intra diurnal and seasonal variation.

In the studies included in this thesis, two different approaches to analysis of the diurnal profile were examined. In the first study visual examination of the profiles were performed based on the theory of a seasonal difference in the potential drivers of the profile, and therefore also of diurnal pattern. In the second, a statistical clustering approach was applied, to examine whether this method could reveal district typical patterns and a correlation with potential drivers could be found.

Seasonal variation in the diurnal grass pollen profile
This study was performed on pollen counts from the campaign in Aarhus, described in Chapter 6, with the objective to analyse the variability in the diurnal time of peak in grass pollen concentrations and whether this depend on the seasonal progression of different grass species flowering. For this, an inventory of species likely to be found in and around Aarhus was compiled, with respect to reported diurnal and seasonal times of emission and productivity. A second objective was to examine whether potential seasonal differences in the profile were driven by differences in meteorological parameters.
For full descriptions of all applied methods see Chapter 6 and *Manuscript II*.

Only days without rain and with pollen counts above 20 grains m^{-3} were examined, due to the effect of washout on the diurnal profile (McDonald, 1962), and the lack of defined pattern on days with few pollen. A total of 157 profiles from the three stations, recorded on 69 different dates, were included in the analyses.

Peaks were identified according to the following criteria on all 24 hour profiles of bi-hourly counts;

- Peaks should be minimum 50 grains m^{-3}.
- The maximum peak was defined as the "primary peak".
- Secondary peaks were defined as being ≥ 6h before or after the primary peak and with a trough of minimum 50 grains m^{-3}.
- If peaks were ≤ 4 h apart and of equal magnitude, they were considered a single wide peak with the intermediate time stamp.
- Equal peaks > 6 apart were considered multiple peaks.
- Peaks just after midnight occurring where a larger peak was registered just before, was rejected, as part of the previous day, and therefore surpassed.

Profiles were grouped by year and site, and three different types of profiles were seen to dominate at different parts of the season. A peak matrix was developed to better visually interpret data (Appendix VI). The season was hereafter separated into A) Early season with twin morning and evening peak (n=37), B) mid-season with single evening peak (n=58) and C) late season with single late morning or early afternoon peak (n=62) (Figure 9.1). The periods did not begin and end at the same time of year during the campaign, neither were they identical between stations.

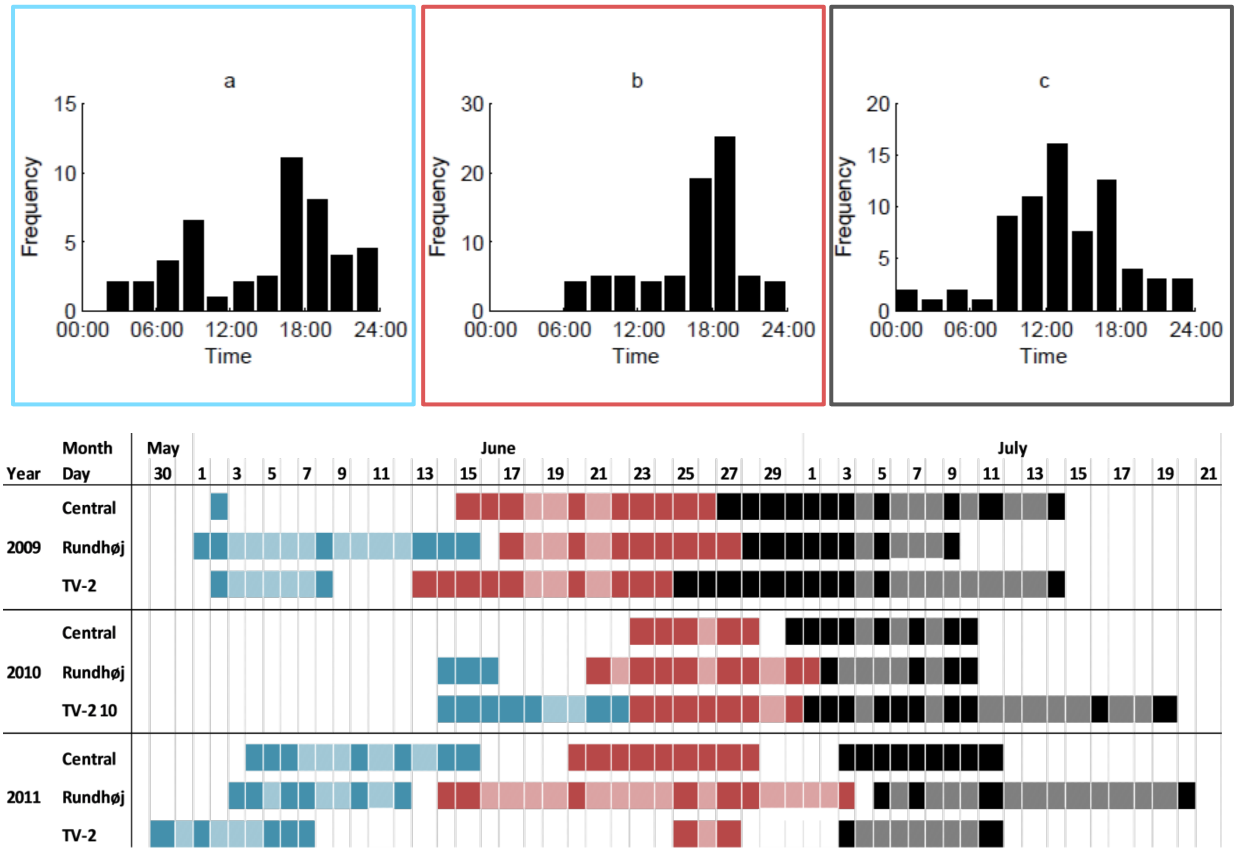


Figure 9.1 Below: Separation of the season into three parts depending on dominating diurnal pattern (lighter colours representing dates within the periods with no data (<20 grains m-3 or rain). Above: Frequency distributions of time of peak in the three periods.

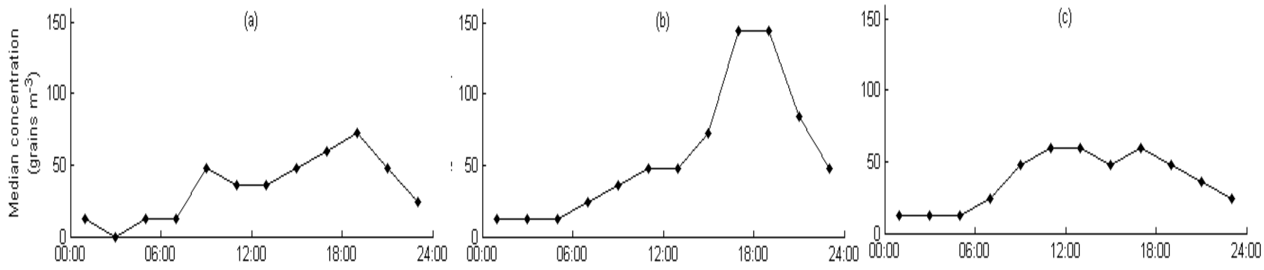


Figure 9.2 Median diurnal concentration profiles in the three periods of the season.

Difference between the stations in the three periods was tested by Anderson-Darling test, and significant differences were found for period 2 between TV2 and the other stations. When examining data, this difference was found to be due to a difference in peak time being 17:00 at TV2 and 19:00 at the two other stations, i.e. overall similar patterns. It was therefore considered appropriated to pool the data from the three stations.

The frequency distribution of time of peak is seen in Figure 9.1(above). According to Anderson-Darling test the distributions differed significantly. Period A has a bi-modal distribution with morning (9:00) and evening (19:00-21:00) peaks. Period B has a uni-modal appearance with evening peaks (17:00-21:00). Period C also has a uni-modal appearance, but with peaks distributed across a wider time-span during all day (09:00- 17:00) however with the majority at midday (13:00).

Average profiles for wind speed, temperature and Vapor Pressure Deficit (VPD) for the days included in each period, were examined for differences by time of day. Temperature was found to be significantly higher all day for Period C compared to the two other periods. It could have been speculated that the higher temperatures would lead to earlier flowering, however the opposite is seen as the peaks are earlier in the colder Period A.

VPD can be considered a proxy for the drying capacity of the air, and higher VPD could be speculated to induce earlier anther dehiscence. A significant increase in VPD was seen associated with the shift from a high frequency of evening to midday peaks from Period B to C. However, no significant decrease was seen between the shift from early to late peak in Period A to B.

Wind speed is both known to affect emission and dispersion. However, no differences in this were seen between the periods.

Analyses of the wind direction at the time of peak revealed peaks occurring during winds from all directions. However, in Period A, morning peaks are all from west. With TV2 and Rundhøj being main contributors to this period, this is consistent with the results in the previous study (Chapter 8), where the main source areas are to the west from both stations, and to the east of Rundhøj, resulting in the shortest distance. This could be an indication of morning flowering in this period, inducing morning peaks when the wind is from the direction of main source area.

Figure 9.3 *Festuca arundinacea*, *Dactylis glomerata* and *Lolium perene*, three prolific species likely to be found in and around Aarhus. Pictures taken in Cordoba, Spain and Ishøj, Denmark.

Grass species are very similar in morphology and allergic potential, but to some extent, they thrive and flower at different conditions. The compilation of a grass species inventory comprised of species listed in the Danish Flora register (Frederiksen and Rasmussen, 2006) as "common" or "very common" along roads, railroads and in parks, the areas likely to be the source of grass pollen in Aarhus as determined in Chapter 8 (Skjøth et al., 2013b). Species were included if listed as common for Copenhagen, a similar large Danish city, in an inventory produced by Hald (2011). A total of 18 species fell within these criteria. Productivity and/or flowering pattern of the different species were reviewed from existing literature, and found for 12 of the species likely to be present in Aarhus (Aboulaich et al., 2009;Beddows, 1931;Clark, 1911;Emecz, 1962;Evans, 1916;Jones, 1952;Ogden and Hayes, 1969;Prieto-Baena et al., 2003;Smart et al., 1979;Smart and Knox, 1979). Pollen production per inflorescence has been seen to vary by approximately 3 orders of magnitude between species (Aboulaich et al., 2009;Prieto-Baena et al., 2003), and it is likely that a few prolific species could dominate the pattern. Leön-Ruiz et al found four species to be the most contributing to the patterns in Cordoba, Spain (2011). Of the species with flowering reported to coincide with the time of the evening peaks found in Period A and B, *Dactylis glomerata* was the most productive, and for the time of midday peaks as seen in Period C, *Festuca arundinacea* and *Lolium perenne* was among the species reported to be high producing. Differences among the local distribution of prolific species could also account for differences between sites. Also the difference in location within the city could affect flowering time. In this study up to 4 days difference in time of periods were seen between sites. The urban heat island effect has previously been shown to affect the flowering of grasses in the city centre compared to outskirts of a city (Rodríguez-Rajo et al., 2010).

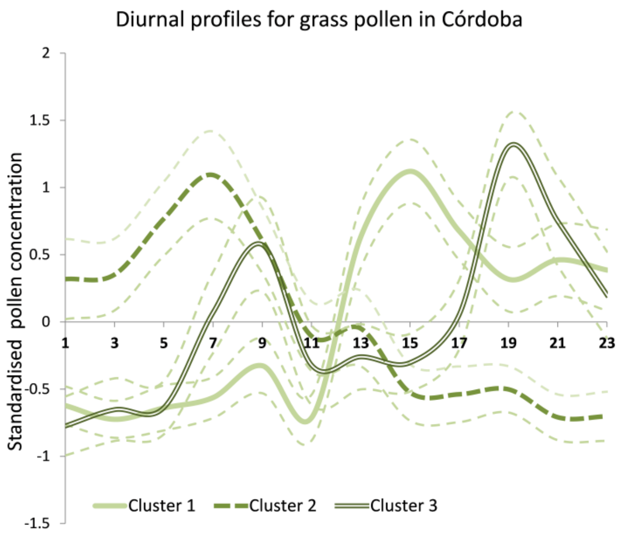
By choosing to allow for differences in timing of the periods between stations, the assumption of the differences in species being the main driver in the differences becomes apparent. Time of flowering could very well depend on highly local conditions of soil type, water availability and competing species,

whereas the meteorology will be the same, although the above mentioned conditions may induce different dependency on meteorology even between sites not far apart.
Periods were seen to last approximately 2 weeks, which is consistent with the duration of a flowering phase of individual grass species (Leon-Ruiz et al., 2011).

Clustering of diurnal profiles

In order to investigate the variability in the diurnal grass pollen concentration profile further, a second approach was examined. In this study, ten years of pollen counts from Copenhagen, Denmark, and four years from Córdoba, Spain were examined. The objective was to investigate whether statistical clustering may define distinct diurnal patterns of grass pollen concentrations, and if so, whether these were related to meteorological variables and vary between sites. The study was initiated during a stay at Córdoba University. For full descriptions of all applied methods, material and sites see Chapter 6 and *Manuscript III*.

Pollen counts from 2008 to 2011 for Córdoba and from 2001 to 2010 for Copenhagen, from days without rain and with daily count above 20 grains m^{-3} were applied as in e.g. Peel et al. (2014a). Pollen concentrations were standardized to eliminate the effect of the magnitude (Oteros et al., 2013a). The standardized bi-hourly values were analysed using hierarchical cluster analysis and the potential number of diurnal profiles at each site was examined graphically via a dendogram. For each site three clusters were estimated to illustrate sufficient variability, as an increased number of clusters did not provide additional distinctively different profiles. The analysis evaluates the "distance" of each diurnal profile, to the centroid of the cluster to which it belongs. Profiles with a high distance to the cluster center were discarded, as the objective was to find distinctive different profiles. For Córdoba 61% of profiles were included and for Copenhagen 73%. Figure 9.4 and Table 9.1 shows the mean profile for each of the three clusters with 95%-CI, for the two sites and the number of profiles included.



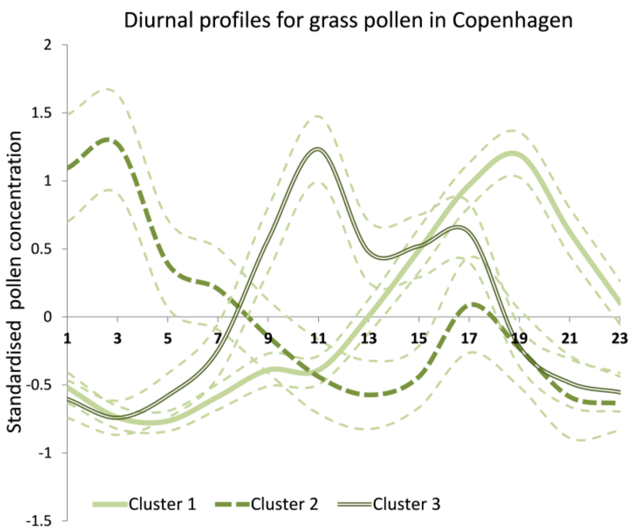


Figure 9.4 Three clusters of standardized diurnal pollen concentration profiles at Córdoba (left) and Copenhagen (right), with 95%-CI-intervals indicated.

	N, days >20 grains m-3 & no rain	N, days included in clusters	Cluster 1	Cluster 2	Cluster 3
Córdoba	*184*	*112*	*45*	*37*	*30*
Copenhagen	*259*	*190*	*109*	*24*	*57*

Table 9.1 Number of diurnal profiles included in the cluster analysis for Córdoba and Copenhagen.

For both sites Cluster 1 represents the most frequent profile, with a single peak around 15:00 in the afternoon for Córdoba (40%) and in the early evening around 19:00 for Copenhagen (57%). Cluster 2 represents a single peak in the early morning around 7:00 in Córdoba (33%) and a nighttime peak around 03:00 in Copenhagen (14%). Cluster 3 represents dual peaks, with the primary around 19:00 in the evening and secondary around 9:00 in the morning in Córdoba (27%), and with the primary peak around 11:00 and secondary around 17:00 in Copenhagen (30%).
For Córdoba, the different diurnal profiles are more equally distributed in the three clusters, than for Copenhagen. Here, the very frequent pattern of Cluster 1, is identical to the most frequent peak occurring in Aarhus, during the main season, as seen in the previous section (Peel et al., 2014a).

In Córdoba, the grass season stretches over a period of highly variable meteorological conditions. An early season in March, which depends strongly on the availability of rain, where the species flowering at this time are reacting very homogenously to weather conditions. Here only a few species flower and if there is no water available they will not flower at all. A mid-season with high pollen concentrations, many species flowering, which react differently to weather conditions, therefore the diurnal profile may be affected very differently from year to year depending on the distribution of the species flowering at this time. The late season in the very warm summer is again highly depending on rain. If no water is available, the season will stop abruptly, but if there is rain, the flowering can carry on. This time is dominated by a few species with similar reaction to weather conditions (based on conversations with the team in Córdoba responsible for the phenological studies).
If the different profiles were primarily driven by a succession of different flowering grass species as speculated in the previous study (Peel et al., 2014a), this should be evident in the analysis of the meteorology at this site.

Meteorological data for temperature, humidity, global radiation, wind speed and wind direction were linked to the dates of each cluster. Data was not available for all dates, and meteorology was therefore analysed for a total of 86 days for Córdoba and 166 days for Copenhagen.
The differences between daily mean meteorology in the three clusters are shown in Table 9.2. Analysis was also performed on hourly data, to investigate whether differences were seen for specific parts of the day, potentially affecting either flowering or dispersion. No differences of explanatory value were found. For Córdoba the only statistical significant difference in meteorology was in global radiation. This could be due to Cluster 1 having the highest proportion days in the early season, also indicated as a slightly lower temperature.

	Córdoba				Copenhagen			
	C1	*C2*	*C3*	*Sig.*	*C1*	*C2*	*C3*	*Sig.*
DOY	*136.82 (18.58)*	*143.86 (17.69)*	*144.91 (18.39)*	*0.09*	*174.06 (13.15)*	*177.6 (15.37)*	*175.74 (14.61)*	*0.47*
Temperature (°C)	*20.13 (3.64)*	*21.33 (3.45)*	*21.98 (3.1)*	*0.14*	*17.11 (3.09)*	*17.98 (2.89)*	*17.69 (2.69)*	*0.20*
Humidity (%)	*55.94 (11.43)*	*52.99 (10.22)*	*55.23 (7.52)*	*0.56*	*66.78 (9.74)*	*69.64 (10.18)*	*68.05 (7.94)*	*0.72*
Global radiation (W/m^2)	*286.34 (42.82)*	*314.00 (43.98)*	*314.47 (31.83)*	*0.02**	*272.64 (59.46)*	*246.25 (71.24)*	*255.12 (69.81)*	*0.19*
Wind speed (m/s)	*1.54 (0.48)*	*1.69 (0.57)*	*1.82 (0.6)*	*0.29*	*3.55 (0.95)*	*3.16 (1)*	*3.45 (0.98)*	*0.17*
Wind direction (°)	*351.38 (248º to 54º)*	*9.30 (256º to 77º)*	*8.20 (264º to 58º)*	*0.40*	*267.34 (90º to 78º)*	*211.6 (35º to 333º)*	*221.2 (54º to 45º)*	*0.01**

Table 9.2 Mean (sd.) for the meteorological variables related to the three clusters at Córdoba and Copenhagen, and statistical significance level for the difference between clusters.

For Copenhagen, the only statistically significant difference in meteorology was in wind direction with the most frequent winds related to Cluster 1 profiles coming from West, whereas more south-westerly winds were associated with Cluster 2 and 3. West of the sampler location is a large residential area with lawns, a large road with grass berms and Lersøparken, a green recreational area. All potential grass pollen source areas, however highly dependent on management (Skjøth et al., 2013b). The proximity to sources in this direction may therefore be affecting the pattern in this Cluster, potentially dominated by afternoon flowering species. This is, however, not possible to conclusively determine without phenological studies from the site, containing information about species composition and diurnal and seasonal time of flowering.

For Córdoba, the mountain-valley breeze (Hernandez-Ceballos et al., 2013) is dominating the diurnal wind direction profile for all clusters, resulting in no significant difference in this variable. The source area can therefore largely be expected to be the same for all the three clusters. However, even minor shifts in wind direction could affect the source area distribution, and it has been seen that areas in close proximity can affect the pollen concentration (Skjøth et al., 2013b). Further analysis would be required to fully investigate the importance of proximity to source areas as the driver behind the different profiles. Also a source map for grass pollen in Córdoba could aid in future analysis. The location of the trap, is at the outskirts of the city, and there are many grass areas in the surroundings.

Even though the two sites have great differences in species composition, local relief and meteorology, the most frequent profile at both sites was a single peak in the afternoon or evening.
The lack of any evident correlation with the day of year for either site does not support the theory of the succession of flowering grasses as the main driver. This is, however, possibly masked by the simplicity in the analysis. A further analysis of time of peak should include time of season, since timing and duration of season can vary from year to year (Peel et al., 2014a). A peak matrix indicating high/low pollen concentration was developed for Córdoba and Copenhagen data (Appendix VII),

however no apparent shift in time of peak was seen upon initial examination. Further analysis could include a more detailed investigation of the time of peak, applying a method identical to the one applied in Peel et al (Peel et al., 2014a)

Implications of results

The potential implications of the measurement method was discussed in Chapter 6, e.g. the issue of comparing data from several traps, assuming comparable replication of actual pollen concentrations, and will not be discussed further here.

In the two presented studies, large variation of the diurnal grass pollen profile was seen at all three investigated sites. However, in Aarhus dual early and late peak-patterns were seen to be more frequent in the beginning of the season, evening peaks were seen to be very frequent in the middle and peaks occurring around midday, but more variable in the late season. The approach applied in the study of pollen counts from Aarhus had the benefit of visual interpretation allowing for individual differences between sites and years in grouping of profiles. This revealed seasonal differences.
As described in Chapter 3, previous studies also separated the grass season in periods of either month by month or defined as pre- and post-peak periods (Khwarahm et al., 2014;Mesa et al., 2003;Mullins et al., 1986;Munoz Rodriguez et al., 2010;Norris-Hill, 1999;Smith and Emberlin, 2005). The majority of these also suggest the difference between the successions of flowering grass species, to be affecting the variability.

The timing of the three periods seen in Aarhus was not uniform. This could be why a purely statistical clustering of diurnal profiles did not show a similar dependency on time of season at the two other sites. Although no conclusive confirmation of the flowering of successive grass species being the driver was found, no contradictive indicators were seen either. Further analysis of source areas and phenological analysis of the contributing species is needed to better examine these associations. A potential methodology could be to apply HYSLPIT trajectories to determine the potential source areas (Hernandez-Ceballos et al., 2011).

Although a simple correlation with separate meteorological parameters was not found in either study, meteorology is likely to be a significant factor in determining the diurnal pattern (see Chapter 3). Both in the effects on emission and dispersion. The effects are however not simple, and may depend on a vast number of combined factors. Different thresholds will also to apply to different grass species, as well as different soil- and nutrient compositions at the individual source areas could affect these thresholds. This will complicate the correlation of a measured pollen concentration compiled of grains originating from variable source areas, with one meteorological measure.

The seasonal shift in time peak found in Aarhus was not seen in a simple high/low matrix of pollen counts from Copenhagen. This may be due to the difference in proximity to major hot spots/sources. In Aarhus, the pollen samplers were closer located to agricultural areas, and this may lead to a clearer and more uniform signal. In Copenhagen there are further distances to major hot-spots and the signal may be more dominated by diffuse sources and dispersion processes.

The two diurnal studies in Danish cities indicate, that the most frequent profile is consistent with peaks in the early evening around 19:00, which is also the time of day with the most frequent peaks in the main part of the season. The combined results therefore support, that although there are great variability, this time of day appear to present the greatest risk of high exposure in both east and west Denmark. This is in contradiction to the current main advice of maximum grass pollen midday in Denmark. Also in Córdoba, previous average profiles for the entire season has reported maximum levels around midday (Alcázar et al., 1999b;Cariñanos et al., 1999;Galan et al., 1989).
For this site, the current study indicates the most frequent peak to be in the afternoon. However, this study do not examine whether this is also the profile related to the days with the highest counts, and since the DOY analysis indicate a large portion on days in the early season in this cluster, further analysis is required to establish the time of highest risk of high pollen exposure.

Results from the two studies on diurnal variation was published in the popular science journal "Miljø & Sundhed" (Environment & Health)(Ørby et al., 2013) in addition to the two manuscripts included in this thesis.

Chapter 10

Seasonal variation in response - The priming effect

Some degree of priming is inevitable. The airways will be affected by all we inhale, and by the processes these substances initiate, be it air pollutants or allergens. The question posed in this study is, to which degree the inevitable priming by natural pollen exposure, is affecting the allergic response. And whether we should consider this priming, when setting the pollen levels described as high, and not have identical limits throughout the season. This effect could be measured by a number of outcomes, affecting any of the allergic symptoms (see Chapter 4). In this study we chose to focus on the bronchial response.

Previous studies have primarily focused on asthmatic subjects, however with the majority of those with pollen allergy only affected by AR (Linneberg et al., 2002), and following the previously mentioned theory on the "united airway" (Feng et al., 2012;Grossman, 1997), this study focus on determining whether the priming effect could also be seen in participants with AR and little or no asthma.

The objective of the current study was therefore to determine whether the bronchial response is greater following priming by the natural seasonal exposure than outside season in AR participants with mild or no asthmatic symptoms. Secondary objectives were to assess the applied non-linear regression model, and the associations between the bronchial response and baseline characteristics.

The methods applied in this study are described in detail in Chapter 7, and in *Manuscript IV*.

The four-parameter non-linear regression model was fitted to data from 85 SIC´s, and estimates of PD_{20} were produced with 1) the assumption of no difference between the SICs performed outside and at the end of the pollen season, and 2) allowing for a difference in PD_{20} between the two exposures times. No statistical significant difference between the two models were found (birch p=0.30, grass p=0.39), indicating no significant influence of seasonal priming effect.

Individual modelled log-dose response curves showed a good fit to the measured data (Figure 10.1), and no systematic errors in plots of residuals were seen.

Although only 7 of the 36 participants reported asthmatic symptoms more than a few times a year, approximately 80% of SICs resulted in a reduction of more than 20% in FEV_1. This supports the theory of the "united airway", with bronchial involvement also seen in participants with primarily AR. With a high percentage of challenges with above 20% reduction in FEV_1, the data was also estimated to be sufficient for modelling a PD_{20} estimate.

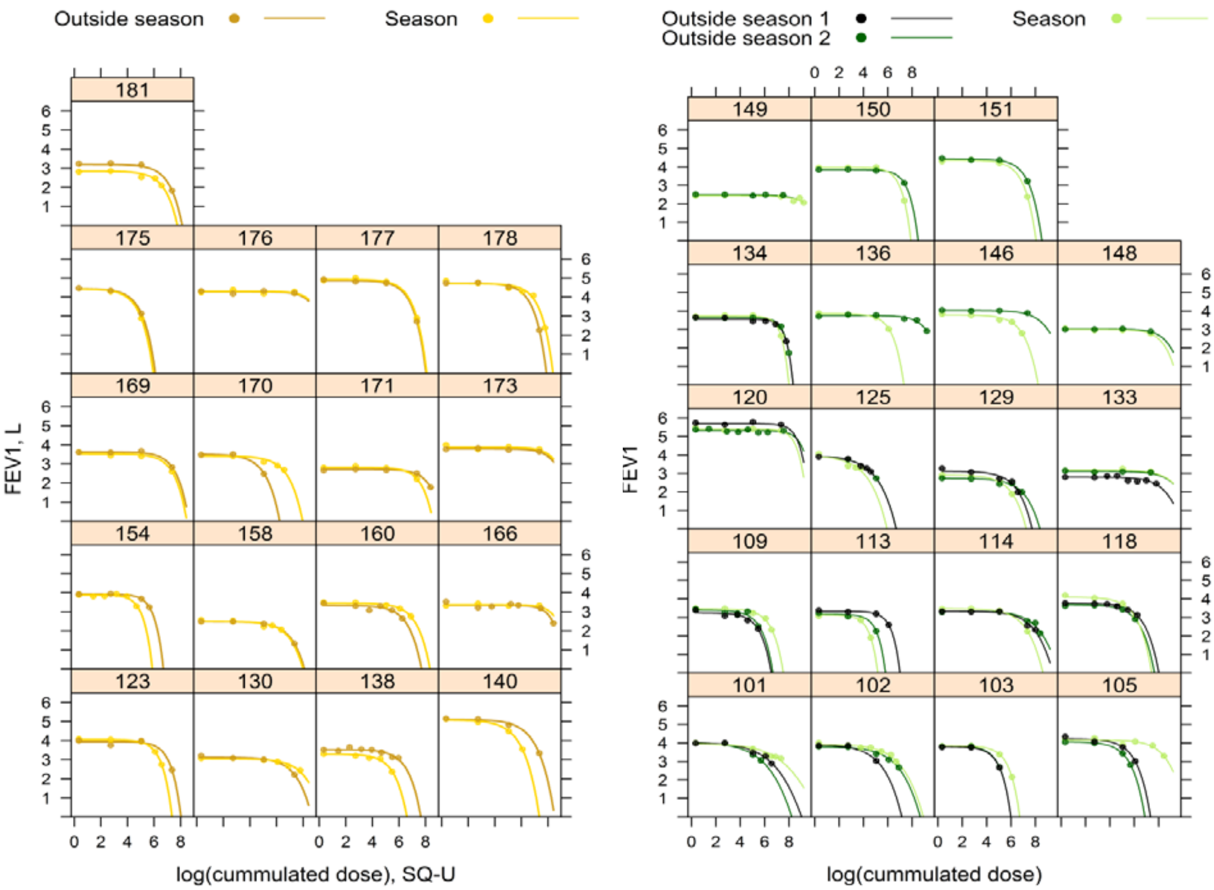


Figure 10.1 Individual modelled dose-response curves for all SICs. Measurements are shown as dots, and numbers indicate the ID´s of the 36 participants. Left; Challenges with birch allergen, right; challenges with grass.

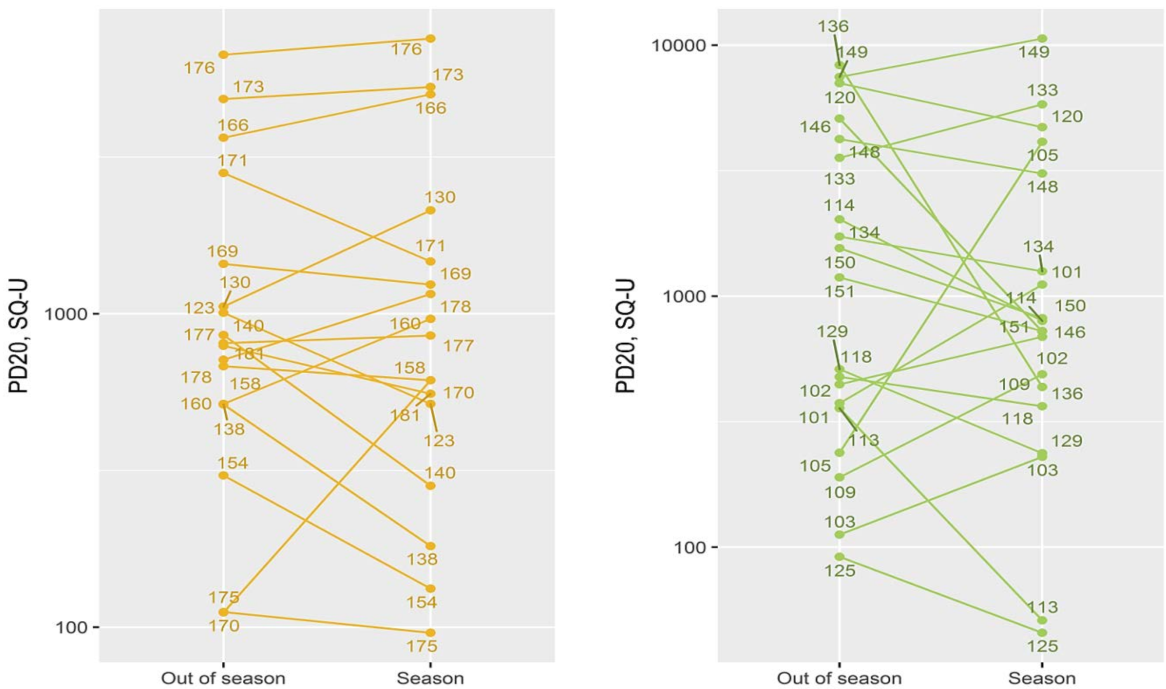


Figure 10.2 Individual PD_{20}-estimates for Out-of-season and seasonal challenges. Left, birch, right; grass.

Individual PD_{20} estimates (Figure 10.2) were lower at the end of the season that outside season for 12 of the 19 grass participants, however only for 9 of the 17 birch participants. This indicates a lower tolerance at the end of the season for the participants challenged with grass.

Figure 10.3 and Table 10.1 shows the PD_{20} estimates and the log-dose-response curves based on the best fit of the non-linear regression model to the measurements from the 85 SICs. For birch the two estimates are very similar. For grass the PD_{20} estimate for the seasonal challenge was approximately 20% lower than out of season, however this difference was not statistical significant, nor if the model and test is run on only those with above 20% reduction in $FEV_{1,}$ indicating that this is not an issue of non-responders affecting the modelled estimates.
Curves are shown for seasonal and out-of-season challenges, for birch and grass allergen challenges separately. The curves show no significant differences between exposure times for either shape or magnitude.

SIC	*Birch PD_{20} (95% CI) ,SQ-U*	*Grass PD_{20} (95% CI) ,SQ-U*
End of season	*840 (464 -1519)*	*792 (400 - 1564)*
Outside season	*889 (494 -1599)*	*1020 (512 - 2031)*
% difference	*6 (-39 – 36) %*	*24 (-48 - 59) %*
p	*0.77*	*0.45*
Baseline FEV_1, p	*0.98*	*0.91*

Table 10.1 PD_{20} estimates and 95% CI-intervals based on the non-linear four-parameter regression model.

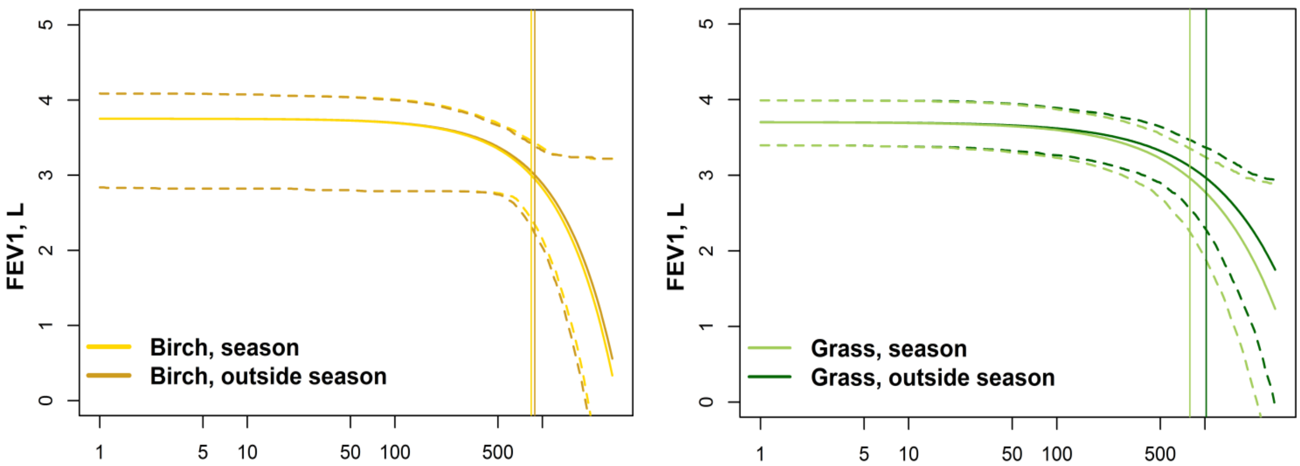


Figure 10.1 Modelled dose-response relationships for birch (left) and grass (right) with 95% confidence intervals. The vertical lines indicate the modelled PD_{20} estimates.

Implications of results

A tendency towards priming effect were seen for the grass challenges, however none of the results were statistical significant. This is not consistent with our hypothesis of seeing a priming effect in all AR participants. There are several potential contributing factors for the lack of significant findings, one being that seasonal priming may not affect the allergic bronchial response in AR participants. However, with the majority of participants having a bronchial response, one would expect priming also to affect PD_{20}. A more likely explanation is a combination of the previously discussed methodological issues (Chapter 7), and priming not affecting all with AR (Chapter 4).

One of these methodological issues is the effects of pre-priming. In this study there were two issues where pre-priming potentially affected the outcome. Two of the grass participants were also sensitized to birch, and 21 of the 36 participants were sensitized to HDM. Running the model without the two birch atopic grass-participants, alter the results by an additional 2% difference in PD_{20}% between challenges, and the p-value to 0.42, i.e. almost identical results (1069 (481;2375) SQ-U outside season and 814 (377;1758) SQ-U at the end of the season). It was not possible to run the model for a subgroup comprising only those without HDM sensitization. For birch, all but 3 had positive SPT for one or both tested HDM-allergens. For grass, this was true for 7 of the 19 participants. The differences in individual PD_{20}-estimates for out-of-season challenges show a 3-fold increase of estimates for the 7 with HDM sensitization, compared to the 12 without (p=0.06), indicating no pre-priming of HDM sensitization on the bronchial response. However, future studies should exclude participants with HDM sensitization.

Whether or not pre-priming by homologous spring flowering tree pollen has occurred in our study population was not possible to determine, however since many with birch atopy also suffer from these, it cannot be excluded. Future studies should consider excluding participants with sensitizations to these pollen, since pre-priming can blunt the results.

The natural seasonal priming of the birch participants may also have been affected by the abrupt ending of the pollen season in 2012. Since dates for the challenges were already planned, the early ending of the season, resulted in two challenges being performed 3 weeks after the season ended (ID 169 and 176). An earlier study on the effect of repeated exposures by Ihre and Zetterström found that the PD_{20}-values were almost returned to pre-challenge conditions 3-6 weeks after allergen exposures (Ihre and Zetterstrom, 1993). We therefore cannot be sure if the seasonal priming effect is still present at this time.

The asthmatic status of our participants may also have influenced the findings in this study. As previously discussed, the majority of earlier studies on the priming effect have been performed on those with allergic asthma, and hyper responsive airways (Bruin–Weller et al., 1996;Crimi et al., 1990a;Dente et al., 2000;Ihre and Zetterstrom, 1993;Paggiaro et al., 1990). However, a few studies do find effects of seasonal exposure in AR patients with no or mixed asthmatic status on either specific or non-specific challenges (Crimi et al., 1990b;Madonini et al., 1987;Walker et al., 2001). In this study the focus was on the much larger group of AR patients (Linneberg et al., 2000), who only have mild or no asthmatic symptoms.

Previous studies investigating the effect on the of repeated or seasonal allergen exposure on the bronchial response are highly variable in applied methods, choice of outcomes to evaluate and the resulting conclusions. The overall indication is however that priming effect could affect allergen PD_{20}, however not in all participants or measures.

Crimi et al (1990a) did not find any effect of seasonal exposure by Paeritaria or HDM on PD_{15}, however they did see an priming effect on the magnitude of the LAR for Paeritaria, although the co-sensitization theoretically ensured pre-priming. This supports our finding of no effect of cross-sensitization to HDM. Another study by the same author on a mixed groups of birch atopic AR and allergic asthmatic participants found a significant three-fold decrease in PD_{15} after seasonal exposure, a two-fold greater LAR, and an almost doubling of birch specific IgE antibodies, but only in those participants not suffering from asthma in a degree where they required steroids during the study period (1990b).

Dente et al (2000) investigated 15 asthmatic participants allergic to grass pollen, and found a three-fold significant decrease in PD_{20} during season compared to outside season, but only for those with dual response, ie- also LAR. The LAR did however not change, as in the study by Crimi et al (Crimi et al., 1990b).

Another study on the response pattern of 27 asthmatic participants allergic to grass pollen by Paggiaro et al (1990), also included investigations of 10 participants in- and out of the season. Here a significantly decrease in PD_{15} was found for only those who shifted from a DUAL response outside season to EAR in season.

We did not have the possibility of analyzing LAR in this study, as discussed in Chapter 7, however it appeared that our participants has a mixed response patterns. If this is a determining factor for the effect of priming, the mixed status of our group may have blunted the differences.

The association between baseline characteristics and PD_{20} showed a statistical significant correlation with the size of the SPT, with a 26% (-37; 13) decline in PD_{20} being associated with a 1 mm increase in weal size (p= 0.002). No other statistical significant associations were found. The association with SPT has previously been seen (Cockcroft et al., 1987;Paggiaro et al., 1990), and was therefore also expected here. The lack of finding for birch SPT may be due to the smaller weal sizes for this group, resulting in larger error on measurements.

It was expected to find associations between PD_{20} and non-specific bronchial hyper-responsiveness, as seen in other studies (Barnig et al., 2013). The lack of finding may be due to the method of determination of methacholine PD_{20}, based on only 44% of participants having a more than 20% fall in FEV_1, rendering this estimate perhaps not sufficiently well described (Chapter 7). It could also be due to not all those with AR, will have similar associations between non-specific and specific responsiveness (Bruin–Weller et al., 1996).

The conclusions from this study are based on the results from the non-linear regression model, where a good fit to measurements were shown. The PD_{20} estimates can therefore be considered reliable for individuals with AR with mild or no asthma, and indicate that although a priming effect appears to affect some, it is not evident in the entire group. As discussed in Chapter 7, the set-up of the study failed to account for the analysis of LAR response patterns, co-sensitizations, and induced potential errors by issues concerning the challenge protocol for non-responders, as well as the timing of challenges and pollen seasons. These issues may all have affected the results.

The focus of this study was to investigate whether seasonal priming is affecting the bronchial response in those with AR and mild or no allergic asthma. The motivation for this was to determine whether the levels of pollen concentrations currently applied in pollen information services, should be altered at the end of the pollen season compared to the onset of the season.

Future studies could include multi-center analysis on control groups from clinical studies. Many studies on drug and treatment efficacy record symptom scores or clinical measures throughout the pollen season compared to outside pollen season, on a control group as well as their study population. One example in a study on grass immunotherapy by Walker et al (2001), who also reports results on decreasing methacholine PD_{20} in season compared to outside season for the control group. This data could be applied in a multi-center study of seasonal variation in symptom development (de Weger et al., 2011).

This study could not confirm that individuals with AR should be warned at different levels of pollen concentrations at the end of the season, although a tendency towards priming was seen in some participants. Based on the literature, it appears that priming does affect the bronchial response, by either altered tolerance or altered response pattern. However the response is not identical for all, and certain endotypes appear to be present, defined by e.g. differences in response pattern, level of IgE or degree and seasonal variability of non-specific bronchial responsiveness. Future studies on the priming effect should therefore include measures of all these variables.

Chapter 11

Co-exposure to pollen allergen and ozone

Co-exposure of allergens and air pollutants is inevitable, however, the degree and effect of it is not fully known. The two studies included in this chapter examine the potential degree of co-exposure, the peak times and the effects of the co-exposure of birch- or grass-allergen and ozone.

Potential for co-exposure to air pollutants and birch or grass allergen

Airborne concentrations of both pollutants and pollen are essentially controlled by three factors; emission, dispersion and deposition. Both emissions of air pollutants from anthropogenic sources and the natural sources of pollen emissions have typical annual and diurnal patterns. This is also true for the meteorological drivers of dispersion. In this study, we therefore aimed to analyse the pattern of pollen and pollutant concentrations, in order to determine potential coincidences of peak concentrations that could lead to aggravated allergic reactions.

The analysis compared 1) the annual patterns of variation in pollutant concentration with the timing of the grass and birch pollen seasons, and 2) the diurnal pattern and concentration magnitude of pollutants with that of grass and birch pollen, on peak and non-peak pollen days. Peak days were defined as days with a daily count above a species specific threshold corresponding to the classification of "high concentration levels" applied by the Danish Asthma and Allergy Association, being above 50 grains m^{-3} for grass and 100 grains m^{-3} for birch. As in the previous studies, days with rain were excluded from the analysis. On this basis, 40% of days during the grass pollen season, and 30% during birch pollen season, were eliminated from the analysis.

The analysis included pollen and pollutant data within the time-period 1997-2012. The analysed pollutants were SO_2, O_3, NO_2, PM_{10} and $PM_{2.5}$, measured at an urban background station and two street level stations. See *Manuscript V* for further details on methods.

Ozone concentrations were at all times higher at background than street level. This is consistent with the reciprocal relationship between O_3 and NO_2, where NO_2 is formed through a reaction between O_3 and NO, a gas which is abundantly available at street level from vehicle exhaust. The inverse relationship between the two gasses was also evident in the diurnal patterns at street level. Ozone concentrations were highest during daytime, and lowest around early morning, when NO_2 peaked.

The annual variation in O_3 showed maximum concentrations from late April to early June, coinciding with the birch season, and slowly declining throughout the grass season (Figure 11.1). NO_2, SO_2, PM_{10} and $PM_{2.5}$ showed little annual variation however, a tendency for higher NO_2 street-level concentrations during the birch pollen season was observed (*see Manuscript V*).

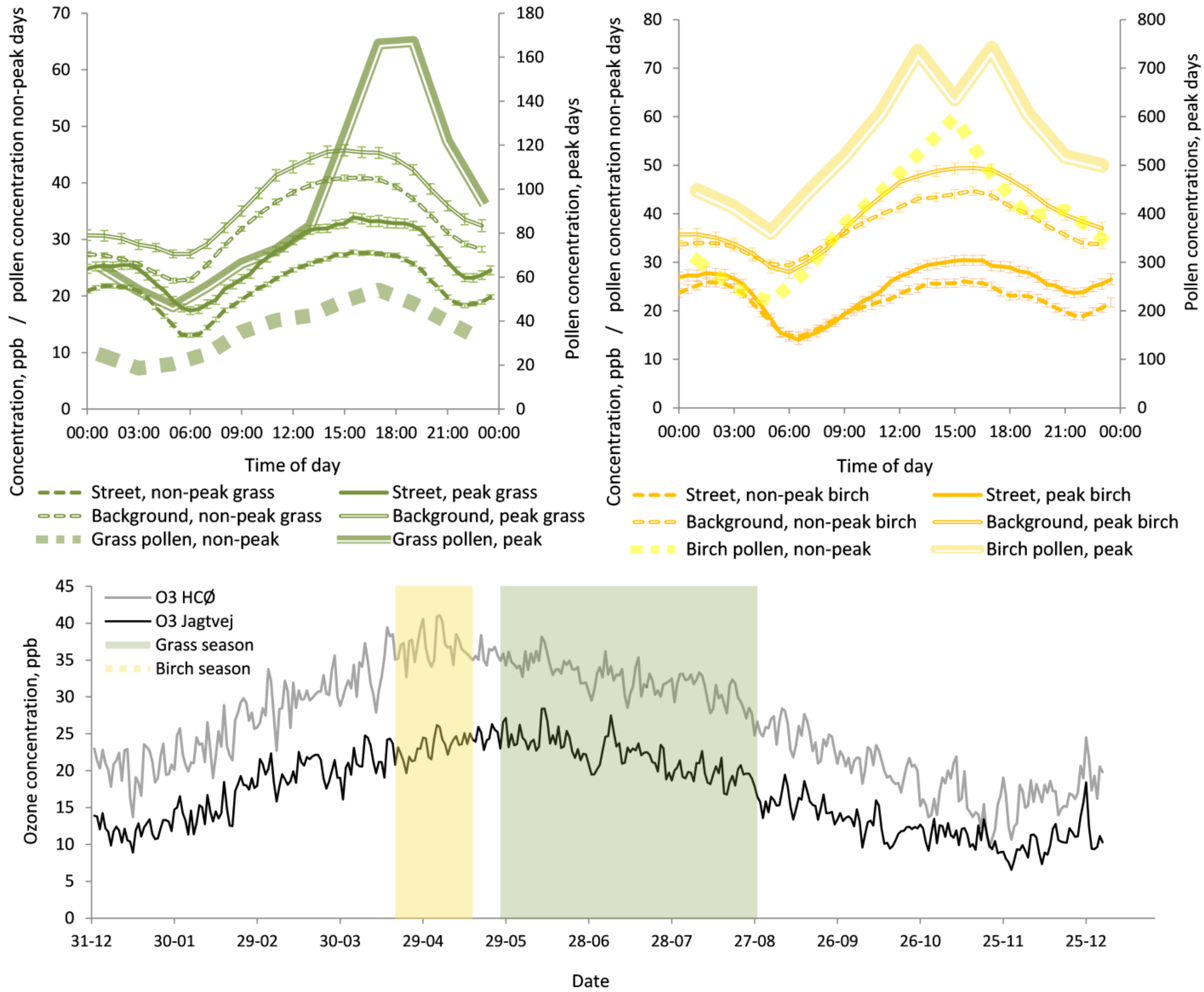


Figure 11.1 Annual and diurnal patterns of ozone. Above; Background and street level averaged from 1997-2012 with indications of the mean time of the birch and grass pollen season. Below left; Ozone levels averaged for peak and non-peak birch pollen days. Below right; Ozone levels averaged for peak and non-peak grass pollen days (Ørby et al., 2015).

Peak pollen days represented 48% of included days for the birch pollen season and 20% of the included days for the grass pollen season. The average diurnal patterns were similar on peak and non-peak days (Figure 11.1). For grass pollen, the dominating pattern indicated peak concentrations in the early evening around 18:00, and for birch, peak concentrations were seen in the afternoon around 15:00.

For the gasses, the diurnal patterns were similar in shape for peak and non-peak days during both grass and birch season. O_3 was the only gas with diurnal peak concentrations coinciding with peak pollen concentrations, with highest amounts in the afternoon and early evening at both street- and background level (Figure 11.1). Ozone levels were also statistical significant higher on peak pollen days for both pollen taxa at street level and for grass at background level. Correlations between O_3 and grass pollen concentrations were all statistically significant (r: 0.16-0.32), whereas a more mixed signal was seen for birch (see table 1 in *Manuscript V* for further details).

Discussion of results

The peak O_3 concentrations in spring may be due to stratospheric intrusions or production in photochemical reactions – the latter increasing due to the higher levels of sunlight at this time. More intense sunlight may also be the driver behind the coincidence of higher ozone concentrations and peak pollen days, where the light and heat may also be facilitating increased flowering and thus pollen release (Galán et al., 1995;Smart et al., 1979). The O_3 concentrations measured in this study are well below the levels typically applied in clinical studies of 120 ppb- 400 ppb (Hernandez et al., 2010;Jörres et al., 1996;Molfino et al., 1991;Peden et al., 1995;Peden et al., 1997;Vagaggini et al., 2002). However, it has been suggested that there is no lower limit to the effects of O_3 on the respiratory system (Húnová et al., 2013;Van Bree et al., 1995), and the levels encountered here could therefore potentially cause aggravated responses to pollen allergen.

NO_2 peak concentrations do not coincide with peak pollen concentrations for either annual or diurnal pattern. The diurnal pattern has an inverse relationship with O_3 and predominantly negative correlations between NO_2 and pollen concentrations were seen. Daily NO_2 concentrations were although higher on peak days, however the levels seen in this study of maximum 143 ppb, were substantially lower than the limit of potential aggravated allergic response of 200 ppb suggested in a review (Hesterberg et al., 2009).

SO_2 concentrations were very low, close to the detection limit, with peak hours in the early morning, not coinciding with pollen peaks. Although SO_2 has been seen to have an effect on the allergic response (Devalia et al., 1996), these have been observed at levels much higher than those encountered here.

Diurnal pattern in PM were similar for both birch and grass, and on both peak and non-peak days, with an early morning peak at street level, and invariant profiles at background level. PM_{10} concentrations were significantly higher on peak pollen days, at both street- and background level, whereas $PM_{2.5}$ only had significantly higher concentrations on peak pollen days at street level. PM_{10} were significantly correlated with grass pollen on peak days at background level and $PM_{2.5}$ with birch pollen on peak days at street level.

The higher PM concentrations on peak days are not likely to be due to pollen fragments, as the majority of allergen has been found in the fraction above PM_{10} in a study on birch pollen (Buters et al., 2010), and the same is likely to be true for other pollen types. During the birch season, the higher levels may be due to ammonium-type particles, which also peak during warm conditions (Skjøth and Geels, 2013), and during the grass pollen season, fungal spores may contribute, as they are as little as 3 μm (Reponen et al., 2001). The limits of PM for aggravating effects on the allergenic response is be very dependent on the chemical composition of the particles

The only other study that was found on the correlations between pollen and air pollutants, was conducted by Voukantsis et al (2009), who applied computational intelligence methods on measurement from Finland, and also found correlations between birch pollen and ozone concentrations. They also saw correlations between ozone and Salix pollen, and between ozone and particulate matter, with Alnus and Picea pollen, pollen types not investigated in this study, but also present in Denmark.

The results from this study indicate that high ozone concentrations coincide both annual and diurnal with high grass and birch pollen concentrations, and that ozone levels are higher on peak pollen days. Although no clinical studies have proven an aggravating effect at the concentrations present in Copenhagen, neither has any lower limit been seen. Particulate matter was seen to be higher on peak pollen days, and has also been proven to induce adjuvant allergic responses. However, no coinciding peaks were found. Further information on the chemical composition of the particles would be needed to conclude whether these constitute a risk factor for aggravated allergic reactions. Neither NO_2 nor SO_2 were seen to have coinciding peak times with pollen, or to be at levels high enough to potentially induce adjuvant allergic responses.

Bronchial effects of co-exposure to ozone and birch or grass allergen

Following the above conclusions, the main objective of this clinical study was to examine whether simultaneous exposure to potentially naturally occurring levels of ozone could induce an aggravated response to allergens. As in the study on the priming effect of seasonal exposure, secondary objectives were to assess the application of a non-linear regression model, and the associations between the bronchial response and baseline characteristics.

As previously discussed, ozone may induce inflammation in the upper and lower airways, hereby having a potential priming effect on the bronchial response to allergens. This effect may also be evident in individuals with AR, with primarily upper airway symptoms, according to the theory of the "United airway". The methods applied in this study are described in Chapter 7, and in *Manuscript VI*.

When examining the priming effect of ozone, pre-priming by outdoor ozone exposures prior to study days could affect results, and monitoring data from a nearby background station were therefore analysed. One exceedance of the EU target value of 120 μg m^{-3} (61,125 ppb) for the protection of human health occurred within 14 days before each study day (Danish Air Quality Monitoring Program (Hertel et al., 2007)). Three participants were challenged 13 days after this exceedance. This was neither a prolonged nor very high episode, and therefore no influence from outdoor ozone concentrations is assumed. No significant difference in baseline FEV_1 between SICs with allergen alone and with co-exposure to O_3 was seen (Table 11.1).

SIC	Birch PD_{20} (95% CI)	Grass PD_{20} (95% CI)
Allergen alone	739 (426-1283) SQ-U	1092 (517- 2305) SQ-U
Co-exposure to ozone	906 (517- 1593) SQU	836 (397- 1759) SQ-U
p	0.35	0.14
Baseline FEV1, p	0.81	0.96

Table 11.1 PD_{20} estimates and 95% CI-intervals based on the non-linear four-parameter regression model.

The non-linear regression model was fitted to data from 85 SIC´s, and estimates of PD_{20} were produced with 1) the assumption of no difference between the SICs performed with co-exposure to ozone and those without, and 2) allowing for a difference between the two exposures. No statistical significant difference between the two models were found (birch p=0.17, grass p=0.08).
Neither was any statistical significant differences found between the PD_{20} estimates for the two exposures, for either allergen (Table 11.1).

The majority of SICs resulted in a reduction of more than 20% in FEV_1. For allergen alone, 82% of birch and 76% of grass participants dropped more than 20%. For the SIC´s during co-exposure to ozone, the numbers were 71% of birch and 74% of grass participants. The data were therefore estimated to be sufficient for modelling a PD_{20} estimate.
Figure 11.2 shows the estimated log-dose-response curves based on the best fit of the four parameter non-linear regression model to the measurements from the 85 SICs. Curves are shown for both allergen alone and co-exposure to ozone, for birch and grass allergen challenges separately. The curves show no significant differences in either shape or magnitude.
The association between baseline characteristics and PD_{20} showed a statistically significant correlation with the size of the SPT, with a 1 mm increase in weal size being associated with a 27% (-40; 12) decline in PD_{20} (p= 0.049) (Figure 11.3) . No other statistically significant associations were found. This is consistent with the finding in the study of seasonal priming effect, and has been discussed in the previous chapter.

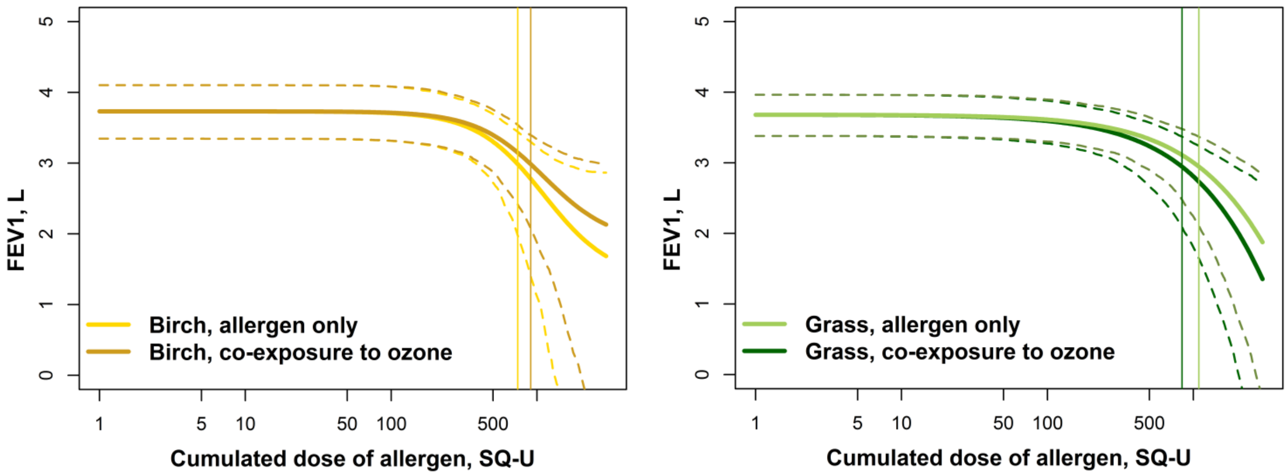


Figure 11.2 Modelled dose-response relationships for birch (left) and grass (right) with 95% confidence intervals. The vertical lines indicate the modelled PD20 estimates.

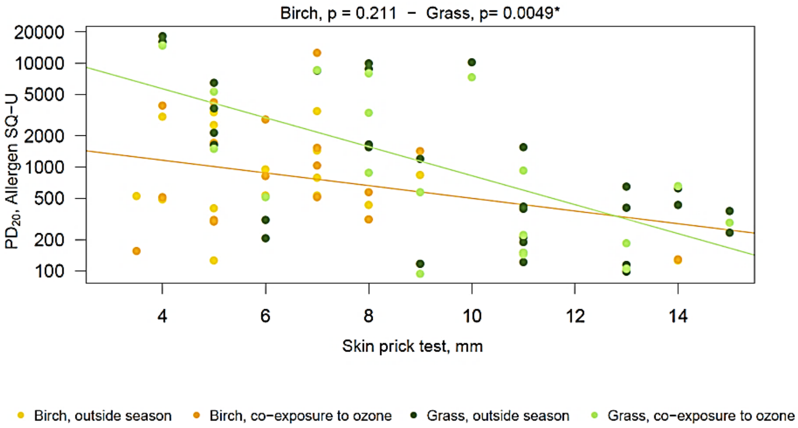


Figure 11.3 Association between SPT and allergen PD20.

Methodological issues affecting this study have been discussed in Chapter 7. The issue of the potential order effect, that could have induced a carry-over effect in the co-exposure to ozone challenge, did not appear to affect the results, and did not induce a lower PD_{20} in these.

The effects of co-exposures to ozone and allergens can either be studied as the effects of natural exposures estimated by modelled or measured ozone and pollen amounts, or in a controlled setting. In this study, the climate chamber provided a controlled environment of uniform and identical ozone exposures throughout the study (120ppb (sd. 1.8)). The weakness of this exposure setting is the limitations related to exposure time. In this study, participants were exposed for approximately 20 minutes prior to the onset of the SICs, and thereafter during the time of the examinations and challenges, for approximately 3 hours, i.e. solely simultaneous exposure.
Previous similar studies on co-exposure of ozone and pollen allergens are not consistent in results (see Chapter 5).
Effects of ozone exposures on the allergic response was found in studies with 120 – 250 ppb, showing significant lower doses required to elicit a defined reduction in FEV_1 (Jenkins et al., 1999;Jörres et al., 1996;Kehrl et al., 1999;Molfino et al., 1991). For all, but the study by Molfino et el., there are major differences from these to the current study. All participants were mildly asthmatic, exposure concentration was higher, exposure duration longer and participants were exercising, increasing inhalation. Also, the majority of participants were HDM allergic, i.e. potentially in a constant state of airway inflammation.
Other studies have, as this study, not been able to show significantly higher bronchoconstriction responses to allergen after exposure to ozone (Ball et al., 1996;Chen et al., 2004;Hanania et al., 1998), as well as some did not find an effect in parts of their study, e.g. examining a group of AR participants (Jörres et al., 1996) or concentrations of 100 ppb (Jenkins et al., 1999).

The short exposure duration, and simultaneous exposure, may have been a contributing factor to the lack of significant response seen in this study. As ozone exposures of the magnitude and duration

applied in this study, has been seen to have little or no effect on FEV_1 (Kehrl et al., 1999;McDonnell et al., 1997), any aggravated response would primarily be induced by the increased inflammatory state. A lag between ozone exposure and allergen challenge may therefore have induced different results. Chen et al and Ball et al also performed challenges immediately after exposures (Ball et al., 1996;Chen et al., 2004), and found no increased response, where Hannania et el performed challenges the following day, and also did not find a significant effect of the exposure to 120 ppb of ozone (Hanania et al., 1998). So the lag-time may be of importance, but the magnitude of the lag and concentration level is most likely relevant.
There is little doubt about the cause and effect of ozone as a primer, however, how much, how long, and who it affects, appears to still be "up in the air".

The results from this study found no statistical significant differences in PD_{20} or any significant differences in the shape or magnitude of the dose-response profiles. Therefore, it appears that there is no additional effect of simultaneous co-exposure to 120 ppb of ozone on the bronchial reactivity in allergic rhinitis participants with little or no allergic asthma.

Implications of results

The main objective of the studies of co-exposure to allergen and air pollutants was to determine whether there are potential risks of adjuvant effects on the allergic response from amounts of co-exposure experienced in Denmark. The results from the two included studies indicate that this is not the case.
There are many conditions attached to this conclusion, both regarding the timing and duration of exposure, and regarding the asthmatic status of those being exposed.
It was unclear from the current studies whether particulate matter is a risk factor for aggravated allergic responses in Denmark. For this to be further investigated, composition analysis needs to be conducted.

Although the level of ozone encountered in Denmark do not appear to elicit aggravated allergic responses, the concentrations encountered in mid and southern Europe are much higher, with events of 85 ppb not being uncommon (Jonson et al., 2006), and with multiple high O_3-episodes resulting in more than 25 days per year exceeding the EU target value for human health (maximum daily 8–hour mean > 120 $\mu g\ m^{-3}$) (Guerreiro et al., 2016). Potential co-exposure in south Europe from higher ozone concentrations may coincide with the earlier seasons for hazel or alder in these regions, allergenic species shown to be cross-reactive with birch, and with other species such as grass.

These studies focus on simultaneous co-exposure, but either allergen or ozone could act as a primer, and shifted co-exposures in any order could result in an aggravated response (see Chapter 5). The duration of ozone exposure may also be of importance, and perhaps the effect is not seen after short duration of exposures, and during the exposures, as in the here presented study. A lag may be needed to see the effect of the potentially increased inflammation.

Future studies could potentially examine the effect of long term exposure, for example in a large scale register based study comparing pollen sensitized persons living under different ozone exposures. However, since pollutants may affect the allergenic response by other mechanisms than a direct effect on the respiratory system, methods for differentiating between these would have to be applied. As discussed in Chapter 5, pollutants may also affect the plants and pollen production, degrade the pollen exine increasing the bio-availability, or even alter the allergen into a different and more allergenic protein (Shahali et al., 2009).
Also, the effect of prior exposures to prolonged and high allergen exposure could be relevant and future studies on for example persons with allergies towards indoor allergens could be of relevance.

This study examined the pollutant levels compared to a single pollen pattern. This is slightly contradictive to the findings in the previous studies on the variability in the grass pollen profile. However, as the profile here seen, is presenting a single evening peak, this pattern is consistent with both the most frequent pattern seen in Copenhagen, and in Aarhus during the peak season, and can therefore be considered a valid foundation for estimation of the main peak incidents of co-exposures.

Even though co-exposure to pollen and pollutants has been examined and discussed for decades, and adjuvants effects of this cocktail effect has been found for some compounds and concentrations, currently pollen and pollution warning systems do not interact. Although our study did not support the idea that air pollution is an important factor to account for in a Danish symptom forecasting system, it appears from literature, to be very relevant in other locations of the world.

Part V - Concluding remarks

Chapter 12

Evaluation, conclusions and further investigations

Impact of methodological issues on the conclusions

Pollen concentration measurements

The methodological issues regarding the pollen sampling was discussed in Chapter 6.
Pollen sampling is continuously performed according to this method, and it is a reliable, resistant, and consistent source of a proxy for airborne pollen concentrations. However, there are issues with the method, which needs consideration when evaluating the results from the included studies.

Results from the study in Aarhus could have been affected by variability between different traps, and if there are differences, the pooling of the data could affect the diurnal patterns, as measurements from the three traps do not contribute equally to the three sections of the season. However, an inspection of data revealed no relevant differences for this analysis.

The pollen concentrations in the cluster analysis are all standardized, and any overestimation of the magnitudes as a result of a potential error in flow measurement, would not affect the pattern to a degree, where the results are highly affected.
Any error introduced by the counting of only a part of the slide, would affect the pattern on low count days the most. In the included studies, days with less than 20 grains m^{-3} were excluded. However, this could have affected the pattern more on low count days, i.e. therefore more at the end and beginning of the season and a potential effect cannot be excluded.

Although the method could have introduced bias and errors in the pollen counts, the potential issues related to the method of pollen measurements are as a whole not estimated to have affected the presented conclusions.

SIC measurements

The methodological issues regarding the SICs was discussed in Chapter 7.
The method of performing SIC has been evaluated in a number of studies, and general recommendations are presented. However, during the planning and during completion of a clinical study, conflicting considerations and practical issues, may result in not all recommendations being possible. The here presented study was in addition considered a pilot study, and some of the issues discussed in Chapter 7, should be addressed before future studies are performed.

The decision to apply a variable protocol based on response was based on considerations regarding safety and avoidance of fatigue. This could have affected our results, as the maximum dose was not given in all relevant cases. The effect of this issue could have had an impact on the results, as it affected 20 of the 121 conducted SIC´s.

Timing of SICs, both related to season and intervals between SICs were not optimal. The recruitment of poly-sensitized subjects could blur the effect of the allergen of interest. However, as the timing-issues only affected a few challenges, and as no effect were seen for HDM co-sensitization , these issues are thus not expected to have had a major impact on the conclusions of the study.

The group was heterogeneous in asthmatic status, and this could have affected the results, as an effect of both seasonal allergen priming and ozone may have been seen in those more hyper-responsive. Subdividing according to asthmatic status would potentially have revealed an effect. Measurements of LAR would also have aided the analysis, and could potentially have revealed endotypes of responding participants. However, as the focus was to investigate whether an effect could be seen in AR patients as a group, the objective of the study was met.

Although issues related to the applied method could have affected the estimated PD_{20} and dose-response curves, these are as a whole not believed to have significantly affected the presented conclusions according the presented objectives.

Conclusions and further investigations

The aim of this thesis was to combine and add to the research on pollen within Health and Environmental Science, to achieve a better understanding of a few pieces in the puzzle for symptom relief for those with pollen allergies.

For this, four major objectives were set. The first objective was;

- *To investigate whether there are large intra-urban variations in grass pollen concentrations, and if these could be linked to variations in the source distribution.*

Daily grass pollen concentrations were seen to vary between three traps in Aarhus, and not to correlate with measurements at the nearest operational monitoring station. The variation in pollen concentrations could be linked to the spatial distribution of grass pollen sources in the city of Aarhus. This was confirmed by analysis of air movements and by building and analyzing a detailed map of potential sources. The local variations are therefore most likely due to the non-homogeneous distribution of sources and thus proximity to sources will be the main factor for local concentrations.

This confirms, that the daily pollen count at Viborg and Copenhagen solely provide the daily pollen concentration in close vicinity of these exact sites. Placement of pollen traps in all regions of the country, will therefore not contribute to a complete knowledge of daily local pollen concentrations for the public, or be applicable as exposure proxy in clinical studies linking to daily symptoms. However, expansion of the current monitoring will provide a much needed and substantial improvement in data for future pollen and symptom forecasting systems.

The current study is to the author's knowledge the only local-scale study applying source analysis including management and Remote Sensing images.
Currently, only one year of data has been analysed, and further studies should include all three years of monitoring, as well as expanding the analysis to include birch pollen concentrations and source map for this taxa. Previous studies have mainly focused on larger scale source-mapping, and the development of local scale mapping and modelling of local scale pollen concentration variation is an important field for exposure assessments.

The second objective was;

- *To analyse the variability in the diurnal time of peak in grass pollen concentrations and whether this depend on the seasonal progression of different grass species flowering.*
    - *To further investigate the diurnal patterns grass pollen concentrations, via examining whether statistical clustering may define distinct diurnal patterns, and if so, whether these are related to meteorological factors and vary between sites.*
    - *A further objective in both studies was to examine whether potential differences in the profile were driven by differences in meteorological factors.*

The diurnal pattern of grass pollen was found to be highly variable, as seen in data from three sites in Aarhus, Copenhagen and Córdoba. Although the diurnal peak was found to occur at all times of the day, a single early evening peak was the most frequent pattern in Copenhagen, and also the most frequent pattern in Aarhus during the peak pollen season. For Córdoba peaks in the late afternoon were seen to be the most frequent. Current advice for allergen avoidance should therefore focus on these hours, and on emphasizing the high degree of variability.
Separating the season into periods or clusters provides a better foundation for analysis of the drivers, as the relative importance of them may shift. However, no clear connection with meteorology was seen in either analysis, and although our results support the hypothesis of the importance of differences between grass species, further studies will need to be performed to confirm this.
Previous studies on variability of diurnal pattern of grass pollen has not tested frequency of the patterns to establish the dominating patterns. The main-season, peak day-patterns may therefore dominate the pattern for the whole season.
The current studies are the only presenting diurnal variations of grass pollen concentrations for Denmark, and the only applying cluster analysis on grass pollen patterns.
Further studies should include phenological recording of grasses around the monitoring site as this could support theory of the succession of flowering species as the main driver.
In the methods applied in this thesis, only patterns have been examined. Further studies could also examine the variability between days of moderate and high pollen concentrations.
For Aarhus, the pattern was seen to change over season. No effect of DOY was seen in Copenhagen or Cordoba, however, the analysis need to be expanded to allow for the variability between years, if this is to be fully examined for these sites.

The third objective was:

- *To investigate whether the natural seasonal priming effect will result in greater bronchoconstriction at the end of the pollen season compared to outside pollen season.*

There does not appear to be a significantly larger bronchial response to pollen allergen by the end of the season compared to outside the season for all individuals with AR. There was however, a tendency towards priming in those challenged with grass. This is consistent with previous studies where a priming effect is neither seen in all AR patients. Based on literature as well as the current results, it therefore appears that priming does affect certain endotypes of individuals with AR.
Current advice given to AR patients should therefore be, that priming may occur for some, and patients should observe own pattern of response and act accordingly.
The study adds on current knowledge by suggesting a novel methodology for estimating PD_{20}, and providing dose-response curves for both grass and birch allergen, in and out of season.
Future studies should further examine whether the priming effect will have an impact on the response of certain endotypes, e.g. those with LAR, high non-specific bronchial responsiveness or high IgE.

The fourth objective was:

- *To analyse whether seasonal and diurnal patterns of pollens and pollutants peaks coincide, and is above known thresholds of exacerbated responses.*
    - *To further investigate the impact of air pollutants on the allergic response, by investigating the effect of co-exposure of potentially naturally occurring levels of ozone, by specific allergen challenges and either co-exposure to allergen or with allergen alone.*

The co-exposure to grass or birch allergens and air pollutants in Denmark was *a priori* estimated to be a potential risk factor for ozone. However, the clinical study applied concentrations higher than those experienced in Denmark, and found no aggravated bronchial allergic response in induviduals wiht AR.
The study supports previous findings on the effects of co-exposure to ozone and grass or birch pollen, where short exposure time of low levels are not seen to induce aggravated bronchial response.
The concentrations of ozone may however be higher in Southern Europe, as well as longer duration of exposure and more severe asthma may induce an aggravated allergic response in some individuals.
Air pollutants should therefore not be the first priority to include in a future symptom forecasting system in Denmark, but may however be important in other parts of the world.
Also, this study did not find any known thresholds of concentrations of particulate matter (PM) and aggravated allergic effect, and this have been shown to affect the allergic response via several mechanisms. Future examinations of e.g. the composition of PM in Denmark, is needed to determine whether this poses a threat to individuals with AR in general.
As for the study on priming effect, this study adds to the field with a novel estimation of PD_{20} and dose-response curves for birch and grass.

Summarized conclusion

There is large spatial variability in grass pollen concentrations, also within the urban area, and this is linked to the distribution of grass sources, where management is an important factor when considering source potential. Also the diurnal pattern of grass pollen is highly variable, and a single average profile will not reflect this. Seasonal periods or clustering can reveal the most frequent patterns, and also potentially improve further analysis of likely drivers. In this study, simple correlation with meteorological parameters or estimation of potential variation in the grass species time of flowering did not however render any conclusive results. Peak concentrations in Denmark were most frequently seen in the early evening.

For individuals with AR, no systematic effect of either seasonal allergen, or ozone priming were seen on the bronchial response. However, both mechanisms are well established and they may affect certain endotypes of individuals with AR. The novel application of a non-linear model, was a good fit to data, and could be applied in future studies.

The combined information for individuals with pollen allergies is therefore that, the daily pollen count is not representative of the local experienced exposure, and no time of day is without risk of high exposures. However, peak time for grass pollen, is most frequently at 17:00-19:00.

The bronchial symptoms is not affected of seasonal priming in individuals with AR, neither is the effects of ozone air pollution. However, both could have an impact, and individuals with AR should observe own pattern of response and act accordingly.

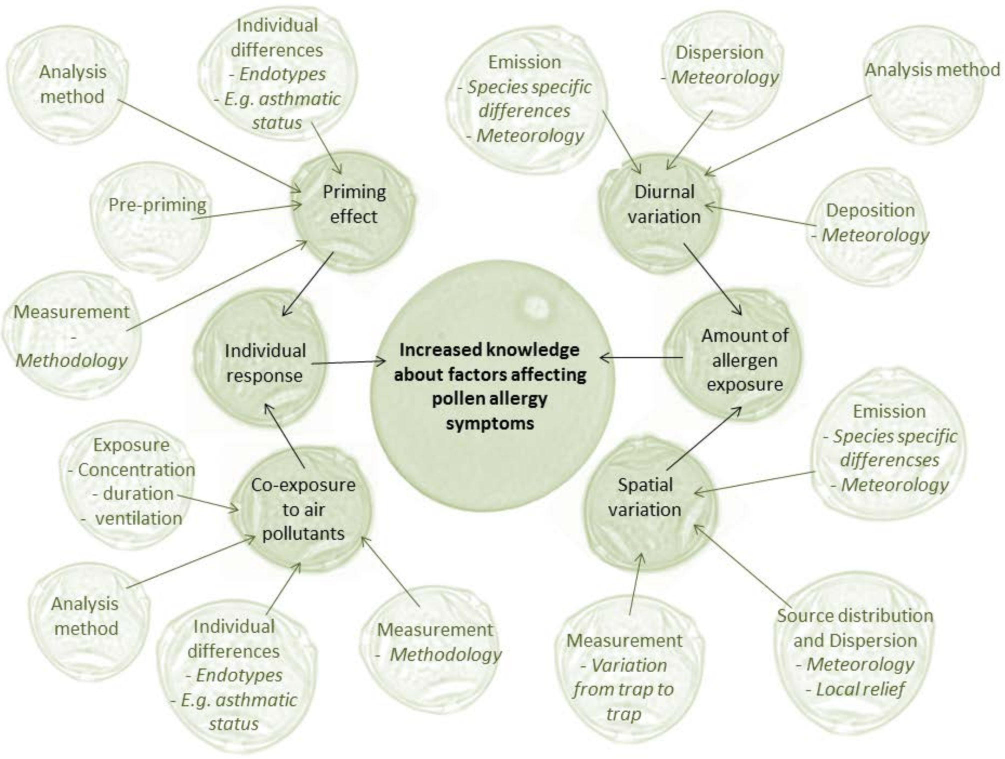


Figure 12.1 There are many aspects affecting the symptoms in those with pollen allergy. This thesis has presented results, considerations and suggestions for future research regarding some of these.

Concluding remarks

Ultimately, the goal for much research in this field is to aid in symptom relief for those with pollen allergy. However, the path to this goal is paved with a multitude of challenges, and maybe the most direct track, it yet to be discovered.

If we consider all the steps in the chain of processes resulting in the symptoms, we still encounter many unknowns. Pollen counts is, at best, indicative of allergen exposure, due to high spatial variability, and potential presence of free allergens. Even if exact allergen dose could be determined, this is not equal to symptoms severity. Symptoms are highly individual, and affected by a range of factors. If we wish to relief the symptoms, the current best option is medication and allergen avoidance, and both would benefit from a prediction of the exposure and symptom severity for the days ahead.

Hyposensitization and vaccination is a short-cut to symptom relief, but not possible or efficient for all, and the medication can be associated with side effects. We therefore still need to proceed in the search for other paths. One of these is a functional symptom forecasting system.

Systems like this need improved models, and for this, we need improvements in input data and to fill some of the knowledge gaps concerning the mechanisms. It is important also in aerobiology to focus not only on correlation but also causality. We can determine many statistical correlations, however without the driving mechanisms the results will not be transferable or at worst case, they may just be the results of the method applied.

It is my belief, that a cross-disciplinary understanding is necessary to better determine which gaps to prioritize, and to combine the many scientific fields. Many pollen researchers are therefore, to some degree, cross-disciplinary.

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Appendix I

Dactylis glomerata, ES

Inflorescence 1	1-1-1	1-1-2	1-1-3	1-2-1	1-2-2	1-2-3	1-3-1	1-3-2	1-3-3
Count	452	434	440	485	497	333	341	287	373
Total pollen	5424	5208	5280	5820	5964	3996	4092	3444	4476
Average 1 (std. dev)	4856	(884)							
Inflorescence 2	2-1-1	2-1-2	2-1-3	2-2-1	2-2-2	2-2-3	2-3-1	2-3-2	2-3-3
Count	323	212	353	263	330	400	368	286	272
Total pollen	3876	2544	4236	3156	3960	4800	4416	3432	3264
Average 2 (std. dev)	3743	(706)							
Inflorescence 3	3-1-1	3-1-2	3-1-3	3-2-1	3-2-2	3-2-3	3-3-1	3-3-2	3-3-3
Count	250	262	300	334	704	198	360	300	442
Total pollen	3000	3144	3600	4008	8448	2376	4320	3600	5304
Average 3 (std. dev)	4200	(1800)							
Average (std. dev)	4266	(1268)							

Dactylis glomerata, DK

Inflorescence 1									
Count	828	308							
Total pollen	9936	3696							
Average 1 (std. dev)									
Inflorescence 2									
Count	789	496							
Total pollen	9468	5952							
Average 2 (std. dev)									
Inflorescence 3									
Count	80	128	84	85	156	85	359	439	160
Total pollen	960	1536	1008	1020	1872	1020	4308	5268	1920
Average 3 (std. dev)	2101	1587							
Average (std. dev)	3690	(3167)							

Alopecurus pratensis, DK

DK									
Inflorescence 1	1-1-1	1-1-2	1-1-3	1-2-1	1-2-2	1-2-3	1-3-1	1-3-2	1-3-3
Count	271	225	317	339	362	313	276	279	304
Total pollen	3252	2700	3804	4068	4344	3756	3312	3348	3648
Average 1 (std. dev)	3581	(489)							

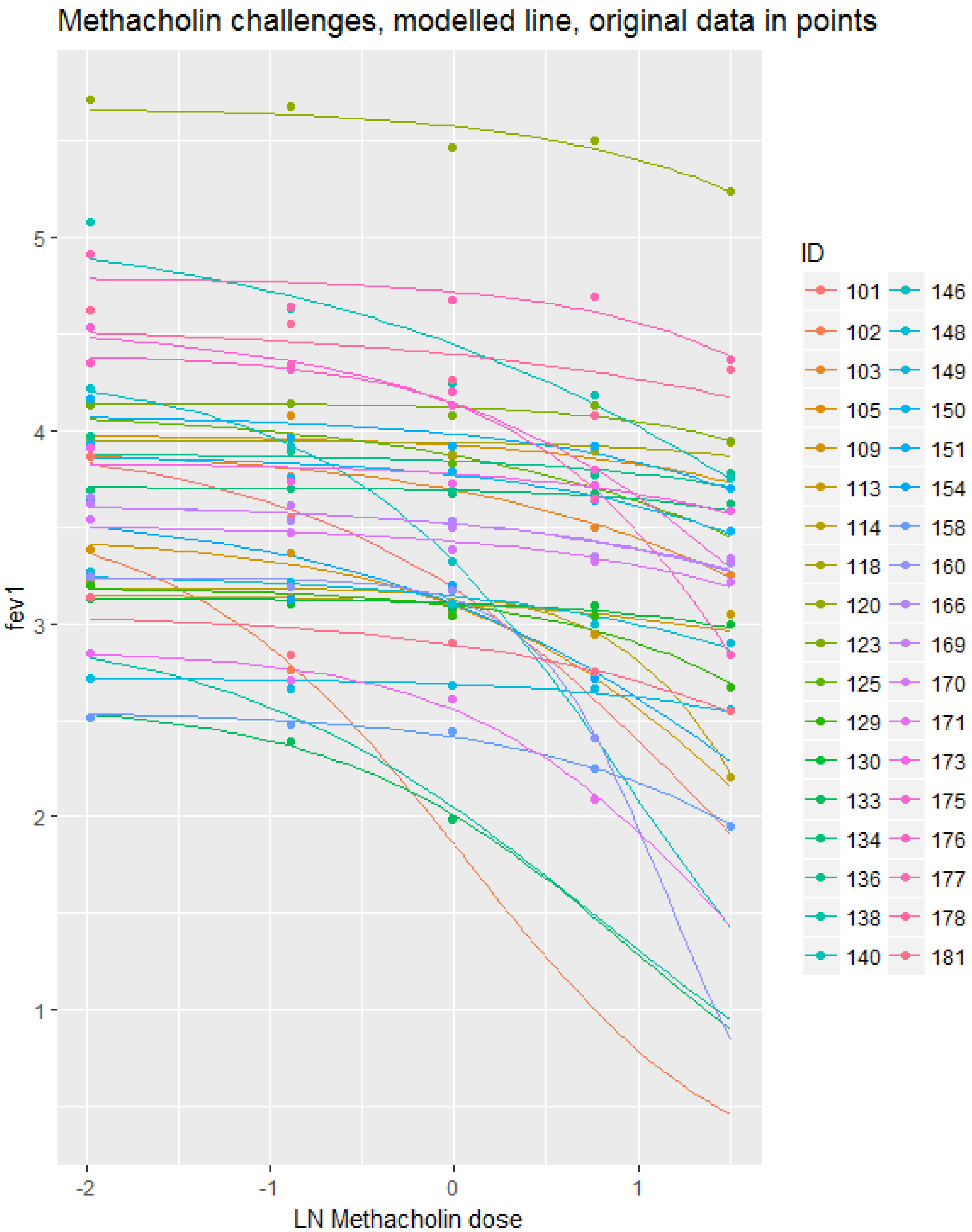
Appendix I Partial results from an initiated study on pollen production in grasses. Estimation was planned to be made of pollen amount in anthers from species growing in minimum two of the three countries; Denmark, UK and Spain. Due to the discovery of uncertainty of the method, the results were not complete, and only pollen amount for some of the anthers were estimated.

Appendix II

	ID	Height	Weight	M/F	Filtered air 1	Filtered air 2	Ozone	Age	PD_{20} M.	% pred. FEV_1.	Grass	Birch	N. all.	HDM
Grass	101	189	79	M	24-11-10	27-05-10	07-12-10	25.3	0.8	77	15		6	6/10
	102	170	58	F	23-11-10	18-05-10	07-12-10	21.0	0.3	112	11	4	5	**
	103	170	61	F	23-11-10	18-05-10	07-12-10	21.0	4.3	108	9		3	**
	105	193	80	M	01-12-10	18-05-10	07-12-10	20.8	13.3	75	6		2	**
	109	168	64	F	24-11-10	26-05-10	13-12-10	23.5	1.6	102	11		1	**
	113	168	62	F	23-11-10	19-05-10	07-12-10	20.0	15.4	97	11		2	**
	114	160	60	F	28-02-11	27-05-10	13-12-10	24.3	3.2	110	5		3	*/3
	118	173	69	M	01-12-10	19-05-10	08-02-11	25.5	20.8	94	14	2	4	**
	120	194	90	M	24-05-11	19-05-10	08-02-11	28.5	10.4	106	4		1	**
	125	187	70	M	24-11-10	19-05-10	07-04-11	24.5	4.7	79	13		1	**
	129	175	68	M	28-02-11	18-05-10	08-02-11	23.0	4.8	71	13		2	**
	133	164	64	F	01-12-10	26-05-10	07-12-10	26.0	0.7	98	5	3	4	4/9
	134	169	53	F	24-11-10	26-05-10	13-12-10	26.0	16.0	107	8		3	3/*
	136	171	87	F	24-03-11		07-04-11	27.0	19.0	107	8		6	4/8
	146	181	80	M	23-03-11		07-04-11	24.3	0.7	85	10		4	*/7
	148	182	75	F	23-03-11		24-05-11	25.0	7.0	74	7		3	**
	149	169	95	F	23-03-11		24-05-11	29.3	11.2	75	8		6	4/7
	150	182	70	M	24-03-11		07-04-11	20.3	7.8	83	11		1	**
	151	173	90	M	24-03-11		07-04-11	24.0	8.9	101	9		1	**
Birch	123	175	70	F	22-02-11		16-03-11	24.1	12.0	108	13	6	4	6/6.5
	130	155	65	F	22-02-11		10-03-11	27.7	13.2	108	9	7	4	*/4.5
	138	176	115	M	30-03-11		15-03-11	26.0	0.4	84	6	3.5	5	7/7
	140	183	102	M	01-03-11		15-03-11	21.1	1.6	105	12	5	7	8/9
	154	174	63	F	01-03-11		12-04-11	25.1	1.3	110	11	8	8	6/7
	158	169	65	F	02-03-11		16-03-11	23.1	3.1	74	10	4	7	3/5
	160	163	56	F	30-03-11		10-03-11	23.0	1.8	106	7	6	5	**
	166	177	87	F	08-02-12		23-02-12	23.1	9.1	91	10	7	5	**
	169	170	66	F	23-02-12		08-03-12	29.1	9.8	103	7	7	5	*/9
	170	170	67	F	08-02-12		29-02-12	22.6	9.4	99	12	5	6	6/6
	171	168	106	F	08-02-12		29-02-12	24.2	1.4	81	11	5	8	5/8
	173	178	74	M	23-02-12		08-03-12	25.6	11.8	85	15	4	5	5/8
	175	189	74	M	23-02-12		08-03-12	25.2	2.2	85	6	14	7	5/5
	176	176	77	M	08-02-12		23-02-12	27.2	2.2	98	16	5	6	2/5
	177	182	87	M	07-03-12		22-02-12	22.2	8.6	105	7	7	6	5/11
	178	170	70	M	07-03-12		22-02-12	21.1	16.2	114	32	8	8	7/26
	181	170	59	F	08-02-12		29-02-12	23.6	4.4	91	20	9	4	*/*

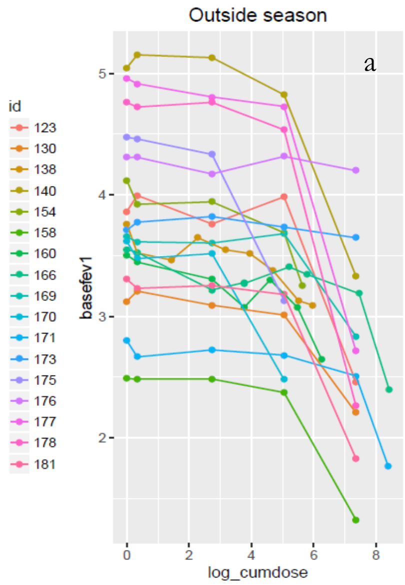
Appendix II Participants characteristics and dates of challenges. M/F: Male/Female. PD_{20}M: Methacholine PD20 estimate. % Pred.FEV1: % predicted baseline FEV_1. Grass, Birch: Size of SPT wheal in mm. HDM: SPT wheal size in mm of *Dermatophagoides farina*/ *Dermatophagoides pteronyssinus,* ** no reaction.

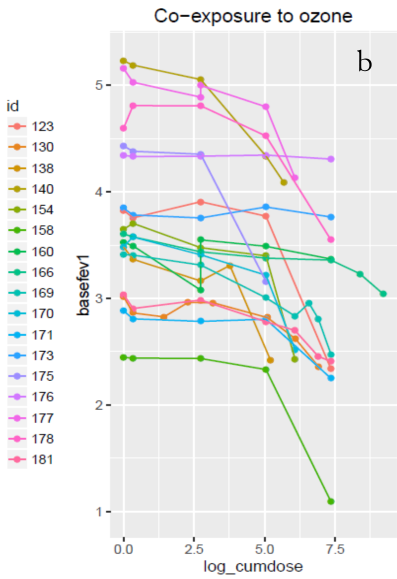
Appendix III

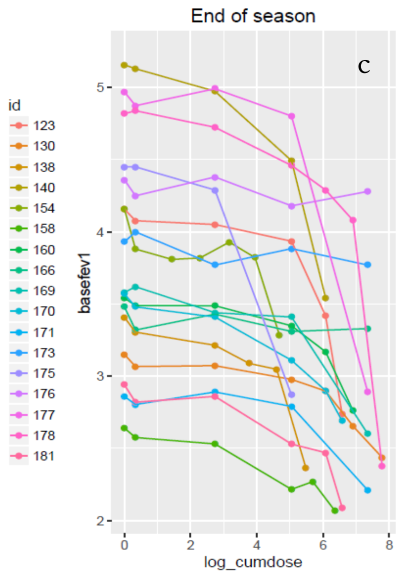


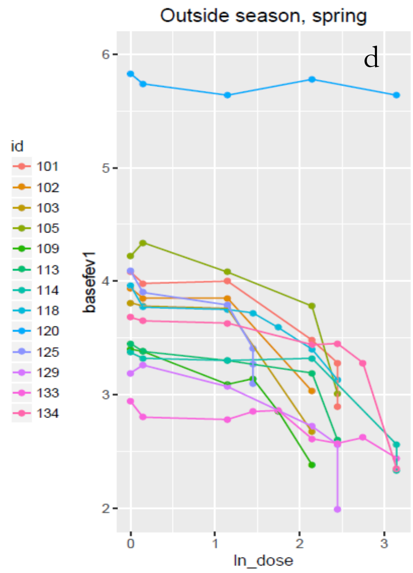
Appendix III Estimated individual methacholine dose-reponse curves based on the non-linear-model. Numbers indicate participant ID´s. Dots are the measured points.

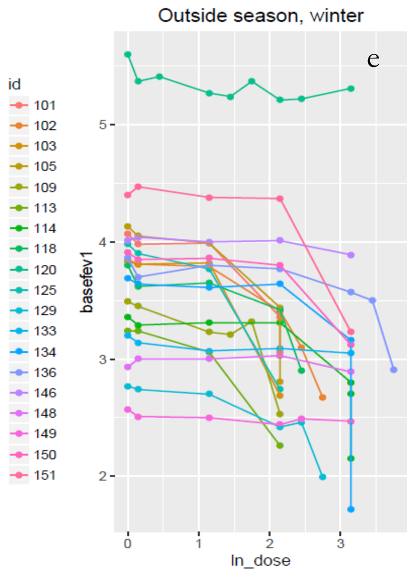
Appendix IV

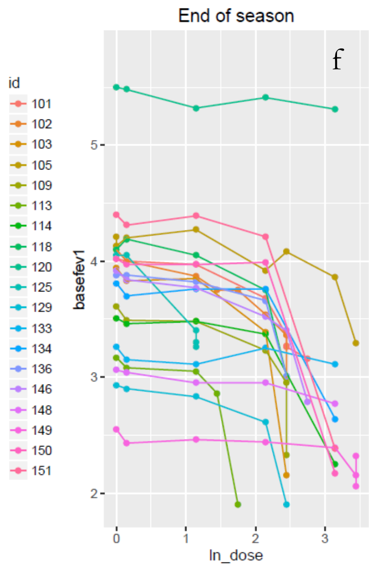


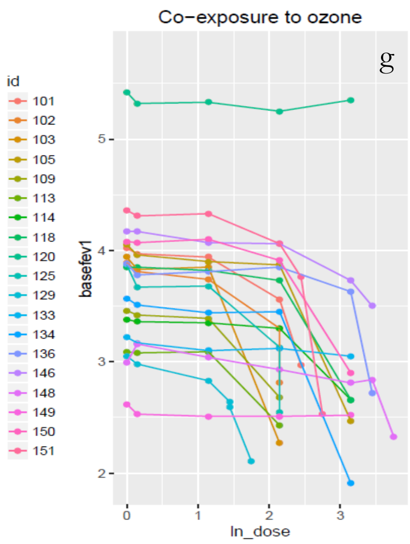






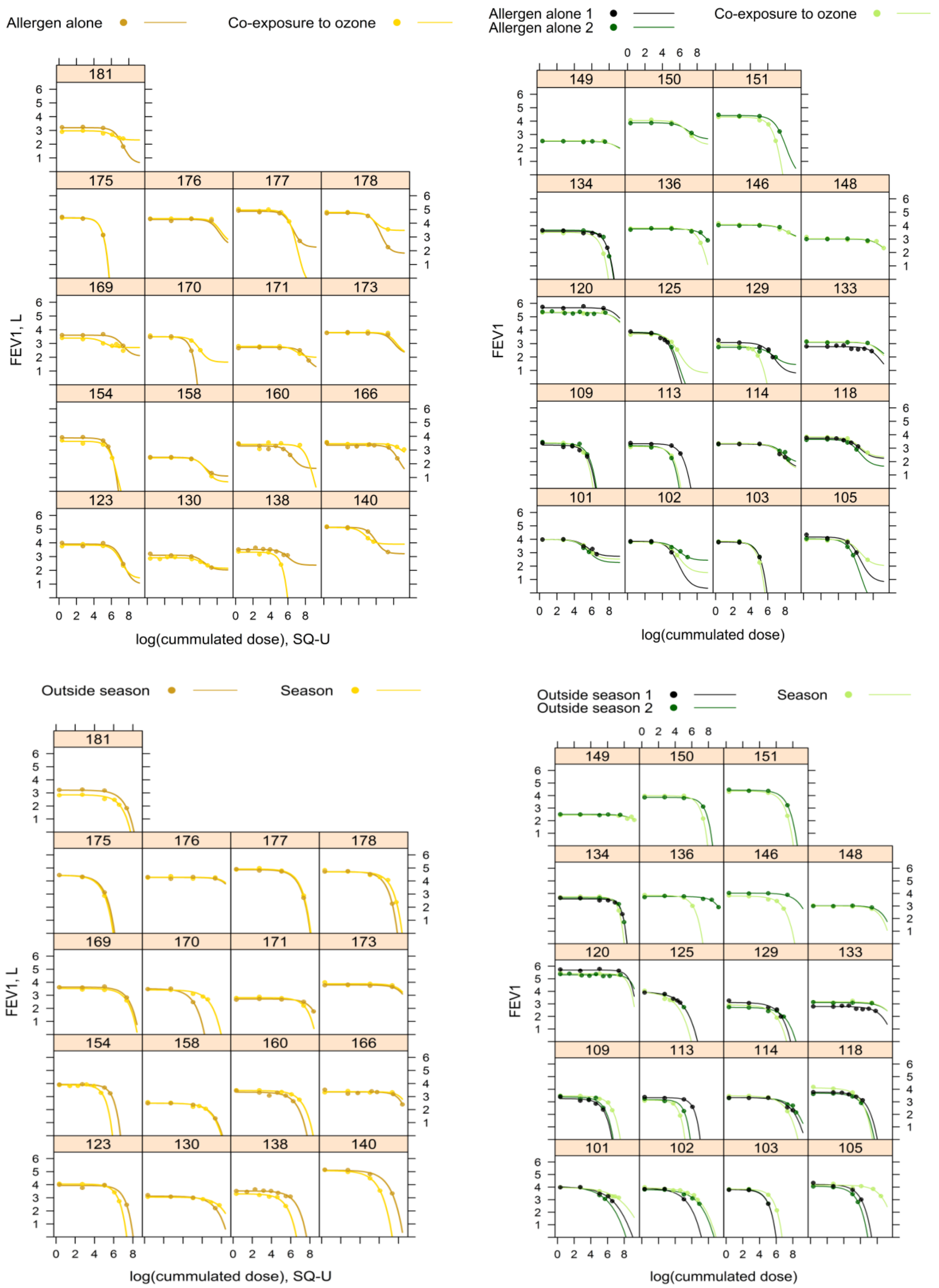






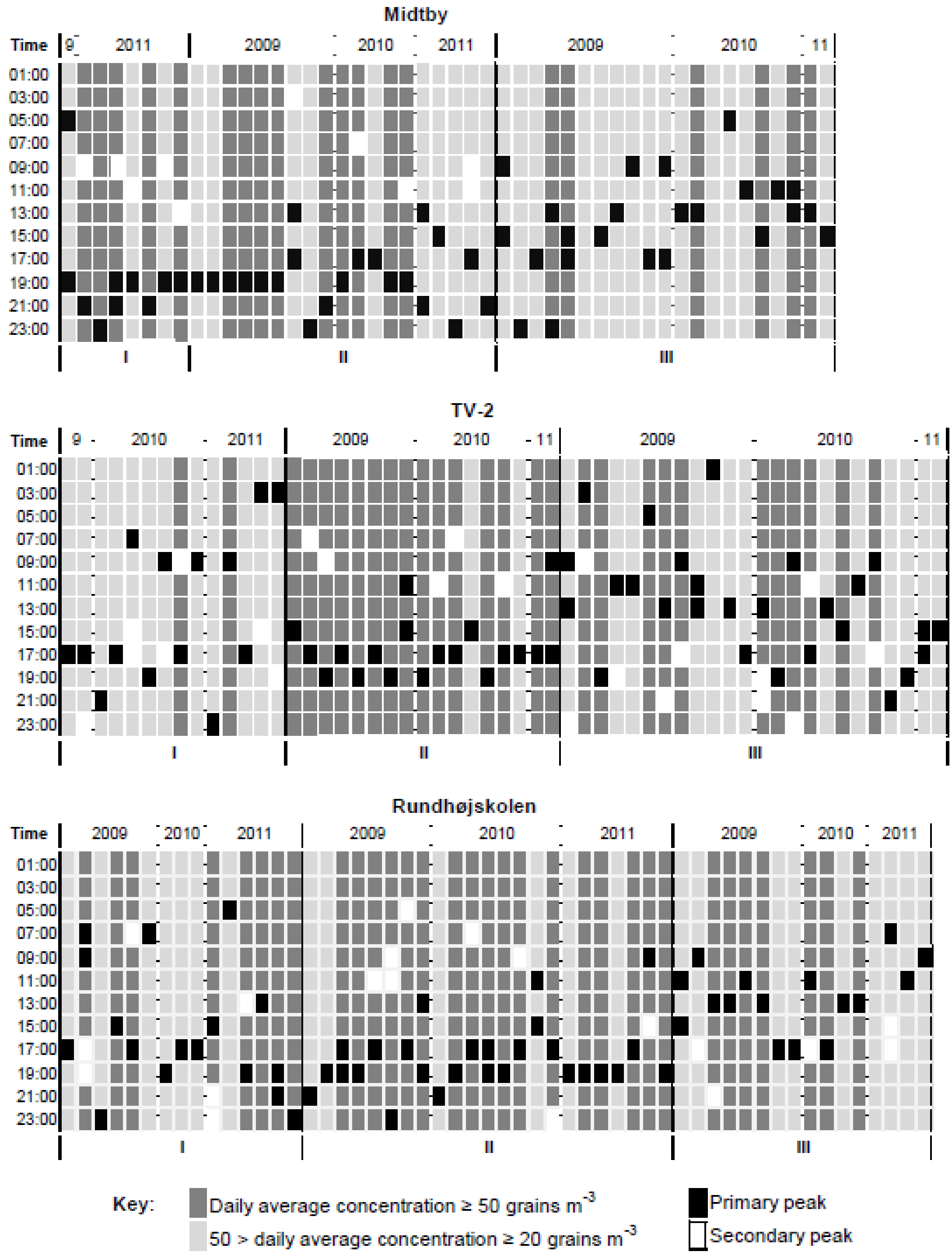
Appendix IV Log. dose-response curves for the SIC´s, not modelled.
a,b,c; birch.
d, e,f,g,; grass.

Appendix V



Appendix V Modelled dose-reponse curves for all SIC´s shown for the two separate analysis; co-exposure to ozone (above) and effect of season (priming effect) (below). Birch, left; yellow, Grass, right; green.

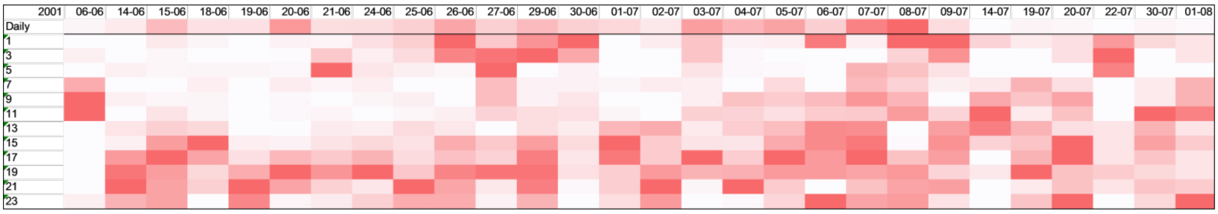
Appendix VI

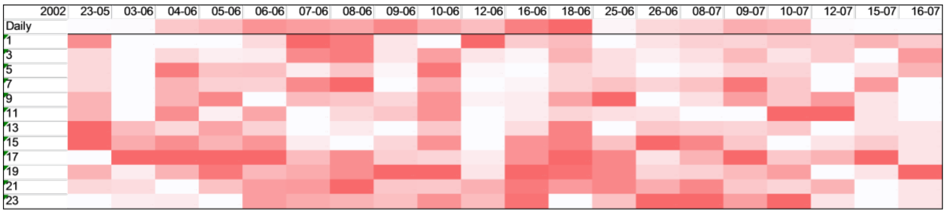


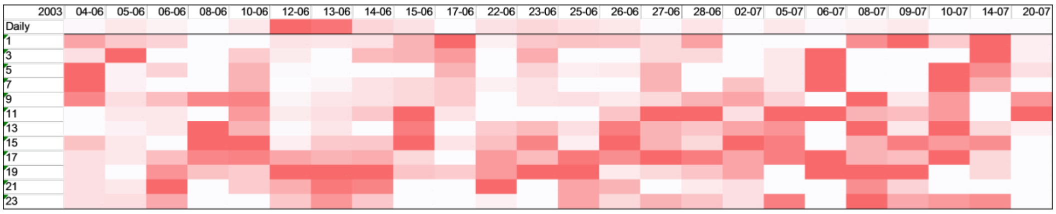
Appendix VI Peak matrix for the three stations in Aarhus 2009-2011. Se Manuscript I and Chapter 9.

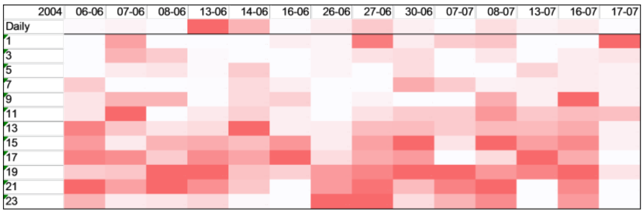
Appendix VII

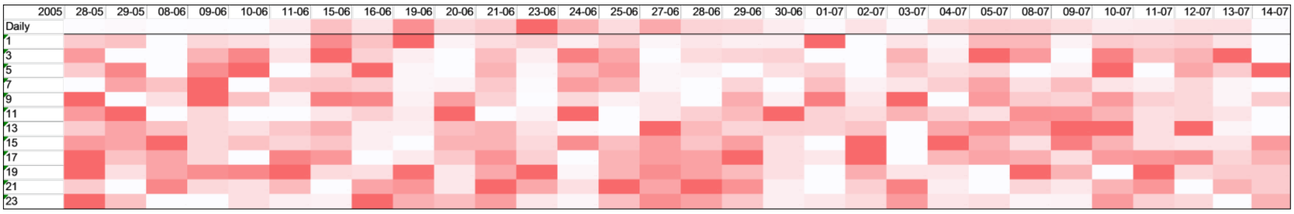
Copenhagen

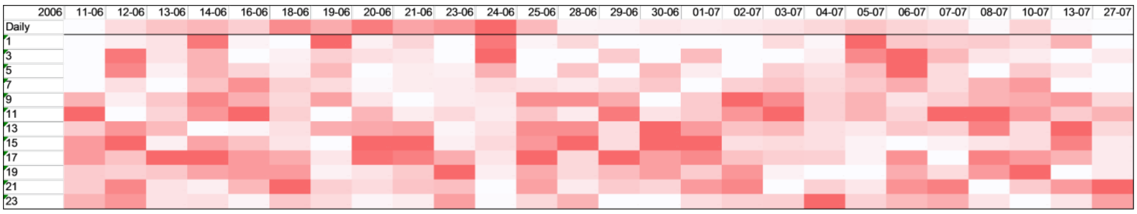


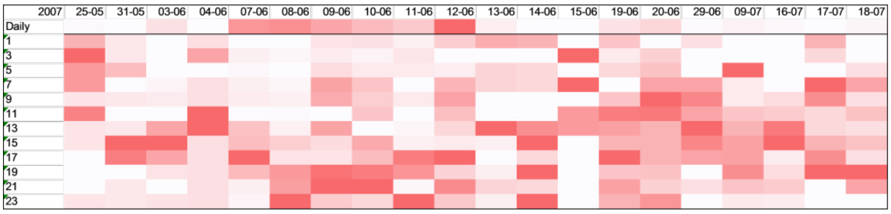








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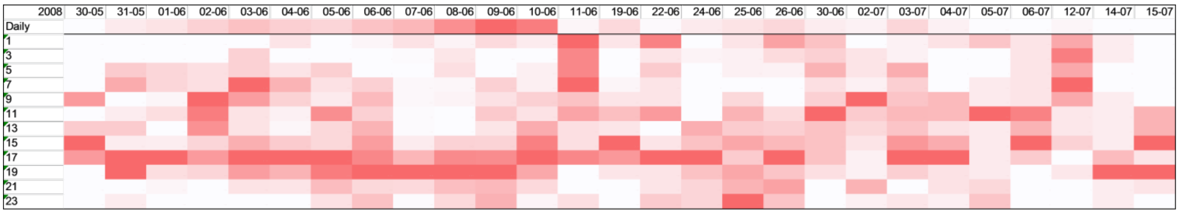
2008
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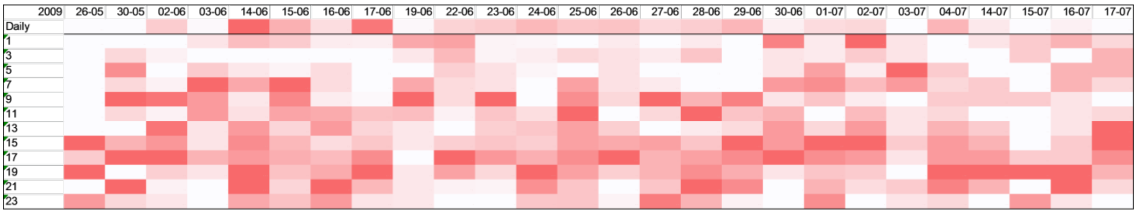
2009
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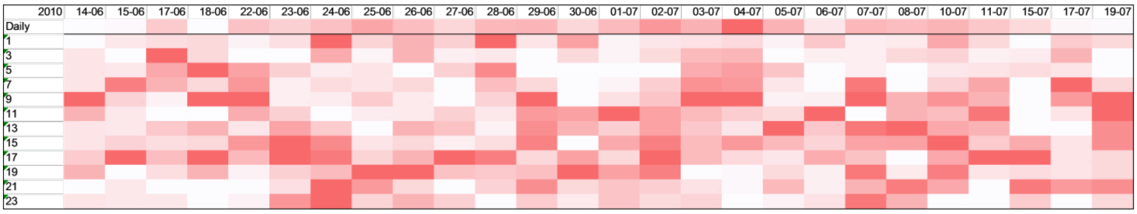
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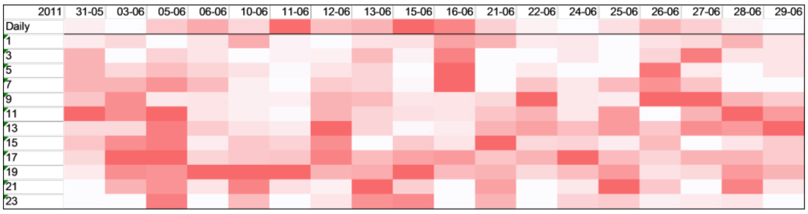
2011
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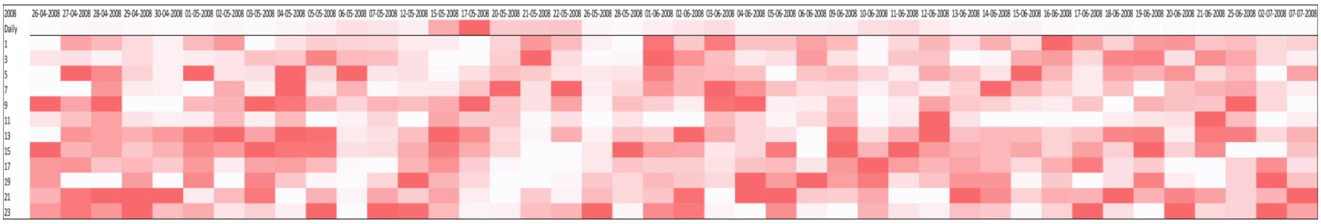
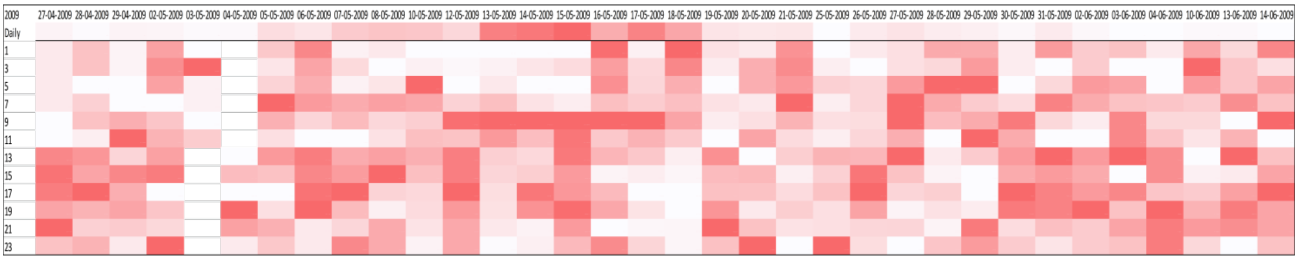
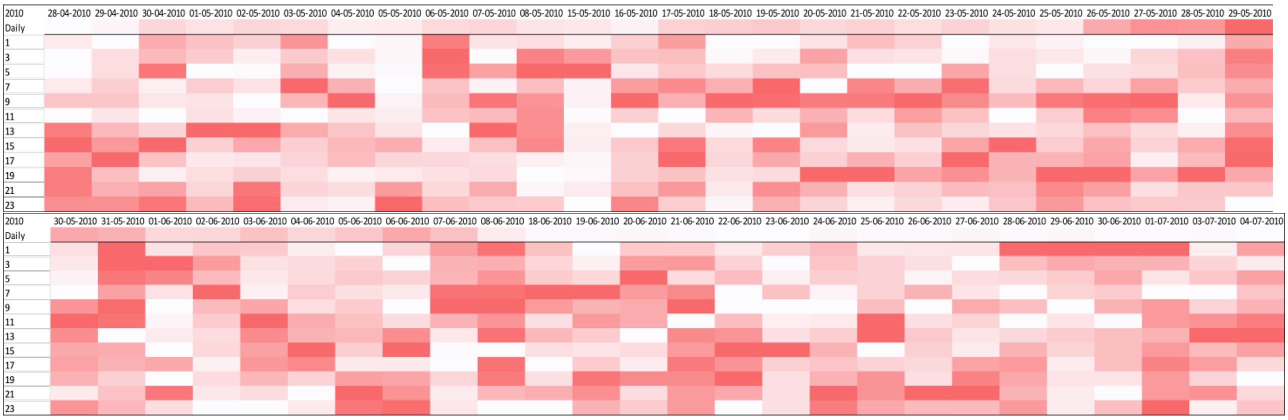
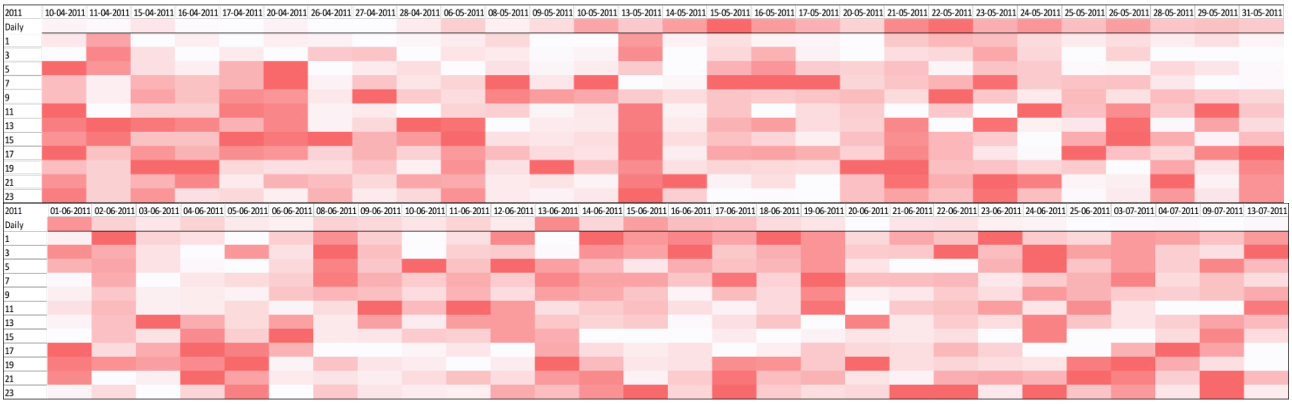
Cordoba









Appendix VII Peak matrix for pollen concentrations measured in Copenhagen 2001-2011. And Córdoba 200-2011.
Top row: Daily count, indicating seasonal minimum (white) to seasonal maximum (red).
Colum's indicate bi-hourly counts, where diurnal maximum is red and diurnal minimum white.
No clear pattern of diurnal peak time shifting through the season is apparent, indicating that the time of peak is influenced by a combination of seasonal shift of primary emission time, and other factors.

Part VI – Manuscripts

Manuscript I

Identifying urban sources as cause of elevated grass pollen concentrations using GIS and remote sensing

Skjøth, C. A., P. V. Ørby, T. Becker, C. Geels, V. Schlünssen, T. Sigsgaard, J. H. Bønløkke, J. Sommer, P. Søgaard and O. Hertel (2013)

Biogeosciences, 10(1), 541-554

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www.biogeosciences.net/10/541/2013/
doi:10.5194/bg-10-541-2013

Biogeosciences

Identifying urban sources as cause of elevated grass pollen concentrations using GIS and remote sensing

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Revised: 17 December 2012 – Accepted: 24 December 2012 – Published: 29 January 2013

Abstract. We examine here the hypothesis that during flowering, the grass pollen concentrations at a specific site reflect the distribution of grass pollen sources within a few kilometres of this site. We perform this analysis on data from a measurement campaign in the city of Aarhus (Denmark) using three pollen traps and by comparing these observations with a novel inventory of grass pollen sources. The source inventory is based on a new methodology developed for urban-scale grass pollen sources. The new methodology is believed to be generally applicable for the European area, as it relies on commonly available remote sensing data combined with management information for local grass areas. The inventory has identified a number of grass pollen source areas present within the city domain. The comparison of the measured pollen concentrations with the inventory shows that the atmospheric concentrations of grass pollen in the urban zone reflect the source areas identified in the inventory, and that the pollen sources that are found to affect the pollen levels are located near or within the city domain. The results also show that during days with peak levels of pollen concentrations there is no correlation between the three urban traps and an operational trap located just 60 km away. This finding suggests that during intense flowering, the grass pollen concentration mirrors the local source distribution and is thus a local-scale phenomenon. Model simulations aimed at assessing population exposure to pollen levels are therefore recommended to take into account both local sources and local atmospheric transport, and not to rely only on describing regional to long-range transport of pollen. The derived pollen source inventory can be entered into local-scale atmospheric transport models in combination with other components that simulate pollen release in order to calculate urban-scale variations in the grass pollen load. The gridded inventory with a resolution of 14 m is therefore made available as supplementary material to this paper, and the verifying grass pollen observations are additionally available in tabular form.

1 Introduction

Grass pollen is the most widespread pollen allergen in Europe (Emberlin et al., 1999, 2000; Jato et al., 2009; Laaidi, 2001; Smith et al., 2009), and furthermore grass pollen allergy is the most frequent pollen allergy in Europe (D'amato et al., 2007; WHO, 2003). Atmospheric concentrations of grass pollen are commonly forecasted and published in order to facilitate self-care among grass pollen allergic subjects. The forecasting of grass pollen concentrations is generally done with statistical models (Chuine and Belmonte, 2004; Garcìa-Mozo et al., 2009; Laaidi, 2001; Smith and Emberlin, 2005, 2006). Statistical or empirical forecast models are, by their nature, limited to the area where they are produced

(Stach et al., 2008). Whether this area covers only the specific urban area where the pollen trap is placed or the area has a larger geographical extent is usually unknown.

Urban areas have previously been reported to be important sources of birch (*Betula*) pollen in the urban environment. Here parks, gardens and small woodlands are considered an important source of increased birch pollen concentrations within the city (Skjøth et al., 2008b). Similar to birch trees, grass areas are commonly found in or near urban areas (Pauleit and Duhme, 2000a). Grass pollen grains from wild grass species usually have a size of 30–40 µm in diameter (Brown and Irving, 1973) and about 50–60 µm for the crop rye (Durham, 1946), whereas birch pollen is only about 20 µm in diameter (Mäkelä, 1996). Both grass and birch pollen are nearly spherical and must be expected to have a density slightly less than water (Gregory, 1973). Hence, according to Stokes' law, grass pollen has a settling velocity which is about four times larger than the settling velocity of birch pollen, which is about 1 $cm\,s^{-1}$ (Skjøth et al., 2007; Sofiev et al., 2006a), resulting in shorter suspension time in the atmosphere for grass pollen.

Grass pollen is released just above ground level. This also differs from the case of birch pollen where the release height is 5–25 m above ground. An increased release height of an air pollutant will in general decrease the concentration near the source but widen the footprint area (Seinfeld and Pandis, 1998), and this principle also applies to the pollen distribution in the atmosphere. Figure 1 illustrates this principle by displaying the calculated pollen concentration near a source, using the Gaussian principle. The source is set to emit 1 million pollen grains – amounts that are commonly found in grasses, trees and weeds: rye (*Secale*), birch (*Betula*) and ragweed (*Ambrosia*) (Fumanal et al., 2007; Pohl, 1937). Grass pollen thus has a larger settling velocity and lower release height compared to birch pollen, and as a result the difference between surface and rooftop concentrations for grass pollen is much higher that the difference found for birch pollen, which has also been documented in experiments (Alcazar et al., 1999; Rantio-Lehtimaki et al., 1991). Consequently, two hypotheses can be formulated:

– The low release height, the large surface/rooftop variations and the potential presence of grass pollen sources in or near the urban area suggest that large intra-urban variations will be present for grass pollen in the air.

– Intra urban variations in grass pollen concentrations are linked to local-scale variations in source distribution.

We will investigate these two hypotheses individually with use of the following three components:

– A dedicated intra-urban grass pollen measurement campaign for the pollen season 2009.

– Mapping the potential source areas using remote sensing and GIS.

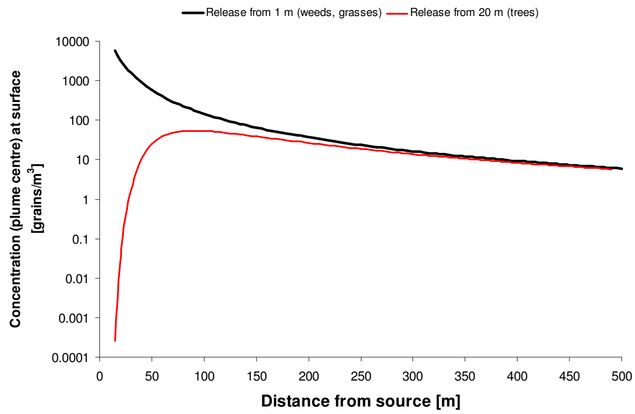

Fig. 1. Local-scale concentration profile of the pollen near the surface after the release of 1 mio pollen grains from trees (20 m) and weeds/grasses (1 m). Overall concentration is calculated using neutral meteorological conditions and a wind speed of 5 $m\,s^{-1}$.

– Linking the source map with possible local air mass transport by using measured wind directions.

2 Methodology

2.1 Pollen data

In this study we investigated the measured pollen concentrations from three pollen traps in the city of Aarhus, Denmark (Fig. 2). In the southern part of Aarhus, sampling was performed from the roof of the school Rundhøjskolen (56°20′ N, 10°30′ E), 60 m above sea level. In the central part of the city, sampling was performed at the Department of Environmental Science (14 m above sea level), Aarhus University's urban background air quality monitoring station in Aarhus – formerly the NERI station (National Environmental Research Institute) (Ellermann et al., 2007). This site also includes measurements of temperature, precipitation, wind speed and direction. In the northern part of the city, sampling was performed at the top of the TV station building, TV2-Østjylland (56°32′ N, 10°31′ E), 75 m above sea level. The heights of the buildings are 15–20 m, and the surroundings of each of the pollen traps are in general urban (Fig. 2). Additionally, the measured pollen data are compared with data from the operational trap in Viborg, about 60 km to the north-west (Sommer and Rasmussen, 2009).

Continuous monitoring of pollen content in the air was carried out using a Burkhard volumetric spore trap of the Hirst design (Hirst, 1952). Air is sucked into the trap at a rate of 10 $L\,min^{-1}$ through a 2 mm × 14 mm orifice. Behind the orifice, the air flows over a rotating drum that moves past the inlet at 2 $mm\,h^{-1}$. The drum is covered with an adhesive-coated, transparent plastic tape, which traps the particles. Pollen is identified and counted at × 640 magnification on 12 transverse strips every two hours, according to the method

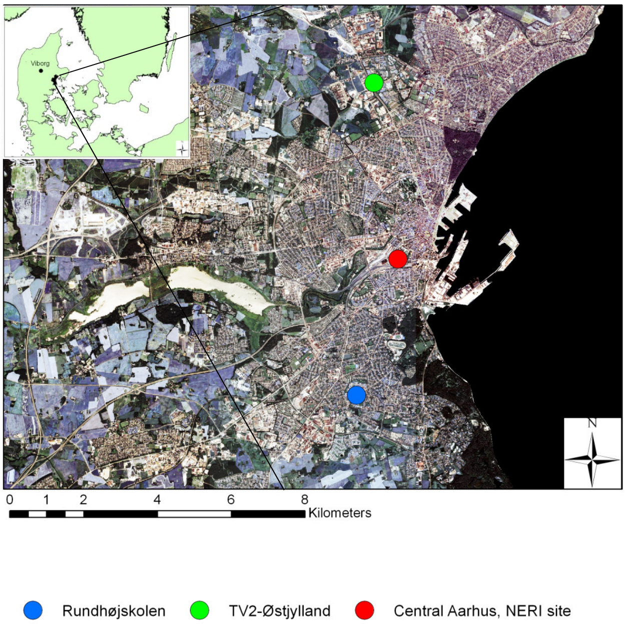

Fig. 2. Municipality of Aarhus, Denmark and location of the three pollen traps in Aarhus and the operational trap in Viborg (upper left).

described by Käpyla and Penttinen (1981). Daily average pollen concentrations are expressed as grains m^{-3}. The total area counted is 65.52 mm^2, which is 9.75 % of the total area of the slide, equivalent to the number of pollen grains in 1.44 m^3 of air.

2.2 Remote sensing analysis

Potential grass pollen source areas in the city of Aarhus are identified using a dataset of six Quickbird satellite images (DigitalGlobe Corporate, 2010) that were taken during summer 2008. The Quickbird satellite images are high resolution datasets (ground resolution of 0.6 m) and provide information in four spectral channels (red, green, blue and near infrared) of 2.4 m ground sample distance (GSD). The 0.6 m resolution is achieved by the fifth band, which is captured as a panchromatic image (DigitalGlobe Corporate, 2010). The potential grass pollen areas are identified as non-woody, but vegetated areas use the following procedure for land cover classification:

1. Every band of 2.4 m GSD is combined with the panchromatic channel using image fusion to achieve high spatial and spectral resolution in every channel.

2. Areas covered by lakes and buildings are erased from the dataset to reduce the possibility of false classification.

3. Calculation of the normalized difference vegetation index (NDVI) (Lillesand et al., 2007) for each satellite image is performed using GRASS GIS for image interpretation and image processing (GRASS Development Team, 2008).

4. Grouping NDVI values into three groups: (1) Non-vegetated areas, (2) Woody areas, (3) Non-wooded vegetation areas, where group 3 is used for further analysis.

The final result of this analysis is a 0.6 m resolution image covering the city of Aarhus and showing the potential grass pollen source areas (Fig. 3). The quality of this NDVI classification has been assessed by an error matrix (Table 2) carried out according to standard methodologies (Lillesand et al., 2007), which in this case separates a limited number of pixels into grass and no grass areas (buildings, lakes, streets and trees).

2.3 GIS analysis

In Denmark the grass pollen season usually extends from May to September (Sommer and Rasmussen, 2009), and it is anticipated that up to 100 species may contribute to the overall pollen load. However, for grass species in large parts of Europe, including Denmark, flowering depends on area management. If the grass areas are managed, e.g. cut on a regular basis or heavily grazed by animals, the grass species do not reach maturity – a stage where they can flower. This means that managed areas do not flower – or have very limited flowering. Otherwise, unmanaged grass areas and areas with less frequent management can have grass that flowers. This rule applies for rural as well as urban areas.

Potential areas for possible grass flowering can therefore be identified through analysis of their management. In Denmark one source of information about management is data from the Danish General Agricultural Register (DGAR). DGAR is administered by the Danish Ministry of Food, Agriculture and Fisheries, and includes a map of field areas and data on crop types. Each field area can consist of several minor fields with different crop types. Data are therefore given as a percentage of the field area covered by each crop. The data from 2008 were obtained from DGAR and classified in six groups according to their probability of being a source of grass pollen: (a) fallow, (b) mixed, (c) seedling grasses, (d) permanent grass, (e) rye, and (f) areas without grass (potatoes, sugar beets, etc.).

Management information is also obtained through analysis of a parcel map of Denmark. Each small land parcel (forest, road blocks, detached houses, cemeteries etc.) has its spatial extent mapped in a GIS-based geodatabase at the National Survey and Cadastre. This database is connected to another GIS database, which contains information about location of roads, buildings and land use for the urban environment. This allows for detailed analysis at the parcel level with respect to both structure and land use. Land parcels containing the following features are considered managed or cut on a regular basis: buildings, parks and cemeteries. Land parcels that

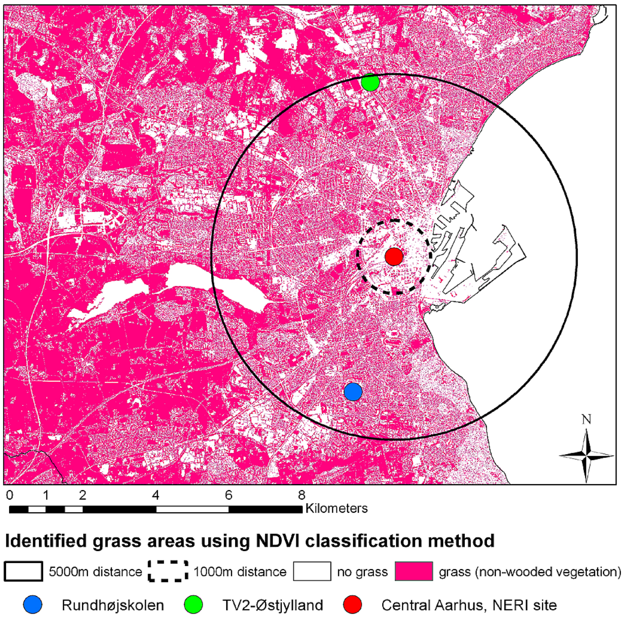

Fig. 3. Possible grass areas in the city of Aarhus based on the NDVI classification methodology described in Sect. 2.2, with a ground resolution of 0.6 m. High density grass areas are displayed as large, intense pink areas and lower density areas as more a of mixture of pink and white pixels. Areas without grass are completely white. Circles are distances from central Aarhus at 1000 m and 5000 m, respectively.

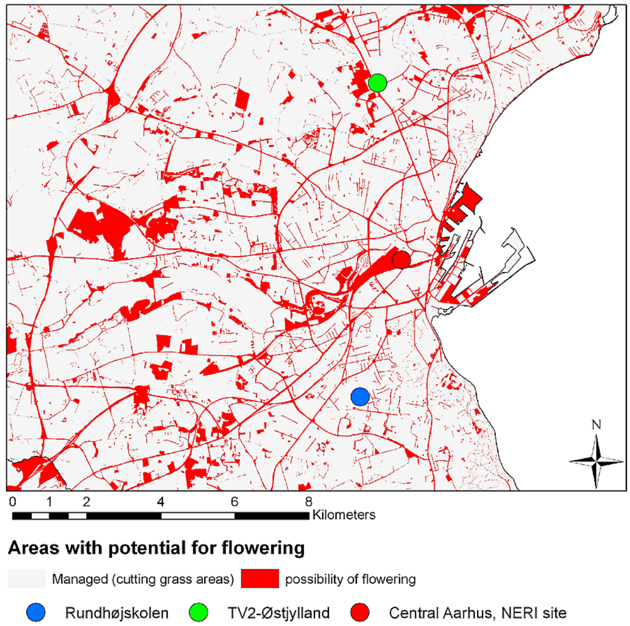

Fig. 4. Flowering possibility according to management criteria of agricultural fields and urban areas according to the method described in Sect. 2.3.

contain the following features are considered unmanaged: major roads (more than 6 m wide), rail roads and associated land, construction sites, unmanaged urban or agricultural land and areas without buildings. Finally, an important piece of additional information is that nearly all major public areas are managed by the municipality of Aarhus. The locations of these public areas are known at the parcel level in the GIS system, and these areas also have a known cutting frequency with respect to the management of the grass areas. These public grass areas are cut either annually (1 time), seasonally (3 times), monthly or more than 12 times per year, where 12 cuts per year roughly corresponds to a cutting frequency of 2 cuts per month during the grass growing season. Areas with a cutting frequency of 1, 3 or 12 cuts per year are considered potential flowering areas, whereas areas with more than 12 cuts per year are considered non-flowering (managed). This information is stored in a management map in the GIS system (Fig. 4). The management map is then converted to a raster data set with the same resolution as the NDVI map (Fig. 3). These two data sets are combined and afterwards aggregated to 14.4 m resolution by using the mean value of all 576 pixels that are fully contained within each 14.4 m grid cell (Fig. 8), which shows the major grass pollen flowering areas within and in the vicinity of the city of Aarhus.

2.4 Meteorological observations and calculations of wind directions

Meteorological observations were obtained from the monitoring site in central Aarhus. The meteorological station is part of the monitoring programme operated by the Environmental Science Department and provides meteorological data on a half-hourly basis for use in integrated monitoring of air quality in Denmark (Hertel et al., 2007).

In the analysis, wind directions were used as an indicator of potential upwind source areas, using a similar approach as presented in Skjøth et al. (2009). Wind directions were obtained from 30 min average raw data for all available grass pollen counts ($n = 1.644$) within the pollen season, i.e. from 25 May till 29 July. For each station, wind directions were sorted by peak days. Peak days are defined as those where the daily average grass pollen count exceeds a threshold of 50 grains m^{-3}. This threshold is based on clinical thresholds at the species level for grass (10 and 50 grains m^{-3}), mugwort (10 and 50 grains m^{-3}) and birch (30 and 100 grains m^{-3}), as defined by Petersen and Munch (1981) and Weeke (1981). The thresholds are furthermore used by the Danish pollen forecasting service, where a daily average level of 50 grains m^{-3} corresponds to the warning level "high". Days with daily average grass pollen counts $>$50 grains m^{-3} were gathered and examined as a group for each of the three stations.

3 Results

3.1 Pollen counts

The observations and subsequent laboratory studies reveal that the grass pollen season in 2009 started on 25 May and ended on 29 July, using the 95 % method (Goldberg et al., 1988). Table 1 and Fig. 5 show that peak pollen concentrations are observed during the period between 2 June and 5 July. After a slight increase in the pollen concentrations on 2 June, the concentrations show a drop for all locations on 3 and 4 June. The concentrations are highest at all stations during the period 14 June to 5 July, with large day-to-day fluctuations. Maximum values are measured at all stations around 14 to 18 June in the range of 121–237 grains m^{-3} (Table 1), with the highest concentration (TV2-Østjylland: 237 grains m^{-3}) occurring on 17 June. The correlation coefficient (Pearson) for the daily pollen counts between each of the stations and the operational trap in Viborg is, for the entire season, between 0.61 and 0.76. Similarly, the correlation for the peak days is between −0.35 and 0.15, and for days with pollen concentration up to 50 grains m^{-3} between 0.68 and 0.88.

3.2 Remote sensing NDVI map

The central urban area corresponding to about 1 km distance from the measurement station in central Aarhus has very limited grass areas (Fig. 3). A secondary, larger urban part within a distance of 5 km from the site in central Aarhus shows a medium density of possible grass areas. Several areas show very low or no pollen sources, corresponding to either water surfaces or wooded areas. The error matrix uses 267 control points and shows that there is some confusion between wooded and grass (non-wooded) areas and that the overall accuracy of the classification is 84 % (see Table 2).

3.3 Flowering map based on management

Analysis of data from the agricultural registers shows that the six groups had the following distribution: fallow = 5.5 %, mixed = 7.1 %, seedling grass = 1.6 %, permanent grass = 23.2 %, rye = 1.9 % and other = 60.6 %, respectively. The fallow, seedling grass and rye areas are considered flowering and sources of grass pollen. These areas are usually found scattered outside the urban area, except for a few cases such as near the trap in the northern part of the city. Near the northern trap, agricultural areas with flowering possibility are located to the west of and in close proximity to the urban area (Fig. 4).

The map also shows flowering at a number of long but narrow areas as well as at a few larger areas within the urban area. These areas are mainly associated with large roads, railroad areas, industrial areas and grass areas near streams and wetlands.

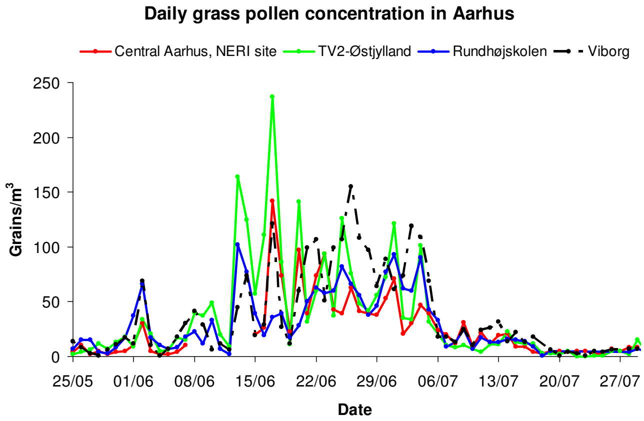

Fig. 5. Daily grass pollen concentrations at the three monitoring sites in Aarhus and the operational pollen trap in Viborg.

3.4 Meteorological data series

Figure 6 shows temperature, precipitation and wind directions measured at the central site in Aarhus.

The average night temperature was 13 °C and the average day temperature was 18 °C during the campaign period. From the beginning of the campaign period, the temperature gradually rose to maximum daily temperatures of 23 °C on 2 June. After 2 June a colder period occurred with maximum daily temperatures around 15 °C, which lasted until 24 June, when the temperature again rose during a period through 5 July with maximum temperatures up to 27 °C.

The pollen season period from 25 May to 29 July 2009 had 33 days with precipitation. From 25 May to 21 June there were 4 precipitation episodes each lasting 1–2 days. These rain episodes were followed by a dry period of 13 days which lasted until 5 July. After this dry period, it rained almost daily for the remaining part of the period through 29 July.

Wind directions for the entire period (Figs. 6 and 7a) are grouped into main directions, with most of the wind directions in these groups appearing in the sectors 225–270 and 270–315 degrees. About 10 % arrives from each of the directions 45–90, 90–135, 135–180 and 180–225. Two to five percent arrives from the northerly directions 315–360 and 0–45. Wind directions during peak days for the northern station (TV2-Østjylland station, Fig. 7b) show that for this particular subset 55 % of the winds arrived from sector 270–315 and almost none from sectors 315–360 and 0–45. Wind directions during peak days at the station in central Aarhus (Fig. 7c) show that about 60 % of the time the wind arrives from the two sectors 45–90 and 270–315. The other sectors have a frequency between a few percent and up to 10 %. During peak days for the trap at Rundhøjskolen (Fig. 7d), about 40 % of the time the wind is from the west (sector 270-315) and between 5 and 15 % of the time from other sectors. The exception is sector 0–45 for which there were no observations.

Table 1. Daily pollen counts in the city of Aarhus and corresponding daily data from the operational monitoring trap in Viborg located ∼ 60 km away. Numbers in bold exceed the critical threshold for severe hay fever symptoms. Underlined, italicised numbers and "–" mark either a reduced or a missing daily measurement, respectively, due to trap failure.

Date	Viborg	Rundhøj skolen	TV2-Østjylland	Aarhus Centre	Date	Viborg	Rundhøj skolen	TV2-Østjylland	Aarhus Centre
25/5	14	7	*2*	4	27/6	**108**	**56**	48	41
26/5	8	15	4	10	28/6	**97**	38	42	39
27/5	3	3	6	2	29/6	**64**	46	**56**	38
28/5	1	4	12	5	30/6	**89**	**77**	**72**	**53**
29/5	6	3	7	2	01/7	**61**	**93**	**121**	**71**
30/5	11	8	13	4	02/7	**74**	**62**	35	21
31/5	17	17	18	5	03/7	**119**	**60**	34	30
01/6	12	37	9	10	04/7	**109**	**90**	**101**	47
02/6	**69**	**66**	34	30	05/7	**69**	43	32	39
03/6	10	17	21	5	06/7	18	33	*36*	24
04/6	1	10	5	2	07/7	18	9	10	20
05/6	7	7	6	2	08/7	13	13	8	12
06/6	18	8	17	4	09/7	25	25	10	31
07/6	30	18	15	10	10/7	10	7	7	9
08/6	41	23	40	–	11/7	24	17	4	22
09/6	29	12	37	–	12/7	26	13	11	12
10/6	6	33	49	–	13/7	32	13	11	19
11/6	12	7	20	–	14/7	14	16	23	21
12/6	6	2	9	–	15/7	22	15	13	9
13/6	45	**102**	**164**	–	16/7	13	14	12	9
14/6	**74**	**77**	**125**	–	17/7	18	9	12	4
15/6	19	39	**57**	20	18/7	0	1	3	3
16/6	29	19	**111**	26	19/7	6	5	3	5
17/6	**121**	36	**237**	**142**	20/7	1	–	2	5
18/6	27	39	**86**	**74**	21/7	4	–	5	4
19/6	12	17	11	23	22/7	3	–	0	5
20/6	**60**	28	**141**	**97**	23/7	1	–	0	5
21/6	**99**	**50**	32	39	24/7	5	–	1	3
22/6	**107**	**63**	**60**	**74**	25/7	4	–	1	3
23/6	**51**	**58**	**94**	**94**	26/7	6	–	5	7
24/6	**99**	**60**	37	43	27/7	0	–	5	5
25/6	**107**	**82**	**126**	39	28/7	7	4	2	8
26/6	**155**	**66**	**76**	**63**	29/7	7	7	15	8
					SUM	2373	1799	2421	1461

	Rundhøj skolen	TV2-Østjylland	Aarhus centre
Correlation with the operational trap in Viborg (all data in season)	0.76	0.61	0.70
Correlation with the operational trap in Viborg (above 50 grains m^{-3})	−0.35	0.15	0.06
Correlation with the operational trap in Viborg (up to 50 grains m^{-3})	0.68	0.74	0.88

3.5 The gridded grass pollen map with main flowering regions

The gridded map of grass pollen source areas (Fig. 8) shows a number of hot spots in the periphery of the urban area. It also shows that one of these hot spots (density 88–100 % grass pollen areas) is located within a few hundred metres of the station TV2-Østjylland to the west and north-west.

Throughout the entire urban area a number of long and narrow areas are seen with low density (1–48 %). Few source areas are seen in the absolute vicinity of the station at Rundhøjskolen, but numerous diffuse sources are seen to the east. A number of pollen sources usually with a medium density (14–67 %) but also with a high density (88–100 %) are found 500–1500 m west of the station in central Aarhus, and almost no sources are found in other directions. The actual

Table 2. Error matrix associated with the NDVI analysis (Fig. 3) of the Quickbird images covering the Aarhus area. User accuracy (User acc.) and Producer accuracy (Prod acc.) are based on standard methodologies according to Lillesand and Kiefer (2007).

	Reference Data						
Classification Data	Grass	Buildings	Lakes	Streets	Trees	Sum	User acc.
Grass	**129**	2	1	9	25	166	78 %
Buildings	1	**25**	0	1	0	27	93 %
Lakes	0	0	**3**	0	0	3	100 %
Streets	0	1	0	**25**	0	26	96 %
Trees	3	0	0	1	**41**	45	91 %
Sum	133	28	4	36	66	**267**	
Prod acc.	97 %	89 %	75 %	69 %	62 %		84 %

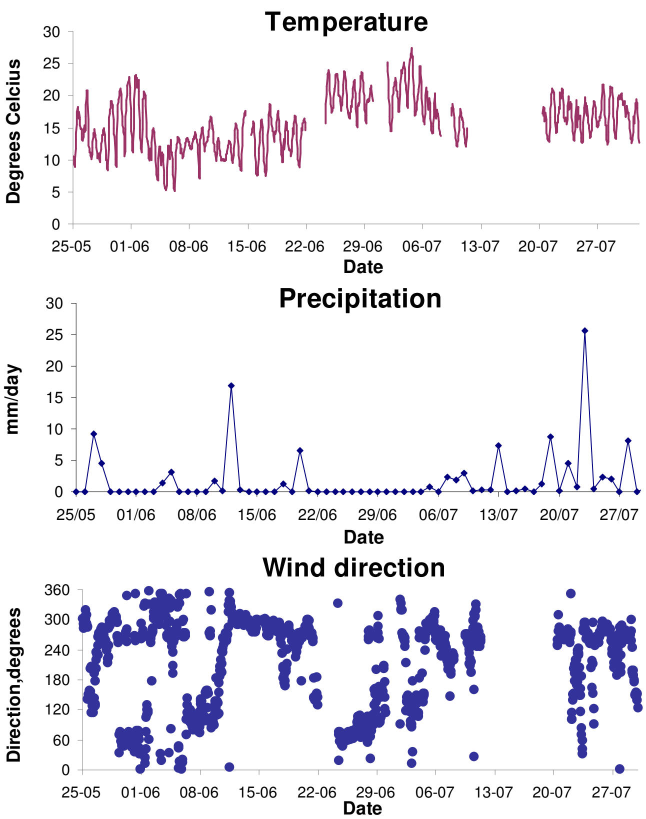

Fig. 6. Meteorological observations of temperature, precipitation and wind direction at the ENVS, AU monitoring site in central Aarhus.

data set is available in the form of a tiff file as supporting information to this article.

4 Discussion and conclusion

The current experiments support the hypothesis that there are large intra-urban variations in the pollen load and that such variations are connected to the source distribution on a local scale.

The highest pollen load is found together with the highest frequency of local sources, and this is observed in the surroundings of the northern trap: TV2-Østjylland (Table 1 and Fig. 8). This result is further supported by the wind direction analysis. Fifty-five percent of the peak days at the TV2-Østjylland station (Fig. 7b) are found when winds are coming from the direction of the high density emission area (Fig. 8), a few hundred metres from the trap. Such a clear pattern of peak days with air masses from only one wind direction is not seen for the two other stations (Fig. 7c and d). These two stations do not have high-emitting areas in their near vicinity (Fig. 8). There was a high correlation between the daily pollen counts at each of the three stations and the operational trap in Viborg during the entire pollen season (Table 1). However, at the same time there was a lack of correlation between the stations on days with elevated pollen counts (Table 1). This suggests that on days with high load, the proximity to local emission sources is very important (Fig. 1).

Analysis of the NDVI map (Fig. 3) shows a high frequency of potential pollen sources almost everywhere except lakes, forest and the city core. This suggests that it is possible to find grass pollen sources almost everywhere. However, only a limited number of all these grass areas will contain grass areas that reach maturity and flower. The flowering of grass is determined by management of the areas. The management map here is based on the assumption that at least 2 cuts of grass areas per month are enough to prevent flowering. In Denmark, it typically takes between 15 days (*Poa trivialis*, *Lolium multiflorum*, *Poa pratensis*, *Festuca pratensis* and *Lolium perenne*) and up to 20 days (*Phleum pratense*, *Dactylis glomerata* and *Festuca rubra*) for typical Danish grass species to evolve from immature flowers into the stage of full flowering. This information is provided through the management plans that are available for Denmark and in particular Danish agriculture (e.g. http://www.dlf.dk/upload/microsoft_word_-_fr_gr_ssernes_blomstring_og_modning.pdf). It is, however, likely

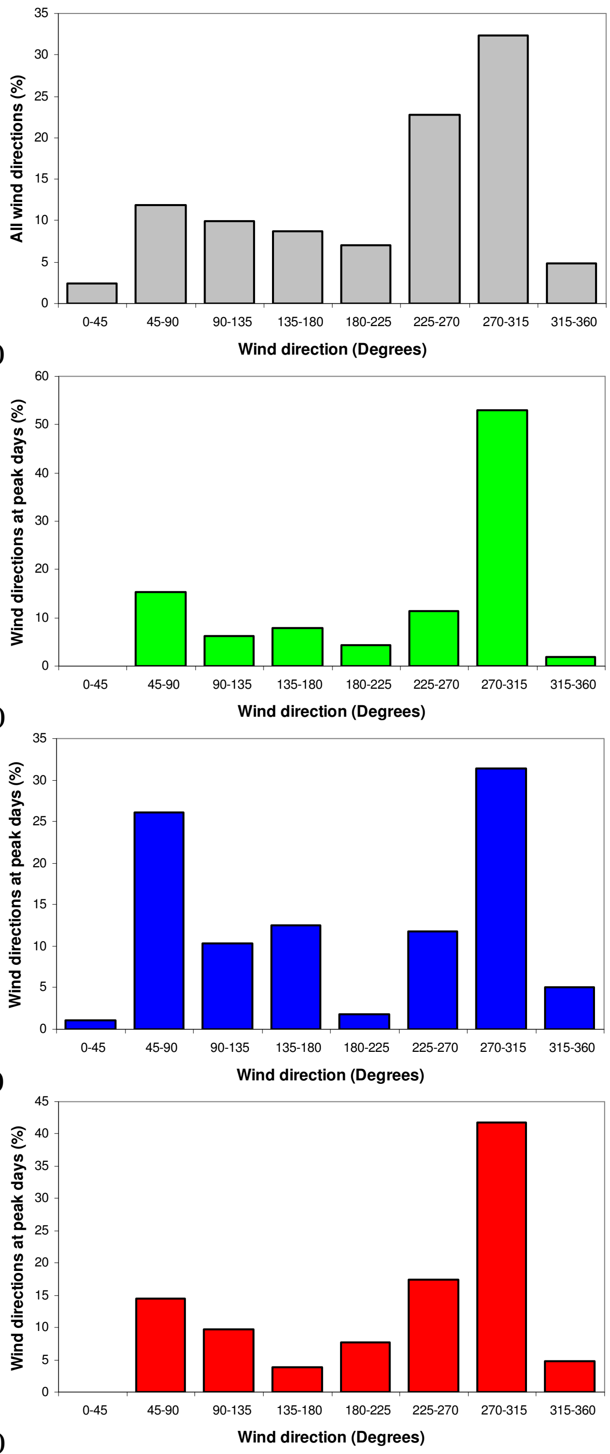

Fig. 7. **(a)** Percentage of all wind directions measured at the trap in central Aarhus ($n = 1224$) from different directions (45° angles). **(b)** Percentage of all wind directions measured at the trap in central Aarhus ($n = 255$) from different directions (45° angles) that were measured at peak days at TV2-Øst. **(c)** Percentage of all wind directions measured at the trap in central Aarhus ($n = 256$) from different directions (45° angles) that were measured at peak days at Rundhøjskolen. **(d)** Percentage of all wind directions measured at the trap in central Aarhus ($n = 103$) from different directions (45° angles) that were measured at peak days at the station in Central Aarhus. Peak days are those when the daily average Poaceae pollen counts exceed 50 grains m^{-3}. Note that the scales on the y-axis are not identical in all four figures.

that not all areas are strictly managed according to the management plan, and special meteorological conditions are likely to advance grass growth and maturation so that small grass areas are able to enter the flowering phase. However, the general picture is that the majority of the managed areas do not release large fractions of grass pollen, especially if they are compared to non-managed areas. The combined use of the management map (Fig. 4) and NDVI map (Fig. 3) indicates areas with a large fraction of flowering in unmanaged agricultural or urban land and a number of areas along roads, etc. These areas with potential flowering grasses are unevenly distributed throughout the urban area. This finding suggests that throughout the city a number of areas contain large gradients in the pollen concentrations – in accordance with our hypothesis.

The pollen observations are urban background concentrations. These measurements are obtained 10–20 m above ground level. However, according to traditional meteorological dispersion theories (Fig. 1), the pollen concentrations will be much higher near the surface when the source is located here. This, in connection with the pollen source map, suggests that elevated concentrations can be found near areas at low height, such as along the large roads seen in Fig. 8 – which adds further support to the hypothesis.

The results also indicate that urban sources should not be considered exclusively. The results show that peak concentrations at the central Aarhus station (Fig. 7c) are observed during easterly winds, despite the fact that the pollen source map (Fig. 8) and the NDVI map (Fig. 3) both suggest that there are no sources in that direction. The nearest sources are about 20–60 km away. This again suggests that either regional-scale or long-range transport of grass pollen can be relevant. The latter is supported by a study of Smith et al. (2005), which shows that grass pollen could originate from sources more than 100 km away. Another study by Smith et al. (2008) adds to this finding that long-range transport is seen only episodically for species such as ragweed (*Ambrosia*). It is therefore likely that a strong signal from long-range transport of grass pollen, which has a much larger settling velocity than ragweed (*Ambrosia*), is even less frequent than for *Ambrosia*. Instead shorter distances are likely to be the relevant scales for grass pollen. According to the definition by Orlanski (1975), the typical distances for mesoscale atmospheric transport can be divided in three groups: meso-gamma (2–20 km), meso-beta (20–200 km) and meso-alfa (200–2000 km), where the latter covers long-distance transport. This study in combination with the existing studies on long-distance transport (e.g. Smith et al., 2008) suggests that the majority of the signal for grass pollen consists of a mixture of atmospheric transport processes on a micro- (below 2 km), meso-gamma and meso-beta scale. From a modelling point of view, this has been identified as one of the major challenges to understand and describe with respect to airborne pollen transport (Sofiev et al., 2013). This highlights the importance of studying local

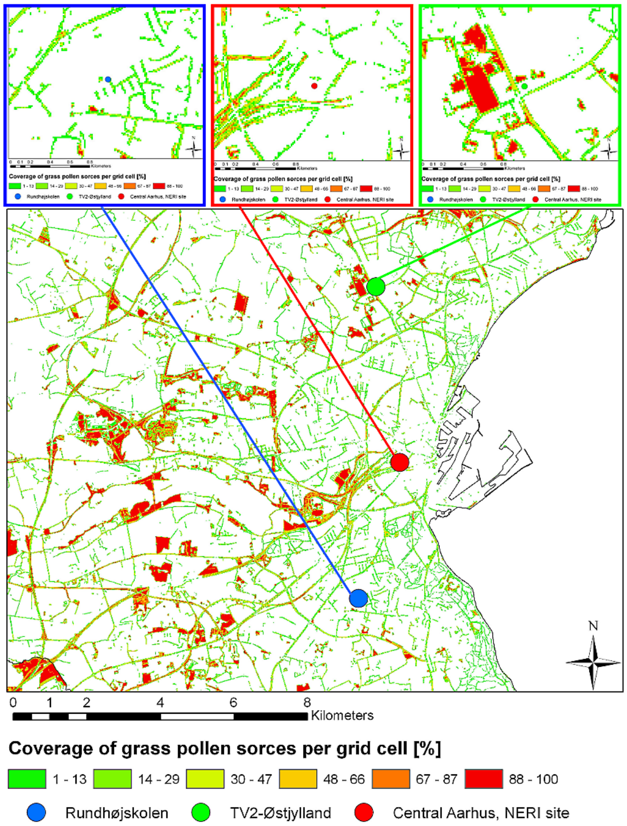

Fig. 8. Inventory of high-emitting grass pollen areas in the city of Aarhus (below) and zoom (top) that provides the vicinity of the three pollen monitoring stations.

sources and also supports our hypothesis about urban gradients in grass pollen concentrations.

The NDVI map (Fig. 3) suggests that the central urban area, forest and lake districts contain very few areas covered by grass, while all other areas could contain large grass pollen source areas. For Denmark these areas are mainly agricultural areas. About two-thirds of Danish land area is used for agriculture (Skjøth et al., 2008a), which in national and international land cover databases such as CLC2000 (European Commission, 2005) is typically indicated as agricultural land. The agricultural register data here show that most of these potential source areas are likely not to be grass pollen sources, as the majority of these areas are used for grown crops without flowering or for permanent grass which is frequently cut or grazed. Only a small fraction of the agricultural areas (fallow, rye, grass seedling) contains significant amounts of grass pollen sources, and according to Figure 4 these areas are unevenly distributed. On the regional scale, these areas are likely to be important for the overall grass pollen level in the region. It is known that management of the agricultural land is generally unevenly distributed on the regional scale (Skjøth et al., 2008a), suggesting that land cover databases such as CLC2000 or GLC2000 (Fritz et al., 2003) or other similar remote sensing products are less suitable as a stand-alone product for identifying these grass pollen source areas. Information about management of these grass covered areas need also to be included for such an identification to be of value. An alternative procedure is the collection of a series of high resolution remote sensing images taken during the entire vegetation period. This will enable mapping of land use and land cover at very high spatial resolution. Such a series of images is usually not available from satellites with high spatial resolution images, such as those from Quickbird. Therefore, series of images must be obtained using other methods such as airplanes, which increases the costs significantly. The most cost-effective solution for producing a grass pollen source inventories seems therefore to be a combination of a high resolution remote sensing images and information concerning the local management of the grass areas in the studied domain.

Clinical exposure studies of grass pollen often use concentrations in the range 1000–8000 grains m^{-3} (Day et al., 2006) – about a factor of 10 larger than what is found in typical peak observations. These levels are chosen to obtain a clear signal from the patients used in the exposure studies. Despite such high concentrations generally not being observed at the stations (Emberlin et al., 1999, 2000; Pashley et al., 2009; Sommer and Rasmussen, 2009), most hay fever patients have severe symptoms from grass pollen allergy every year. However, the observations are obtained at the urban background level at about 10 to 20 m above ground level, and according to traditional dispersion theory in meteorology the concentrations can be much higher at the surface, where people are exposed (e.g. Fig. 1). This is supported by the experiments of Rantio-Lehtmiaki et al. (1991), which show that surface concentrations can be about a magnitude larger than rooftop concentrations. This indicates that there is a large knowledge gap regarding how to link actual observations at rooftop level, symptoms among patients and findings in actual exposure studies. Model simulations of exposure to grass pollen near the surface are considered here a method of reducing this knowledge gap. Such simulations can be done by using local-scale dispersion models. A basic requirement for such studies is access to a highly detailed inventory, such as the one we present in this study (data in supplementary material), as well as verifying observations (Table 1).

This study extends a few other local-scale studies such as the one by Bricchi et al. (2000). They suggest that high gradients in London plane (*Platanus*) pollen are present in the city of Perugia (Italy) and that the zone of influence for single sources is less than 800 m. Grass pollen is released at a much lower height than pollen from London plane (*Platanus*), and as a consequence the influence zone can be expected to be much smaller – in a similar manner as seen in Fig. 1. Urban areas have been shown to contain a significant amount of green areas, which all have the potential to be pollen sources (Pauleit et al., 2002). The composition of the green areas is, however, different between the areas, between cities and between regions (Pauleit and Duhme, 2000a, b). Specific considerations must therefore be taken in urban-scale experiments. Here an urban grass pollen source map is suggested, which to the knowledge of the authors has not previously been performed. This study suggests that local-scale studies of pollen concentrations can be necessary and that the need for these studies depends on the presence or absence of local sources. This information can be obtained by using a combination of remote sensing and management information of the area. Other studies have used tools such as trajectory models (Cecchi et al., 2007; Skjøth et al., 2009; Stach et al., 2007) or regional-scale dispersion models (Helbig et al., 2004; Skjøth, 2009; Sofiev et al., 2006a; Zink et al., 2012). Such tools can be very useful for establishing source–receptor relationships on regional-scale transport. However, for local-scale sources the use of regional-scale models is less appropriate and should be based on local-scale dispersion models such as AERMOD (US-EPA, 2003) or OML (Olesen et al., 1992; Sommer et al., 2009), provided that they can be further developed to describe atmospheric dispersion of pollen. In general this requires validated phenological models and a model that parameterises daily pollen release (e.g. Skjøth et al., 2010). Emission models for use by OML that take into account crop and grass growth are already developed for Danish land area (e.g. Skjøth et al., 2004; Gyldenkærne et al., 2005; Sommer et al., 2009; Geels et al., 2012). It has previously been shown that these emission models can be extended to tree pollen (Skjøth et al., 2009, 2010). It is therefore likely that they can also be extended to grass pollen in order to handle the main grass species, such as *Poa trivialis, Lolium multiflorum, Poa pratensis, Festuca pratensis, Lolium perenne, Phleum pratense, Dactylis glomerata* and *Festuca rubra*, and in particular their flowering. The use of these models has the potential to give further insight into urban-scale concentrations in the grass pollen load, given that source inventories such as the one in Fig. 8 are available for the model applications.

Inventories such as the one given in Fig. 8 can in principle not be validated, as all data are supposed to be used by the inventory (Simpson et al., 1999; Skjøth et al., 2008a). Instead the validity behind the principles and design of these source inventories should be discussed, and if possible the quality of the inventory and its sensitivity to input data should be assessed using other methods, such as cross validation (Skjøth et al., 2010). In the study presented in this paper, the inventory depends on two data sources: a remote sensing product (Fig. 3) from the Quickbird satellite and a management map (Fig. 4). In general, the remote sensing product can be considered state-of-the-art with respect to satellite observations. Quickbird has four spectral bands, as does the Landsat7 satellite. Landsat is used for the Corine2000 product with 100 m resolution (European Commission, 2005). Quickbird has a resolution several orders of magnitude higher than the Landsat7 satellite, and this makes it suitable for urban-scale studies and identification of both large and small grass areas. The NDVI methodology does not distinguish between grass areas and other non-wooded vegetation. This means that the weeds will be misclassified as grass, thus posing a risk for overestimating the abundance of grass areas in the inventory. Nevertheless, the quality of the NDVI map must be considered very high as the overall accuracy exceeds newly released remote sensing products such as the Globcover data set (Bicheron et al., 2008). The NVDI analysis (Fig. 3) furthermore shows that only few grass areas will be located in forest areas. Such areas must be considered unmanaged, but are not considered in the potential flowering map (Fig. 4) as a result of the applied generalised forest cover. However, due to the surrounding forest, it can be assumed that the majority of the pollen from these limited areas will be trapped inside the forest canopy and therefore to a great extent will not be dispersed further into the atmosphere. This is therefore not considered a significant error in the inventory.

The map of potential flowering areas shown in Fig. 4 does not include grass along small agricultural roads between fields, nor does it include field boundaries. This introduces a risk for a small underestimation of the grass pollen areas in the rural areas. Whether it is important for the regional-scale load is not known. However, it is not considered relevant for the main hypothesis of this paper concerning sources and variations in the pollen concentration in the urban area. The map of potential flowering areas (Fig. 4) is based on exact data in combination with assumptions concerning the urban management of private areas. Particularly, one of these assumptions is questionable: Race courses and motor-cross areas are excluded on a regular basis. At least two such areas exist in the urban area of Aarhus. It is known that parts of these areas are managed, but it is likely that this assumption only applies to parts of them, meaning that some fractions of these areas are managed and others are not. This information is, however, not available for our analysis. A better map would be obtained if exact management data for these areas were available, but currently this is not the case, and it is questionable whether a complete survey among all private and public land owners would be rewarded by a significant improvement in the data quality compared to the costs of such a survey.

The analysis is also limited by equipment failures, including trap failures as well as missing data from the meteorological station. However, at least three traps out of four were working each day, so these failures are of minor importance with respect to identifying variations in the daily pollen load. The lack of meteorological data is not critical in this context either. The main period of data loss was 11–20 July; and since this period did not contain any peak days, the data loss does not affect the analysis in Fig. 7b, c and d.

The geographical coverage of the inventory is 20 km × 20 km (Fig. 8). Faegri and Iversen (1992) as well as Avolio et al. (2008) suggest that the typical transport distance for pollen is in the range of 30–100 km. This is, however, an overall estimate related to pollen from trees, weeds, and grasses. Pollen from trees has in general a much longer transport distance than pollen from weeds, due solely to the high release height (e.g. Fig. 1). It is therefore reasonable to assume that the inventory will capture most of the sources that contribute to the pollen load in the Aarhus area. This is furthermore supported by the studies by Bricchi et al. (2000) suggesting that the influence zone for individual sources is less than 800 m. Nevertheless, it should be expected that long-range transport episodes will occur. Such episodes can be systematic from specific locations (Stach et al., 2007) with repeating episodes (Skjøth et al., 2007). But long-range transport is generally episodic (Belmonte et al., 2008; Smith et al., 2005, 2008). As such, it is reasonable to assume that the inventory can be used to explain the majority of local variations in the grass pollen load.

5 Further work

In summary and in compliance with the main hypothesis outlined in the beginning of this paper, the current study identified a number of urban grass pollen source areas. The performed analyses show that these source areas can be expected to contribute significantly to elevated grass pollen concentrations in the urban area, especially when peak concentrations are observed. Therefore it is crucial to include urban area sources in assessments and forecasts of pollen concentrations. The present study is, to the knowledge of the authors, the first published urban-scale grass pollen study which includes an integrated approach in the construction of an actual inventory of grass pollen flowering areas. The applied methodology is novel and uses available remote sensing and land use information. It also shows that the management of the grass areas (Fig. 4) is critical information in order to obtain an inventory for grass pollen source areas. Remote sensing or land cover information cannot stand alone in such an analysis. The next step will be to further develop a local-scale dispersion model, such as the OML or AERMOD models (Sommer et al., 2009; US-EPA, 2003), in order to apply the pollen emission inventory, and use this as a basis for understanding and explaining air movements transporting grass pollen on the local scale. This improved understanding can be used to develop an integrated urban-scale exposure system for co-exposure of allergenic pollen and chemical air pollutants as well as chemical reactions between allergenic pollen and air pollutants. Such an integrated methodology is state-of-the-art and has previously been used in air pollution and cohort studies (Hertel et al., 2006; Sorensen et al., 2003). It is believed that this methodology can be extended to allergenic pollen (Skjøth, 2009) by combining regional-scale models like SILAM (Sofiev et al., 2006a, b) or DEHM (Brandt et al., 2012) with local-scale models like OML or AERMOD in a similar way as applied in the DAMOS system (Geels et al., 2012) – by taking variations in pollen productivity into account (Brostrom et al., 2008) at the species level as well as in the flowering pattern of the main grass species. The applied methodology behind DAMOS has proven highly useful in the assessment of air pollutants for which it is crucial to account for sources that contribute as a result of atmospheric flow pattern on a micro-, meso-gamma and meso-beta scale (Hertel et al., 2013) (in this context see the highly useful definitions of micro-, meso-gamma, and meso-beta scale in Orlanski, 1975). This methodology therefore defines the strategy to explain how various levels of pollen concentrations affect the Danish population – connections that so far have been difficult to explain (Carracedo-Martinez et al., 2008; WHO, 2003).

Supplementary material related to this article is available online at: http://www.biogeosciences.net/10/541/2013/bg-10-541-2013-supplement.zip.

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Manuscript II

Seasonal variation in diurnal atmospheric grass pollen concentration profiles

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Seasonal variation in diurnal atmospheric grass pollen concentration profiles

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Abstract. In this study, the diurnal atmospheric grass pollen concentration profile within the Danish city of Aarhus was shown to change in a systematic manner as the pollen season progressed. Although diurnal grass pollen profiles can differ greatly from day-to-day, it is common practice to establish the time of day when peak concentrations are most likely to occur using seasonally averaged diurnal profiles. Atmospheric pollen loads are highly dependent upon emissions, and different species of grass are known to flower and emit pollen at different times of the day and during different periods of the pollen season. Pollen concentrations are also influenced by meteorological factors – directly through those parameters that govern pollen dispersion and transport, and indirectly through the weather-driven flowering process. We found that three different profiles dominated the grass pollen season in Aarhus – a twin peak profile during the early season, a single evening profile during the middle of the season, and a single midday peak during the late season. Whilst this variation could not be explained by meteorological factors, no inconsistencies were found with the theory that it was driven by a succession of different grass species with different diurnal flowering patterns dominating atmospheric pollen loads as the season progressed. The potential for exposure was found to be significantly greater during the late-season period than during either the early- or mid-season periods.

1 Introduction

Grass pollen is recognised as one of the principle causes of pollen allergy in Europe, with national sensitisation rates of up to 26 % reported for the region (Bousquet et al., 2007). Atmospheric grass pollen concentrations typically fluctuate over the course of a 24 h period, and diurnal patterns can differ greatly from day-to-day. The factors responsible for these differences are, however, not well understood. Pollen forecasts usually attempt to predict daily average concentrations, with this information disseminated to allergy sufferers in order that they might better manage their symptoms. The elicitation of allergy symptoms is dependent on an individual's recent exposure history (Connell, 1969), whilst exposure is directly related to the amount of time spent outdoors (Kailin, 1964; Mitakakis et al., 2000) and the ambient concentration at that time (Riediker et al., 2000). The time of day that pollen concentrations peak may thus be of greater importance to the allergy sufferer than the daily average concentrations typically made available to the public (Käpylä, 1981). A better understanding of what drives variation in diurnal grass pollen profiles may lead to improved advice on how allergy sufferers may best avoid exposure, and would furthermore help to improve the accuracy of pollen dispersion models (Viner et al., 2010).

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It is common practice in aerobiology to produce average diurnal pollen concentration curves based on entire pollen seasons in order to establish typical patterns of variation and times of peaks in concentration. These profiles have been found for grass pollen to vary with location, with single evening peaks (Emberlin and Norris-Hill, 1991; Mullins et al., 1986; Yang et al., 2003), single morning peaks (Galán et al., 1989, 1991; Trigo et al., 1997), two-peak profiles (Rantio-Lehtimäki et al., 1991; Kosisky et al., 2010), and invariant profiles (Gassmann et al., 2002) reported in the UK and Taiwan, Spain, Finland and the USA, and Argentina respectively. Intra-seasonal variation in diurnal profiles has, however, received scant attention in existing published literature. Mullins et al. (1986) compared profiles averaged over two different months (June and July) but found no discrepancies between the two, whilst Norris-Hill (1999) noted four different profiles for the four quarters of the season, and sought to relate these to rainfall patterns.

Pollen concentrations are influenced both by pollen emission and by the meteorological parameters that determine dispersion, transport and deposition (Galán et al., 1995). Different grass species release their pollen at different times of season (León-Ruiz et al., 2011) and at different times of day (Emecz, 1962). Diurnal flowering patterns are furthermore known to change in accordance with meteorological factors in a species-specific manner (Subba Reddi et al., 1988). The changing character of the diurnal grass pollen profile may thus be driven by the weather, the flowering patterns of local grasses, or both.

The objective of this study was to investigate and explain seasonal variation in the diurnal atmospheric grass pollen profile. This was achieved in the following manner:

1. Systematic changes in the diurnal grass pollen concentration profile were shown to occur in the Danish city of Aarhus as the pollen season progressed, with profiles for the early, middle and late periods of the season showing different statistical properties.

2. The potential for exposure during these three periods was estimated and compared in order to test whether the different profiles have the potential to lead to significant differences in exposure.

3. The hypothesis that the seasonal variation was driven by meteorological factors was tested against the alternative hypothesis that it related to a progression of different grass species dominating pollen emissions as the season developed.

4. An inventory of the principle grass species likely to be common in Aarhus was compiled together with available information on their flowering cycles, in order to support the interpretation of results.

2 Materials and methods

Medial time stamps are reported for both pollen concentration and meteorological data, as is the convention in aerobiology, with all time stamps given in Central European Time (UTC +1).

2.1 Site description and data provenance

The study was conducted using data from Aarhus, Denmark's second largest city. Aarhus lies on the east coast of Jutland, the peninsula that constitutes the western part of Denmark, and has a population of around 250 000 (Statistics Denmark, 2012). Aarhus is a green city in which numerous parks and unmanaged[1] natural areas are found. The surrounding countryside consists largely of arable land, with common crops including seedling grasses, permanent grass and rye, all of which are potential sources of grass pollen (Skjøth et al., 2013).

For the years 2009–2011, three temporary pollen monitoring stations were operational in Aarhus during the grass pollen season. The three monitoring stations were situated within 8 km of one another (Fig. 1). Each consisted of a Burkard Seven Day Recording Volumetric Spore Trap (Hirst, 1952) installed at roof level, 15–20 m above ground level. The Central Aarhus monitoring station was situated in the centre of the city on the roof of Aarhus Municipality Department of Nature and Environment, and was surrounded by a regularly managed lawn. The TV-2 monitoring station lay in the northern outskirts of the city on the roof of the TV-2 Østjylland TV station, close to open countryside and less than 100 m from an unmanaged grass field. The Rundhøjskolen monitoring station was situated on top of a school building in the southern suburbs of the city.

Uniform materials and methods were used at the three monitoring stations. Pollen data were collected and processed in accordance with the guidelines of the British Aerobiology Federation (1994). Samples were collected on Melinex tape coated with silicone fluid using standard seven-day drums. Weekly sample traces were divided into seven 24 h sections and mounted on microscope slides using a stain-bearing gelatine mountant. For each daily slide, bi-hourly concentration data were obtained by counting the number of pollen grains deposited along 12 transverse transects at 640 times magnification (equating to 9.75 % of each daily slide) under a light microscope, with counts converted into concentrations in grains m^{-3}.

Three-hour averaged wind speed, wind direction, surface air temperature, dewpoint temperature and precipitation data were obtained from the Flyveplads Kirstinesminde weather station (WMO Station ID 06074), situated just north of Aarhus (Fig. 1), courtesy of the UK Meteorological Office (2012). Three-hour averaged saturated and actual vapour

[1] Management is defined as cutting on a regular basis such that flowering does not occur.

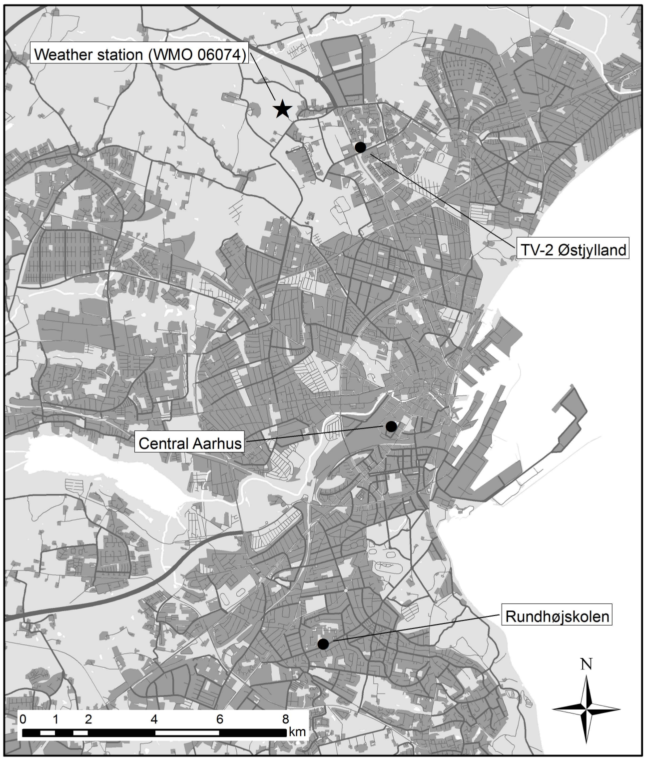

Fig. 1. Map of Aarhus showing the locations of the three temporary pollen monitoring stations and the weather station.

pressures were calculated from ambient and dewpoint temperatures respectively using Eq. (3) of Henderson-Sellers (1984). Vapour pressure deficit (VPD) was then computed using method 1A of Howell and Dusek (1995).

The daily dynamics of population activity were modelled using the time–activity diurnal curve for the population of the USA presented by Klepeis et al. (2001). The proportion of the population outdoors was determined for hours of the day corresponding to pollen data as the sum of the "residence-outdoors", "near vehicle (outdoors)" and "other outdoors" activity categories.

2.2 Data reduction and processing

2.2.1 Pollen data

The Aarhus grass pollen season ran from 20 May to 29 July in 2009, from 6 June to 8 August in 2010, and from 21 May to 27 July in 2011. Here we define the start (end) of the season as the first (last) day that a daily average of ≥ 10 grains m^{-3} was recorded at one of the three monitoring stations.

For each monitoring station and each year, the grass pollen concentration time series was divided into 24 h daily "profiles" (midnight–midnight). All daily profiles with a corresponding daily average concentration of < 20 grains m^{-3} were discarded, on the basis that at low concentrations the resolution of data becomes poor[2]. All profiles that coincided with precipitation were also discarded, since rain removes pollen grains from the air with great efficiency (McDonald, 1962) and may thus strongly influence profile shape. For each of the remaining 157 profiles (48, 54 and 55 for the Central Aarhus, Rundhøjskolen and TV-2 stations respectively, relating to 69 different calendar days), peaks in the diurnal pollen curve were identified according to the following criteria:

1. Each peak was required to have a minimum[3] bi-hourly concentration of ≥ 50 grains m^{-3}. Profiles where concentrations failed to exceed this threshold were considered to have no peak.

2. Overall maximum bi-hourly concentrations satisfying (1) were designated primary peaks.

3. Local bi-hourly concentration maxima occurring ≥ 6 h before or after a primary peak were designated secondary peaks, provided that the trough between the two was at least 50 grains m^{-3} deep.

4. Two candidate peaks of equal magnitude ≤ 4 h apart were considered a single peak and given an intermediary time stamp: for example, a peak bi-hourly concentration of 100 grains m^{-3} occurring at both 17:00 and 19:00 was defined as a single peak at 18:00.

5. Two candidate peaks of equal magnitude occurring six or more hours apart were considered separate peaks.

6. Apparent peaks close to midnight that were associated with an actual peak occurring on the preceding evening were rejected (e.g. a peak at 01:00 was rejected if a greater concentration occurred at 23:00 on the previous evening).

In this manner each profile was characterised in terms of the time at which peaks occurred, with each profile featuring 0, 1 or 2 peaks. These peak-time profiles were then grouped by site and year, and each group arranged in chronological order. Three characteristic profile types were observed to dominate at different points during the grass pollen season, meaning that the season could be divided into three distinct periods:

[2] Profiles with daily average concentrations < 20 grains m^{-3} generally failed to show a well-defined diurnal pattern, with the random nature of sample collection apparently dominating. An alternative (and relaxed) criterion of a daily maximum bi-hourly concentration > 50 grains m^{-3} was considered, but was ultimately rejected as it increased the number of poorly defined profiles. It should be noted that profiles were rejected purely based on data resolution – some sensitive allergy sufferers have been reported to experience symptoms at daily average concentrations as low as 1 grain m^{-3} (Hyde, 1972).

[3] The value 50 grains m^{-3} was chosen because this is generally considered to be the average concentration above which all individuals sensitised to grass pollen experience symptoms (Galán et al., 1995).

Period 1: The early season, characterised by a twin morning and evening peak profile.

Period 2: The middle of the season, characterised by a single evening peak profile.

Period 3: The late season, characterised by a single late morning/early afternoon peak profile.

Dates of transition between periods were determined for each monitoring station and each year as the point where the dominating diurnal profile switched from the character of one period to the character of another, and each profile was thus assigned to period 1, 2 or 3.

For each of the three periods, the peak-time distributions of data collected at the three monitoring stations were then compared using the Anderson–Darling two-sample goodness-of-fit test with adjustment for ties (Trujillo-Ortiz et al., 2007). Results were considered significant at the 95 % level, and are summarised in Table 1. For periods 1 and 3 no differences were found between the three stations. For Period 2 the distribution of TV-2 data was found to differ significantly from those of the Central Aarhus and Rundhøjskolen stations; however inspection of the data showed that distributions were very similar except that the highly dominant modal peak time for the TV-2 station was 17:00, whilst for the Central Aarhus and Rundhøjskolen stations it was 19:00. It was therefore considered appropriate to pool data collected at the different monitoring stations for each of the three seasonal periods. Using these pooled data, the Anderson–Darling test was then used to test for differences between the peak-time distributions of the three periods.

Table 1. Anderson–Darling test (with adjustment for ties) for differences in peak-time distribution between the three monitoring stations for each seasonal period. D is the Anderson–Darling rank statistic and p the associated probability.

		Central/ Rundhøjskolen	Central/ TV-2	Rundhøjskolen/ TV-2
Period 1	p	0.650	0.147	0.435
	D	0.476	1.604	0.832
Period 2	p	0.592	0.004[b]	0.030[a]
	D	0.562	4.535	2.855
Period 3	p	0.783	0.663	0.606
	D	0.234	0.443	0.540

[a] Indicates a significant difference at the 95 % level.
[b] Indicates a significant difference at the 99 % level.

2.2.2 Population exposure

In order to neutralise magnitude-related differences and isolate the qualitative shape of each diurnal profile, pollen concentration data were standardised by dividing each bi-hourly value by its respective daily maxima. A proxy for population exposure was then calculated for every other hour of the day by multiplying the standardised concentration by the proportional outdoor population. For each day, bi-hourly standardised population exposure values were summed to give a measure of the daily total standardised population exposure, and the Wilcoxon rank sum test (normal approximation applied) used to test for differences in daily total standardised population exposure between the three periods.

2.2.3 Meteorological data

Wind speed, temperature and VPD data were selected for days corresponding to the pollen dataset, and divided into three groups corresponding to the three periods of the pollen season defined above. Data for days where periods overlapped between the different monitoring stations were omitted. Data from one further day were omitted due to an incomplete record. Average diurnal profiles were plotted for each variable and each period, and differences in diurnal profile shape were tested for by grouping data by time of day. The Wilcoxon rank sum test was then applied to each temporal group. Results were considered significant at the 95 % level.

2.2.4 Grass species inventory

The inventory of grass species was composed from species listed by Frederiksen et al. (2006) as “common” or “very common” along roads and railways and in parks – the habitats where unmanaged grasses are likely to be found in Aarhus according to Skjøth et al. (2013) – and species found to be common in Copenhagen by Hald (2011). It seems likely that species that are abundant in Copenhagen will also be well represented in other large Danish cities. Data on the pollen productivity and flowering behaviour of constituent species were gathered from available existing literature and added to the inventory.

3 Results

3.1 Pollen data

The availability of pollen data and the way they were distributed between the three seasonal periods can be seen in Fig. 2. The maximum period overlap between stations within a single year was 4 days (the transition from Period 2 to 3 at the Rundhøjskolen and TV-2 stations during 2009). The transition from Period 1 to 2 occurred later in 2010 than in 2009 or 2011; the transition from Period 2 to 3 also occurred later in 2010 than in 2009, but cannot be precisely located for 2011 due to the sparsity of data. Period duration cannot in general be precisely stated due to the volume of missing data, but was typically in the order of 1–2 weeks and appears to have been briefer during 2010 than during 2009 or 2011.

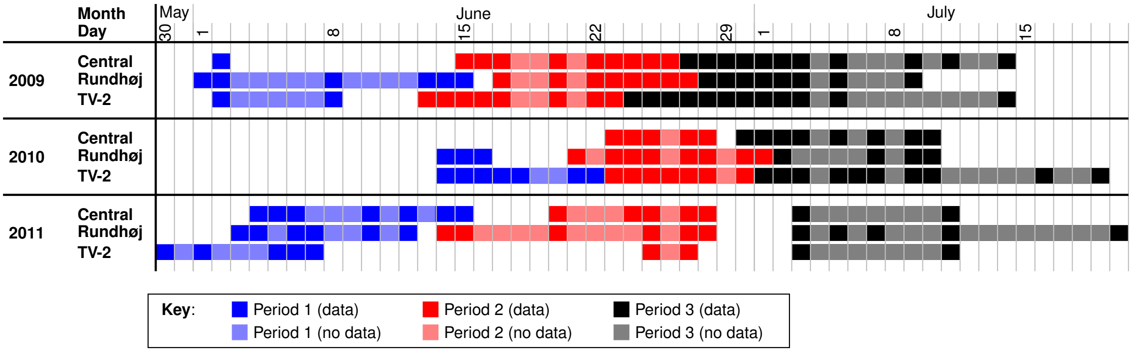

Fig. 2. Assignment of data to the three seasonal periods for the Central Aarhus (Central), Rundhøjskolen (Rundhøj) and TV-2 monitoring stations for each year. Contributing dates (i.e. dry days with daily average concentrations ≥ 20 grains m^{-3}) are in bold, and non-contributing dates for which period affiliation can be projected are faded.

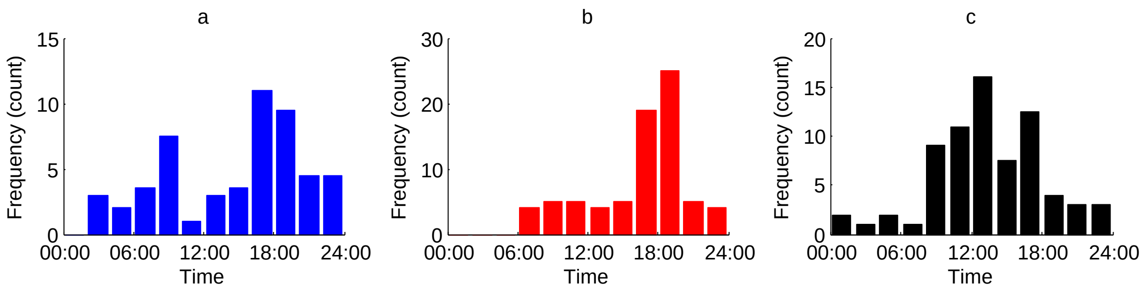

Fig. 3. Peak-time distributions for **(a)** Period 1 ($n = 37$), **(b)** Period 2 ($n = 58$) and **(c)** Period 3 ($n = 62$), where n is number of constituent profiles. Where peaks straddled two time slots, each bi-hourly bin was assigned a value of 0.5.

The peak-time distributions of the pooled data are presented in Fig. 3. Period 1 shows a bimodal tendency, with morning peaks common at 09:00 and evening peaks common at 17:00 and later. Period 2 shows a uni-modal distribution, with the majority of peaks occurring at 17:00–19:00 and otherwise background peak levels between 07:00 and 23:00. Period 3 also shows a uni-modal distribution, but with peaks common between 09:00 and 17:00 and attaining a maximum frequency at 13:00. According to the Anderson–Darling test, the peak-time distributions for the three periods differ significantly from one another at the 95 % level (periods 1 and 2: $D = 2.51$, $p = 0.048$; periods 1 and 3: $D = 3.84$, $p = 0.010$; periods 2 and 3: $D = 11.51$, $p < 0.001$).

3.2 Population exposure

Average standardised population exposure diurnal profiles are presented in Fig. 4 for the three periods, together with average standardised pollen concentration profiles. The standardised population exposure profiles of periods 1 and 2 are reasonably similar, both showing essentially only a single evening peak at 17:00. Period 3, however, shows a different profile, peaking around midday. Average concentration profiles agree qualitatively with the peak distributions of Fig. 3.

The daily total standardised population exposure mean (range) was 0.31 (0.09–0.55) for Period 1, 0.35 (0.17–0.70) for Period 2 and 0.40 (0.20–0.59) for Period 3. According to the Wilcoxon rank sum test, daily total standardised population exposure did not differ significantly between periods 1 and 2 ($W = 1535$, $p = 0.1733$), but did differ significantly

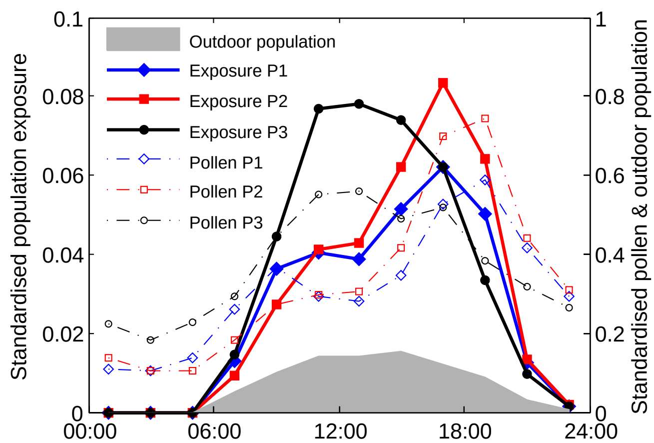

Fig. 4. Mean diurnal standardised population exposure (solid lines) and standardised pollen concentration (dotted lines) profiles for Period 1 (P1), Period 2 (P2) and Period 3 (P3). The outdoor population profile (solid), derived from the results of Klepeis et al. (2001), is also shown.

between periods 1 and 3 ($W = 1282$, $p < 0.001$) and between periods 2 and 3 ($W = 3070$, $p = 0.0061$).

3.3 Meteorological data

Median diurnal profiles for the three periods are presented in Fig. 5, together with the results of the Wilcoxon rank sum test. No significant differences were found between the three periods for wind speed or between periods 1 and 2 for temperature or VPD, with the exception of temperature between 22:00 and 01:00, when Period 2 values tended to be higher. Temperatures during Period 3 were found to be significantly higher than those during periods 1 and 2 at all times of day. VPD was found to be significantly higher during Period 3 than Period 2 between 07:00 and 13:00, whilst no significant differences were found between periods 1 and 3.

Figure 6 shows time of peak plotted against concurrent wind direction. Winds appear to be dominated by two sectors, south-east and west; however within both sectors, peaks are seen to occur at all times of day with the exception of Period 1, where all morning peaks occur under winds from the west. This, however, appears to reflect the fact that almost all peaks during Period 1 were accompanied by westerly winds. Figure 7 shows time of peak plotted against the number of days since rain. There is no apparent relationship between the two.

3.4 Grass species inventory

Table 2 lists the 18 grass species likely to be present in Aarhus. Data on diurnal flowering behaviour was found in existing published literature for 12 of these species. Amongst these are seven species that have been reported to flower at around the time of the early peak during Period 1 (*Alopecurus pratensis*, *Dactylis glomerata*), around the time of the evening peak during periods 1 and 2 (*Arrhenatherum elatius*, according to Jones (1952)), or during the middle of the day when peaks commonly occur during Period 3 (*Festuca arundinacea*, *Lolium perenne*). There are also species that have been reported to flower twice per day at times coinciding more or less with the two peaks of Period 1 (*Anthoxanthum odoratum*, *Holcus lanatus*).

Pollen productivity estimates were found for nine of the species in Table 2, with the number of pollen grains produced per inflorescence ranging from 0.1×10^6 (*Poa annua*) to 11.7×10^6 (*Festuca arundinacea*). Of those species whose flowering times coincided with concentration peak times, three (*Dactylis glomerata*, *Festuca arundinacea*, *Lolium perenne*) were reported to be relatively productive with estimated yields of at least 2.3×10^6 grains per inflorescence, one (*Holcus lanatus*) had received conflicting productivity estimates, two (*Alopecurus pratensis*, *Arrhenatherum elatius*) had unknown productive capacity, and only one species was reported to be relatively unproductive (*Anthoxanthum odoratum*).

4 Discussion

4.1 Drivers of diurnal grass pollen concentration variation

In this study, we have shown that the diurnal pollen concentration profile for the Danish city of Aarhus varies in a systematic manner as the pollen season progresses. Using statistical methods, different diurnal patterns were shown to dominate during different periods of the season: twin morning and evening peaks characterised the early part of the season, a single evening peak the middle of the season, and a single midday peak the late season. That diurnal grass pollen profiles vary from day-to-day is well known (Käpylä, 1981). It has also previously been demonstrated that the grass pollen season can be divided into several periods with different characteristic properties. In their 7-day ahead grass pollen forecast model for the UK, Smith and Emberlin (2005) used different parametrisations for the pre-peak, peak and post-peak periods of the grass pollen season, whilst Sánchez Mesa et al. (2003) obtained greater correlation between meteorological parameters and the daily average grass pollen concentration by isolating the pre-peak period from the remainder of the season. However, as far as the authors are aware, the systematic variation found during this study has not previously been shown to occur.

Atmospheric pollen concentrations are determined by two sets of variables – those that mediate pollen release into the atmosphere, and those that mediate its dispersal from source to receptor (Galán et al., 1995). Pollen emission is regulated by biological and meteorological factors, which restrict it to a limited range of weather conditions and a specific portion of the day (Raynor et al., 1970). For plants that flower during turbulent weather, which can be crudely approximated as the hours of daylight, we would expect flowering and increased atmospheric concentrations typically to coincide. This is especially true for taxa with smooth pollen grains that are relatively easily removed from the anther (Subba Reddi and Reddi, 1985), or relatively large pollen grains whose residence time in the atmosphere is limited (Skjøth et al., 2013). For Poaceae pollen, which is both smooth and relatively large, the timing of peaks in atmospheric concentration can thus be expected in general to follow patterns of local emission.

Pollen primarily enters the atmosphere directly from the anthers following flowering. For grasses, flowering intensity generally follows regular diurnal cycles that differ from species to species, and furthermore vary with changing weather conditions (Emecz, 1962; Jones, 1952; Subba Reddi et al., 1988). Different species of grass flower at different points during the pollen season (León-Ruiz et al., 2011), meaning that as the season progresses, different subsets of the local grass flora are likely to be contributing to the atmospheric pollen load. The systematic variation observed in this study could therefore potentially be driven by two different

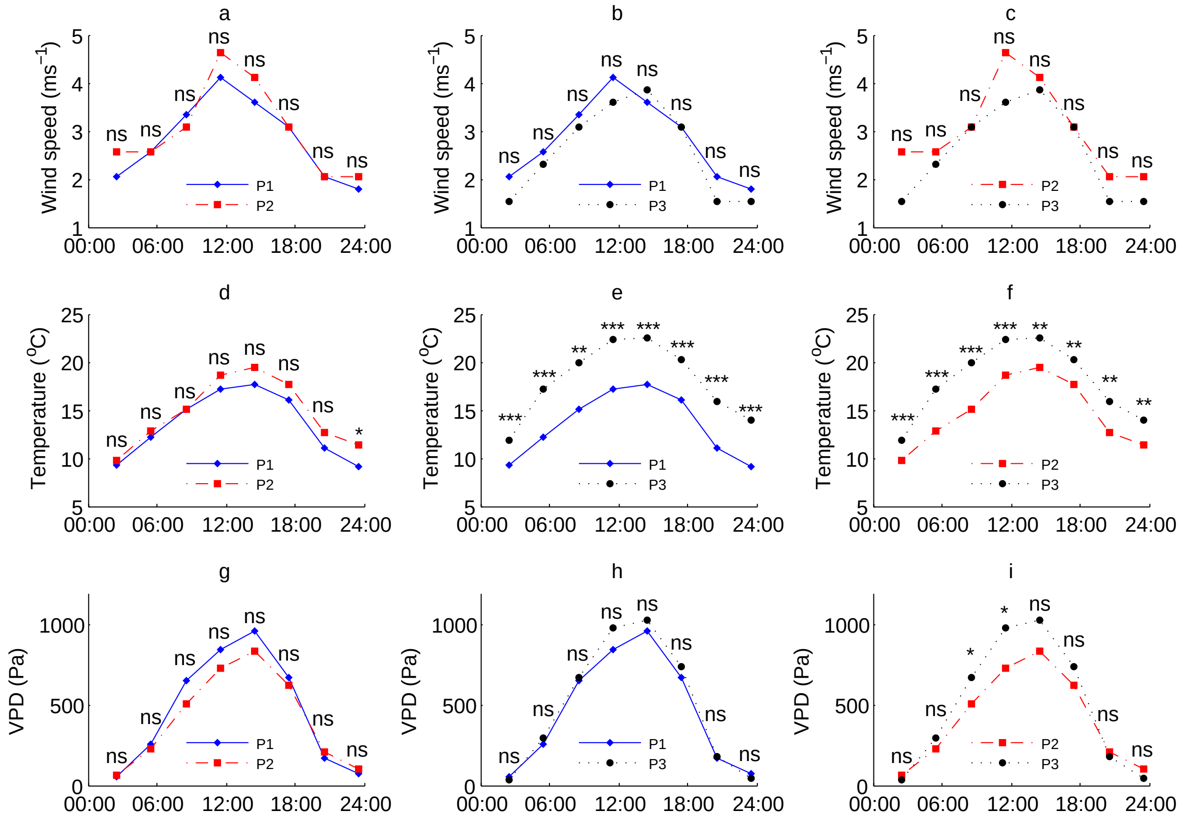

Fig. 5. Pairwise median diurnal profiles for wind speed **(a–c)**, temperature **(d–f)** and VPD **(g–i)** for Period 1 (P1, $n = 16$), Period 2 (P2, $n = 17$) and Period 3 (P3, $n = 24$). Results of the two-tailed Wilcoxon rank sum test are indicated for each three-hour weather data averaging period, with "ns" indicating no significant difference and "$\star$", "$\star\star$" and "$\star\star\star$" indicating significant differences at the 95, 99 and 99.9 % levels respectively.

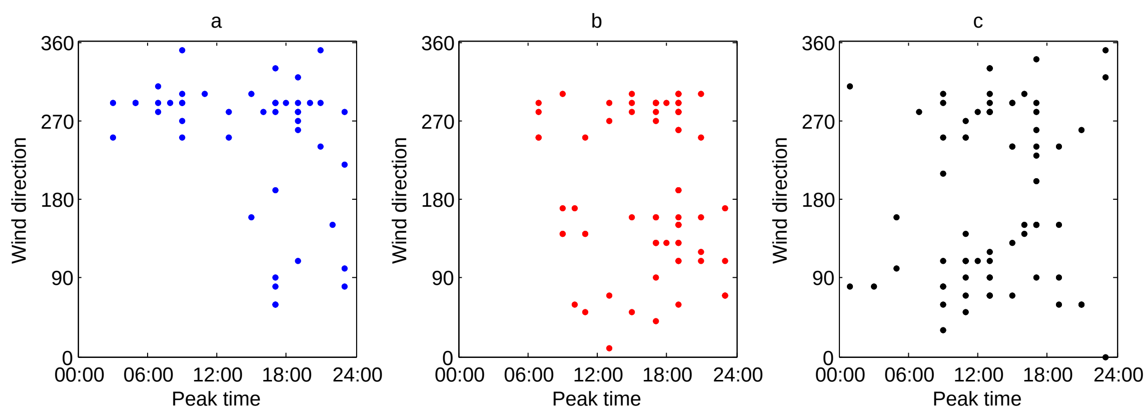

Fig. 6. Scatter plots showing time of the peak against concurrent wind direction for **(a)** Period 1, $n = 37$; **(b)** Period 2, $n = 58$; and **(c)** Period 3, $n = 62$.

factors: a difference between the weather conditions in the three periods of the season, or a succession of different grass species dominating pollen emission as the season develops.

4.2 Do meteorological factors explain the three periods ?

Flowering amongst grasses is in general dependent on a species-specific temperature threshold being exceeded (Emecz, 1962). It is possible that higher temperatures could

Table 2. Grass species that are likely to be present in and around Aarhus. Constituent species are listed by [a] Frederiksen et al. (2006) as "common" or "very common" along roads and railways and in parks, and/or were found by [b] Hald (2011) to be common in the city of Copenhagen. Details of productivity (grains/inflorescence[c,e] or spike[d] – note that an inflorescence consists of one or more spikes (Guinther, 2013)), time of flowering (range gives period of flowering & hour denotes time of peak flowering, unless otherwise indicated), minimum temperature threshold ($^{\circ}$C) that must be exceeded in order for flowering to occur (square brackets indicate temperature that induces maximum liberation), and minimum light intensity (i – foot candles) and duration (d – hours) necessary to initiate flowering. Information is derived from the following sources: [c] Prieto-Baena et al. (2003), [d] Smart et al. (1979), [e] Aboulaich et al. (2009), [f] Emecz (1962), [g] Ogden et al. (1969), [h] Jones (1952), [i] Beddows (1931), [j] Smart and Knox (1979), [k] Evans (1916), and [l] Clark (1911). * Also known as *Agropyron repens*.

Species	Productivity	Time of flowering	Temp	Light i/d
Agrostis capillaris[a]	–	–	–	–/–
Agrostis stolonifera[b]	2 426 609[c]; 777 058[e]	–	–	–/–
Alopecurus pratensis[a]	–	07:52[f]; 06:00–11:00[i]	11[f]	–/10[f]
Anthoxanthum odoratum[a]	621 363[e]	05:00–10:00 and 17:00[i]	–	–/–
Arrhenatherum elatius[a,b]	–	15:00–19:00[h]; 05:00–11:00 and 18:00–19:00[i]	–	–/–
Bromus hordeaceus[a]	245 176[c]; 407 489[e]	06:00[i]	12[i]	–/–
Dactylis glomerata[a,b]	7 971 347[c]; 3 700 000[d];	06:25–08:35[f] (peak); 04:00–10:00[h];	15.5[f]	1600/8[f]
	3 419 469[e]	04:00–10:30[i]		
Elytrigia repens[a,b,*]	–	14:00–18:00[h]; 16:30[i]	23[i]	–/–
Festuca arundinacea[a]	11 697 131[c]	15:08[f]; 06:00[i]	17[f][14[i]]	3600/5[f]
Festuca brevipila[a]	–	–	–	–/–
Festuca pratensis[a]	–	10:00[f]; 06:00[i]	15[f][14[i]]	1200/2[f]
Festuca rubra[a,b]	–	06:00; 09:45–14:30 (peak 12:00–13:00)[i]	–	–/–
Holcus lanatus[a,b]	875 715[c]; 4 500 000[d]	Typically ∼ 06:00–07:00 and ∼ 18:00–19:00 though other patterns also reported[i]	–	–/–
Lolium perenne[a,b]	2 300 000[d]	11:45–15:20[f] (peak); 09:00–12:00[i] 12:00–14:00 & 20:00–22:00[j] (peak)	14–17[f]	2000–5200/1.5–3[f]
Phleum pratense[a,b]	–	05:48–08:54[f] (peak); mostly 06:00–08:00[g]; ∼ 03:00[l]; 02:00–04:00[k] (peak); 04:00–09:00[h] 4:30–10:00[i]	16–17[f]	2200–3000/10[f]
Poa annua[b]	115 511[c]; 142 911[e]	04:30[i]	11[i]	–/–
Poa pratensis[a,b]	–	03:00–08:00[h]	–	–/–
Poa trivialis[b]	2 088 492[e]	–	–	–/–

lead to thresholds being exceeded earlier in the day, bringing the time of flowering forward. Temperatures tended to be higher during Period 3 than during periods 1 or 2 at all times of day; however the earliest peaks occurred during Period 1. Temperatures also showed a tendency to be higher during Period 2 than during period 1 between 22:00 and 01:00. We would expect that if this had any effect, it would lead to earlier peaks during Period 2 that during Period 1; however, the opposite is in fact seen.

Anther dehiscence, the process during which the anthers split open to release pollen, occurs following dehydration (Stanley and Linskens, 1974, p. 24). VPD may be considered a proxy for the drying power of the air, and greater VPD earlier in the day may thus lead to earlier drying, emission and concentration peaks. The shift from an evening to a midday peak as Period 2 transitions into Period 3 is indeed accompanied by a significant increase in VPD between 07:00 and 13:00; however the VPD values that accompanied the earliest

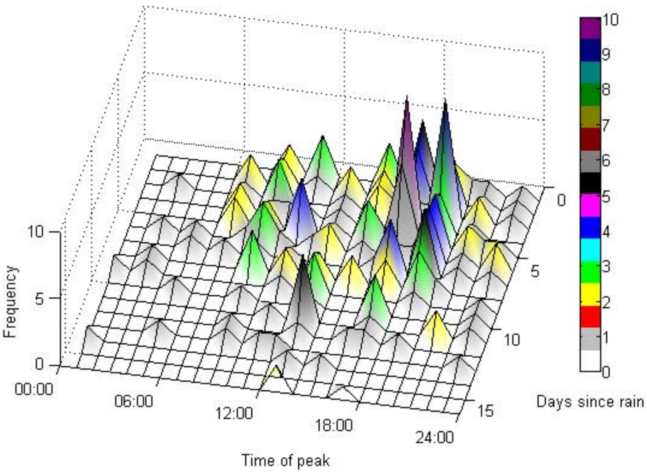

Fig. 7. Frequency map of peak time against the number of days since ≥ 1 mm rain.

peak, which occurred during Period 1, did not differ significantly from those of periods 2 or 3 at any time of day.

Horizontal transport is dependent on wind direction (Stull, 1988, p. 3–5), and if major sources are not found in all compass directions about a monitoring station, it is possible that concentration peaks are the result of wind directions that carry pollen from source to monitoring station. Although all morning peaks were recorded under westerly winds during Period 1, morning peaks are seen to occur under winds from all directions of the compass during periods 2 and 3.

Wind speed is associated both with the primary emission of pollen from the anthers (Emecz, 1962; Lu et al., 2005) and with secondary emission through resuspension (Sánchez Mesa et al., 2003; Sehmel, 1980); however, no significant difference in wind speed was found between the three periods of the grass pollen season. Norris-Hill (1999) proposed that the timing of diurnal grass pollen peaks may be related to the time elapsed since rainfall due to the availability of pollen for resuspension. In this study, we find no relationship between the two.

In summary, we thus find no evidence in support of the theory that the systematic changes in the diurnal grass pollen concentration pattern in Aarhus are related to meteorological factors.

4.3 Could flowering time explain the three periods?

Pollen production can vary hugely between species; indeed, Table 2 shows that pollen production amongst the species common in Aarhus may be expected to vary over at least 2 orders of magnitude. Clearly species that are both abundant and prolific pollen producers will hold greater influence over atmospheric pollen concentrations, and thus it is possible that the diurnal pattern of pollen concentration variation is determined by only a handful of species. León-Ruiz et al. (2011) identified only four locally occurring species as being likely to contribute significantly to atmospheric pollen concentrations in Córdoba, Spain. Of the species likely to be relatively abundant in Aarhus, the times of flowering of seven are expected to coincide with the times of day that peak concentrations tended to occur; of these seven species, at least three are thought to be relatively prolific pollen producers (see Table 2).

It seems probable that the period during which an individual grass species has the potential to dominate pollen emission will largely be limited to the "full flowering" phase of the flowering cycle, the period during which the central 50 % of anthers dehisce. León-Ruiz et al. (2011) found that the length of the full flowering phase varied between species, but was typically in the range 1–2 weeks, i.e. comparable with the typical lengths of the three periods identified in this study. The full flowering phase was also found to be briefer during years when flowering began late. The start of the Aarhus grass pollen season, here defined as the first day with an average concentration ≥ 10 grains m^{-3} at any of the three monitoring stations, occurred later in 2010 (6 June) than in either 2009 or 2011 (20 and 21 May respectively). This coincided with a tendency for later transition dates between the three periods, and also with apparently briefer periods.

It is well known that local-scale variation in micro-climate can cause the onset of flowering to vary by several days. The urban heat island can, for example, advance the flowering of grasses within a city compared with on the outskirts (Emberlin et al., 1993; Rodríguez-Rajo et al., 2010), whilst the onset of flowering in *Platanus* trees has been reported to differ between different areas of the same city (Alcázar et al., 2004). In the present study, the dates of transition between the three periods were found to differ between monitoring stations by up to 4 days, meaning that differences in diurnal pattern were found over distances under 5 km. The resulting overlap between periods at the different monitoring stations could be explained by small differences in the onset and culmination of the main flowering phase of specific species.

The theory that the observed systematic variation in the diurnal grass pollen concentration profile is driven by a progression of different grass species dominating grass pollen emissions as the season progresses is therefore consistent both with the characteristics of the three seasonal periods, and with the flowering behaviour of the grass species thought to have a significant local presence. No contradictory evidence has been found.

4.4 Implication of the three periods on population exposure

One way of preventing or minimising the development of allergic symptoms is to minimise exposure by pursuing allergen avoidance strategies (Custovic et al., 1998). The Danish Asthma and Allergy Association, for example, advise patients to remain indoors around midday (Astma-Allergi Danmark, 2013), the time that grass pollen concentrations are expected to peak at the Copenhagen pollen monitoring station (Sommer et al., 2006). This study, however, reveals that the time of peak pollen concentrations can vary through the season. One way of evaluating the significance of this finding from an allergy sufferer's perspective is to test whether the potential for exposure is likely to differ between the three periods.

The daily total standardised population exposure was significantly greater during Period 3 than during periods 1 and 2 (this does not imply that greater magnitude exposure is expected during period 3 – standardised population exposure is a relative value that can be used for comparing the impact of "typical" days from the three seasonal periods with similar magnitude pollen concentration peaks). That daily total standardised population exposure did not differ significantly between periods 1 and 2 likely reflects the similarity between the exposure profiles of these two periods, with the early peak during Period 1 having limited influence because a relatively small proportion of the population are expected to be outdoors at this time. The increase in exposure risk during Period 3 can be explained by the coincidence of midday peaks in pollen concentration and the proportion of the population outdoors. The clinical impact of increased exposure risk at the end of the pollen season is unclear – according to the priming effect theory, symptom intensity increases following repeated exposure (Connell, 1969), which could lead to increasing sensitivity amongst allergy sufferers as the season progresses; however, in the Netherlands similar pollen concentrations have been found to provoke more intense symptoms during the early than the late grass pollen season (de Weger et al., 2011).

We would not expect the exposure experienced by a cohort of allergy sufferers adhering to allergen avoidance advice to be represented by that of the entire population. The above result indicates that individuals within a population that continue to behave "normally" and do not moderate their behaviour would be expected on average to experience greater exposure during Period 3 than during periods 1 or 2 on days with pollen concentration peaks of equal magnitude. A dynamic avoidance strategy that takes into account this seasonal variation in the diurnal concentration pattern could help an individual to reduce their personal exposure.

It is worth noting that grass pollen concentrations measured at urban background monitoring stations do not necessarily equate to those encountered by the local population – indeed, Peel et al. (2014) found a tendency for concentrations measured at roof level monitoring stations to be greater than those recorded simultaneously at street level within the key exposure environment of the urban street canyon, implying that monitoring station data may overestimate within-canyon exposure. Monitoring station and within-canyon data were, however, also found to be fairly strongly and significantly correlated (Spearman's correlations coefficients of 0.84 and 0.65 for canyons in Aarhus and London respectively), suggesting that the variation in the diurnal pattern detected in the monitoring station data analysed in this study will be replicated at street level.

5 Conclusions

In this study it was shown that the typical diurnal grass pollen concentration profile for the city of Aarhus changes as the pollen season progresses, leading to three distinct periods of the season with different profiles. This variation most likely reflects a succession of grasses flowering and dominating pollen emission as the season progresses. The change in profile shape is expected to lead to a significant increase in standardised exposure potential during the final period of the season.

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Manuscript III

Cluster analysis of variations in the diurnal pattern of grass pollen concentrations in Northern Europe (Copenhagen) and Southern Europe (Córdoba)

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Cluster analysis of variations in the diurnal pattern of grass pollen concentrations in Northern Europe (Copenhagen) and Southern Europe (Córdoba)

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Abstract

From an allergological point of view, Poaceae pollen is the most important type of pollen that the population is exposed to in the ambient environment. There are several studies on intra-diurnal patterns in grass pollen concentrations, and agreement on the high variability. However, the method for analysing the different patterns is not yet well established. The aim of the present study is therefore to examine the method of pattern analysis by statistical clustering, as well as relating the proposed patterns to time of season and meteorological variables at two highly different biogeographical locations; Córdoba, Spain and Copenhagen, Denmark.

Airborne pollen is collected by Hirst type volumetric spore traps and counted using an optical microscope at both sites. The counts were converted to bi-hourly concentrations and a cluster analysis was applied with the aim of determining the most frequent diurnal patterns in pollen concentrations and their dependencies of site, season and meteorological variables.

Three different well defined diurnal patterns were identified at both locations. The most frequent pattern in Copenhagen was associated with days having peak pollen concentrations in the evening (maximum between18h-20h), whereas the most frequent pattern at Córdoba was associated with days having peak pollen concentrations in the afternoon (maximum between 14h-16h). These three patterns account for 70% of days with no rain and pollen concentrations above 20 grains m^{-3}. The most frequent pattern accounts for 40% and 57% of the days in Cordóba and Copenhagen respectively. The analysis clearly shows the great variation in pollen concentration pattern, albeit a dominating pattern can be found.

It was not possible to explain the differences in the patterns by the meteorological variables when examined individual.

Clustering method is estimated to be an appropriate methodology for studying aerobiological phenomena with high variability.

Keywords: Poaceae pollen, bioaerosols, clustering, bi-hourly, aerobiology, meteorology.

Introduction

Grass pollen is one of the most important from an allergological point of view. It is the most wide-spread pollen (Skjøth et al., 2013a) and may be considered as the most important cause of pollinosis in Europe due to a long season (Smith et al., 2014), its wide-spread distribution (Skjøth et al., 2013b) and the generally very high number of sensitizations (Burbach, 2009; D'Amato et al., 2007). Numerous species contribute to the concentration of this pollen {Kraaijeveld, 2015 505 /id}. Different grass species flower at different times of the year (Beddows, 1931; Jones, 1952) and day, this may affect the diurnal patterns in pollen from this family. As an example, *Agrostis* and *Festuca* flower at midday whereas *Anthoxanthum* and *Holcus* flower in the morning or late afternoon (Hyde and Williams, 1945; Peel et al., 2014). A number of studies have demonstrated an afternoon maximum in the concentrations of grass pollen (e.g. Goldberg et al., 1988; Simoleit et al., 2015). Nevertheless, variations in airborne grass pollen concentrations are not solely related to time of anthesis. Due to their transport and dispersion in the air, pollen concentrations also depend on meteorological parameters like urban atmospheric stability and local breezes (Puc, 2012; Pérez-Badia et al., 2011; Muñoz Rodríguez et al., 2010; Kasprzyk, 2006). Convection lift pollen grains to higher elevation and when convection currents cease at the end of the afternoon, suspended particles are subject to gravitational settling which lead to increasing pollen concentrations at lower height.

Airborne grass pollen in Copenhagen (Denmark) and Córdoba (Spain) are likely to have different diurnal patterns, and no previous studies have reported multiple diurnal patterns for either site. This difference is caused by considerable differences in climate and species composition as described by base maps used in the habitat directive (e.g. http://www.eea.europa.eu/data-and-maps/data/biogeographical-regions-europe). Also differences in local relief could affect the patterns, as Córdoba is highly affected by the surrounding mountains, and Copenhagen is located at a flat coastal position.

Grass pollen concentrations originate from an amalgam of species (García-Mozo et al., 2010). However, in Córdoba (Spain) it has been shown that just four Poaceae species are the dominating contributors to pollen concentrations (León-Ruiz et al., 2011; Cebrino et al., 2016) while other regions, such as Leiden in Northern Europe, has a different profile (Kraaijeveld et al., 2015). Finally, meteorological effects on the diurnal profiles for grasses can vary considerable between years, e.g. potentially limiting flowering of specific grass species responsible for the early season profile, as the one observed by Peel et al. (2014). Long term studies and interregional comparisons are therefore important. Previous studies conducted in Córdoba have showed that the diurnal pattern in grass pollen concentration was homogeneous throughout the study years. These patterns showed an increase early in the morning, a moderate decrease in the afternoon, and stable values throughout the night (Galán et al., 1989; Cariñanos et al., 1999; Alcázar et al., 1999). A previous study in Copenhagen has shown the maximum frequency of pollen peak concentrations during the afternoon (Goldberg et al., 1988), whereas a recent study showed seasonal variation in the profile (Peel et al., 2014).

Determining the actual concentrations of pollen including the diurnal pattern is an important element in providing advice to patients on allergen avoidance during peak hours in pollen concentrations (Sommer et al., 2009). These patterns can vary over the season and can be specific to the geographical region. A single unified pattern therefore has limitations. The diurnal pattern and its potential variations are of importance for patients suffering from allergy as well as for doctors that are studying allergic rhinitis or treating and guiding patients. Until now primarily seasonal averaged diurnal pattern in pollen concentrations have been available in literature and the interest in bringing this a step forward and provide diurnal pattern as function of time of season and meteorological conditions is the background for the presented work.

The aim of this paper was to analyse the variation in diurnal patterns of grass pollen concentrations in Copenhagen (Denmark) and Córdoba (Spain). The diurnal patterns in bi-hourly pollen concentration are examined by statistical clustering to objectively reveal groups solely based on pattern and relate these to meteorology and time of season.

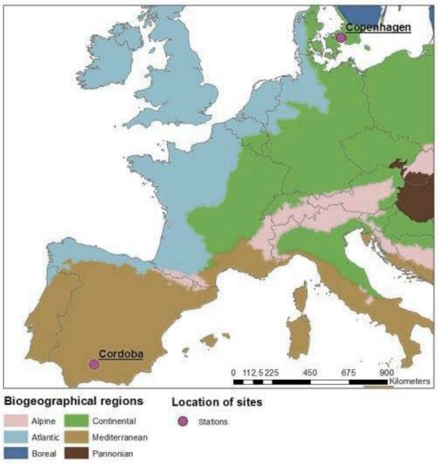

Figure 1 Biogeographical regions and the locations of Copenhagen and Córdoba.

Material and Methods

This study investigates the measured bi-hourly pollen concentrations from two pollen traps in the cities of Copenhagen and Córdoba (Fig.1). Continuous monitoring of pollen in the air is carried out from Hirst type volumetric spore traps (Hirst, 1952). Air is sucked into the trap at a rate of 10 L/min through a 2mm×14mm orifice. Behind the orifice, the air flows over a rotating drum that moves past the inlet at 2 mm/h. The drum is covered with an adhesive coated, transparent plastic tape, which traps the particles through impaction.

Copenhagen is the capital of Denmark. It is situated on the eastern coast of Zealand island (55º40'N, 12º34'E), 20 m a.s.l. The city is in located on low lying flat ground near the coast and subject to low pressure systems from the Atlantic resulting in unstable conditions throughout the year. The area is mainly urban with agricultural surroundings and biogeographically located in the northern part of the continental region with little distance to the Atlantic and Boreal regions. The annual diurnal mean temperature is 8ºC and the annual precipitation is 613 mm with rainfall fairly evenly distributed throughout the year. Weather data is obtained from near the pollen trap, including hourly measurements of temperature, wind speed and direction. Daily precipitation is from the nearby synoptic meteorological site at Kastrup airport (USAF-ID 061800), obtained from the data set Global Summary of the Day exchanged by World Meteorological Organisation. The pollen trap is situated 15 m above ground level on the roof of the Danish Meteorological Institute (55°43 N, 12°34 E). The Copenhagen pollen monitoring station is part of the permanent Danish pollen monitoring network, and is typically in continuous operation from January to October.

The typical grass pollen season in Denmark is from end of May till end of August, peaking at the end of June, with an average annual pollen index of 2200, varying from 588 to 3222 (1985-2009). Peak daily pollen concentration occurred in 2004 with 320 pollen m^{-3} (Sommer and Rasmussen, 2011).

The city of Córdoba is placed in the south of Iberian Peninsula (37°50'N, 4°45'W), 123 m a.s.l. The area has a Mediterranean climate with some continental features. The annual mean temperature is 17.8 °C and the annual average precipitation is 621 mm, with hot dry summers. The nearby area is urban with agricultural surroundings (pasture and crops under rotation), olive plantations as well as shrub and/or herbaceous vegetation. Biogeographically Córdoba is located in the southern parts of the Mediterranean region. Weather data, including hourly measurements of temperature, precipitation, wind speed and direction, were provided by the central service for research support of the University of Córdoba (SCAI), based on readings taken at Rabanales Campus, located around 10 km north-east of the pollen sampler site.

The trap in Córdoba city is located on the roof of the Educational Sciences Faculty, at 15 m above ground level. The typical grass pollen season starts in April and ends in July. The peak concentration is recorded during May. Annual pollen index varies from 1000 to 10000 pollen grains, and daily peak concentrations vary from less than

100 to more than 800 pollen grains m^{-3}. Pollen concentrations were obtained using a standard protocol published by the Spanish Aerobiology Network (REA) (Galán et al., 2007). Córdoba has a special location with a valley-mountain breeze known to affect pollen concentrations (Hernandez-Ceballos et al., 2013; 2014), where winds are towards the mountains in the morning and from the mountains in the evening.

Both locations follow the minimal requirements of the European Aeroallergen Network (EAN) for pollen monitoring (Galán et al., 2014).

For this study, we included data from 2008 to 2011 for Córdoba and from 2001 to 2010 for Copenhagen. Days with less than 20 grass pollen grains m^{-3} were excluded from the analysis in the same way as in Peel et al. (2014) due to the large uncertainty in the daily pattern at very low concentrations. Days with rain were also excluded due to the efficiency of precipitation on removal of pollen from the atmosphere (McDonald, 1962), and the resulting effect on the profile.

Pollen data from Córdoba is counted for every hour. To reduce statistical error, as the orifice of the sampler is 2 mm wide and the drum runs by 2 mm per hour, we have re-calculated data into bi-hourly concentrations. Time stamps have been corrected corresponding to official Danish and Spanish time (UTC+ 1 hour during autumn and winter and UTC + 2 hours during spring and summer).

In the following description of the statistical analysis, all manipulations are performed by using the SPSS 15.0® Software Package and R Software (R Core Team, 2014). The pollen concentrations were standardized to eliminate the effect of the magnitude. The formula for standardizing bi-hourly grass pollen concentrations is presented in (1), where Z_i is the standardized bi-hourly value, x_i is the real bi-hourly value, $\bar{X}$ is the daily mean of the bi-hourly pollen concentrations and SD is the daily standard deviation of the bi-hourly pollen concentration (Oteros et al., 2013).

$$Z_i = \frac{x_i - \overline{X}}{SD}$$

(1)

The standardized bi-hourly values are analysed using hierarchical cluster analysis (HCA). The number of natural daily pollen patterns in every city was examined graphically by a dendogram. Hierarchical clustering analysis was performed using Ward's method, in which the distance of each element, in our case each day, to the centroid of the cluster to which it belongs was evaluated. The mean vector of all standardised bi-hourly pollen concentrations was calculated, determining the multivariate centroid for each cluster. The squared Euclidean distances between each element and the centroid (mean vector) of all clusters were then calculated. Finally, distances for all elements are combined.

"K-means" conglomerate method was applied for generating clusters. Patterns in grass pollen were generated on the basis of similar bi-hourly standardized pollen data. From various types of cluster analysis available, this is deemed to the most appropriate, in that it provides a more flexible approach and does not assume any specific distribution of variables.

The relationship between pollen profiles and daily weather parameters was carried out using an average-comparison method. Tested daily weather parameters were: temperature, humidity, global radiation, wind speed and wind direction. Wind direction was available for Córdoba as the hourly percentage of wind source from each octant and for Copenhagen as the predominant wind direction within 30 minutes. In the case of Córdoba, we selected the most common wind direction every hour as the prevalent direction with the aim of getting degrees' units. We calculated one value of predominant wind direction per day in degrees (0º-360º) by the circular average of the wind directions.

Aerobiological data are often non-normally distributed, which was verified using the Saphiro-Wilk test. Variances and homogeneity was tested by Fligner-Killeen Test (Conover et al., 1981). Due to the non-normality and the presence of outliers (tested by plotting boxplots), a Robust Anova analysis is applied for analysing the correlation between patterns in pollen concentrations and weather parameters. Significant differences in weather variables between clusters were examined applying Tukey test to analyse in which clusters they are present. The analysis is performed using "WRS2" package of R (Mair et al., 2015; R Core Team, 2015).

The effect of wind direction on the clustering pattern was studied by circular statistics (Sadyś et al., 2015, Borycka and Kasprzyk, 2014, Maya-Manzano et al., 2017). The circular average of the prevalent wind direction was calculated for the days associated with each cluster. Circular statistics is performed by using "Circular" package of R (Agostinelli and Lund, 2013; R Core Team, 2015). Differences in wind direction between clusters were analysed by applying circular ANOVA.

Results

A total of 259 days of data for Copenhagen and 184 days for Cordoba met the above listed criteria of no rain and pollen concentrations above 20 pollen grains m^{-3}. Three well defined diurnal profiles were observed in both locations by the above described method.

Days with high distance to cluster centre were not included in further analysis, since these days do not have a well-defined hourly pattern. For Copenhagen, this condition applied to 69 days, and for Cordoba, 60 days.

Figure 2 represents the average and the 95% confidence intervals (CI) of each of the three pre-defined clusters for Córdoba. Great variation is seen between clusters in the time of peak pollen concentrations. Cluster 1 is the most frequent pattern, with 40 % of the cases, showing the typical afternoon peak. Cluster 2 represents 33 % of the days included, and shows an early morning peak, with substantial concentrations before daylight starts (around 7 in Córdoba). Cluster 3 represents 27 % of the days included and has a two-peak pattern with morning and evening peaks.

Figure 3 shows the average and the 95%-CI of the three pre-defined clusters for Copenhagen. Cluster 1 is the most frequent pattern, with 57% of cases, showing peaks recorded during the early evening. Cluster 2 represents 13% of cases, and consists of days with peak concentrations during the night. Cluster 3 represents 30% of cases, and shows a midday-afternoon double peak.

By applying comparison of mean methods, we found relationships between pollen patterns and meteorological variables (Table 1). Meteorological data was not complete for all days. A total of 166 days for Copenhagen and 86 days for Córdoba were included. Differences were seen between the days of the year in which most of the cases of each patterns are observed in Córdoba, however not significant. Cluster 1 has the highest fraction of observations from early in the season, while Cluster 3 has the main fraction of observations during the late pollen season (Appendix 1). This fact could be related to the association of the flowering features of different species to different patterns, but also could be a masking factor for the differences caused by meteorology.

By comparing pollen patterns with weather parameters in Copenhagen we only found a significant difference for wind directions. The main wind direction was from West in Cluster 1 and from South-West in Clusters 2 and 3.

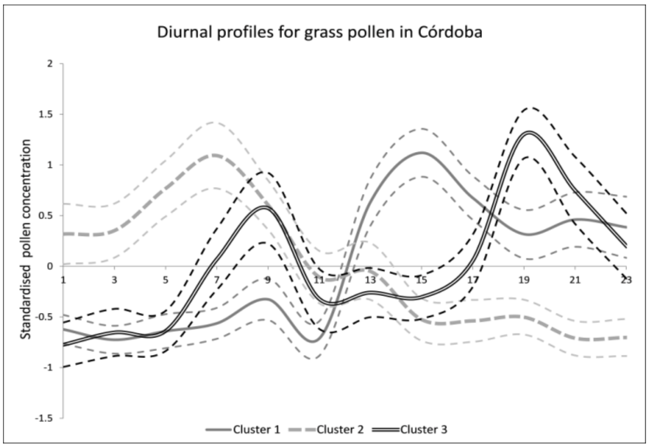

Figure 3 Average and confidence intervals for each cluster of profiles of grass pollen concentrations in Córdoba, Spain.

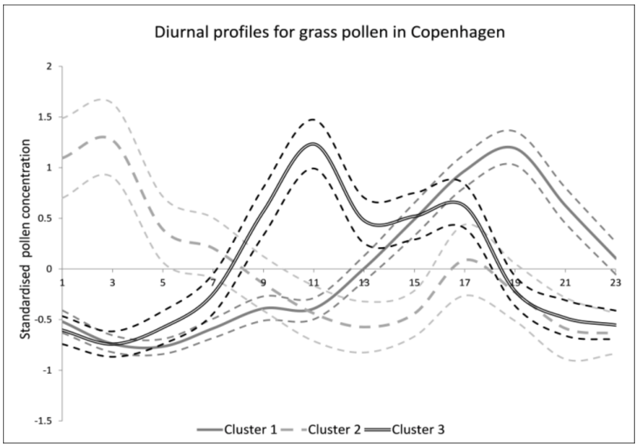

Figure 3 Average and confidence intervals for each cluster of profiles of grass pollen concentrations in Copenhagen, Denmark.

	sig.	C1 Mean (SD)	C2 Mean (SD)	C3 Mean (SD)
DOY	0.09	136.82 (18.58)	143.86 (17.69)	144.91 (18.39)
Temperature (ºC)	0.14	20.13 (3.64)	21.33 (3.45)	21.98 (3.1)
Humidity (%)	0.56	55.94 (11.43)	52.99 (10.22)	55.23 (7.52)
Global radiation (W/m²)	0.02	286.34 (42.82)	314.00 (43.98)	314.47 (31.83)
Wind speed (m/s)	0.29	1.54 (0.48)	1.69 (0.57)	1.82 (0.6)
Wind direction (º)	0.40	351.38 (248º to 54º)	9.30 (256º to 77º)	8.20 (264º to 58º)

Table 1 Córdoba (Spain). Differences in daily environmental parameters between days defined with different hourly patterns in airborne pollen. Robust ANOVA significance. C1, Cluster 1. C2, Cluster 2. C3, Cluster 3. DOY; Day of the Year. Wind direction is calculated by circular statistics approach. Only maximum and minimum are shown for wind direction, not SD, due to the circular properties.

	sig.	C1 Mean (SD)	C2 Mean (SD)	C3 Mean (SD)
DOY	0.47	174.06 (13.15)	177.6 (15.37)	175.74 (14.61)
Temperature (ºC)	0.20	17.11 (3.09)	17.98 (2.89)	17.69 (2.69)
Humidity (%)	0.72	66.78 (9.74)	69.64 (10.18)	68.05 (7.94)
Global radiation (W/m²)	0.19	272.64 (59.46)	246.25 (71.24)	255.12 (69.81)
Wind speed (m/s)	0.17	3.55 (0.95)	3.16 (1)	3.45 (0.98)
Wind direction (º)	0.01	267.34 (90º to 78º)	211.6 (35º to 333º)	221.2 (54º to 45º)

Table 2 Copenhagen (Denmark). Differences in daily environmental parameters between days defined with different hourly patterns in airborne pollen. Robust ANOVA significance. C1, Cluster 1. C2, Cluster 2. C3, Cluster 3. DOY; Day of the Year. Wind direction is calculated by circular statistics approach. Only maximum and minimum are shown for wind direction, not SD, due to the circular properties.

Discussion

It is known that the pollen load varies across the duration of a day, and that methods for predicting the time of the day where maximum peaks are reached have still not been developed (Bogawski and Smith, 2016). Due to pollen grains being biological particles with an important impact on human health, the study of diurnal profiles of pollen is very useful to prevent high exposures (e.g. Sommer et al., 2009). For this reason several papers have focused on hourly pollen information and the parameters mainly influencing this variation, finding great diversity in the daily rhythms of pollination (Beddows, 1931; Jones, 1952; Kasprzyk, 2006; Muñoz Rodríguez et al., 2010; Peel et al., 2014; Pérez-Badía et al., 2011; Puc, 2012; Rojo et al., 2015).

The variation in diurnal pollen patterns is especially clear in the case of multi-species pollen types such as *Poaceae*, and the time of maximum concentration is difficult to predict as an average that only shows one most frequently found pattern without accounting for other possible patterns. Alba et al. (2000) found also that there is not a single diurnal pattern even for pollen measurements originating from a single species (*Olea europaea* L.). They postulated that limiting the visualizing of the average behaviour of airborne pollen (through the average diurnal pattern) limits the analysis of the diurnal pollen pattern, resulting in the understanding to be incomplete. They found that 54% of the observed days fitted a single dispersal pattern, on the remaining days (46%) the pollen dispersal was highly irregular. In our study we found three possible patterns where approximately 70% of the studied days without rain and a daily pollen concentration above 20 pollen grains m^{-3} could be fitted. For 27% of days for Copenhagen, and 32% of days for Córdoba the pattern showed a high distance to cluster centre, i.e. a pattern not fitting any of the three clusters.

Hernandez-Ceballos et al. (2015) found the existence of five different patterns for *Quercus* pollen in Córdoba. *Quercus* pollen is, like *Poaceae,* an important aeroallergen where eight species are found abundantly in many European countries (Skjoth et al., 2008), thereby contributing to the overall pollen load and eventually causing variations in the daily pollen pattern. Our findings on *Poaceae*, as well as the other studies on tree pollen, suggest that variations in the diurnal pattern are a common phenomenon.

Many factors are involved in the variation of the diurnal pattern of pollen concentrations. In the case of *Poaceae*, differences in pollination features of the different species can have an important influence. Several papers report considerable variation in the pattern, linking this to species flowering at different time, peak occurring mostly in the morning or in the afternoon (Kapyla, 1981; Peel et al., 2014). It could therefore be important to determine this by a dedicated phenological study focusing on the species that contribute to the majority of airborne grass pollen concentrations in order to determine the time of the day at which they liberate the pollen, and potential differences in the effect of meteorology on the different species. This can also be expected to be site-specific, and transferable uniform patterns may not be possible, however uniform methods may be developed.

León Ruiz et al. (2011) found that in Córdoba only four species were major contributors to the *Poaceae* airborne pollen curve (*Dactylis glomerata, Lolium rigidum, Trisetaria panacea, Vulpia geniculata*) while Kraaijeveld et al (2015) found a larger number of important species in the Netherlands. Cebrino et al. (2016) support these results and show that the majority of the pollen sources are found locally. Peel et al. (2014) found a relationship between diurnal profiles and the time of season potentially linking this to the flowering of different species. This fact could be explained by the existence of different pollination features depending on the grass species. In this study we did not see a clear difference related to time of season, and could therefore not explain the patterns as being primarily driven by the succession of flowering species.

Another factor that must be taken into account is the distance between pollen sources and the trap (Perez Badia et al., 2011), although this fact could be less relevant for *Poaceae* as these taxa are densely distributed everywhere, inside and around cities. Nevertheless, distance from the pollen source can be also of great importance and show large variations within short distances (Skjøth et al., 2013b). Depending on the distance from the pollen source and flowering phenology, wind direction seems to be determinant for explaining some intra-diurnal variations of pollen loads (Rojo et al., 2015). In our study the wind direction showed significant differences between clusters for Copenhagen, with Cluster 1 having more winds from West and Southwest. This is the most frequent pattern with early evening peaks. A large residential area with gardens, lawns and associated grass covered recreational areas is located approximately 0.5-1.5 km in this direction. However, whether this area is a major source of the pollen will highly depend on the cutting frequency of the lawns and meadows (Skjøth et al., 2013b).

The pollen patterns in Córdoba and Copenhagen were both affected by wind directions although not to a large degree. In Cordoba, the valley-mountain breeze (Hernandez-Ceballos et al., 2013; 2014) is dominating the wind directions for the three clusters of days, and therefore no significant differences are seen here. Differences are therefore unlikely due to differences in source areas for this site, however a separate analysis would be required to establish this. Our result along with the previous results by Norris-Hill and Emberlin (1991) suggest that the foot print area could be an important factor to take into account in further grass pollen studies. Even highly local sources could be of great importance (Skjøth et al 2013). Ideally they should focus on both the variation in the daily pattern as in our study as well as the dominating species and the associated ecosystems found within the atmospheric foot print.

Different grass species are associated with main ecosystems and geographical regions as defined by the biogeographical regions of Europe and used in the habitats directive. This is clearly illustrated in the contribution from a large number of species to the overall grass pollen index found in Leiden, within the Atlantic biogeographical region (Kraaijeveld et al., 2015) and four the dominating species found in Cordoba (Cebrino et al., 2016). In the *Poaceae* family, the liberation of pollen is controlled by factors inherent to each species and occurs in a short period of hours each day but pollen grains can remain in the air where their dispersion is again affected by meteorological parameters (Myszkowska, 2014). These meteorological effects also vary during the day, e.g. as in the valley winds affecting Cordoba and the associated pollen concentrations (Hernandez-Ceballos et al., 2013; 2014). In this sense, Norris-Hill and Emberlin (1991) tried to divide days into categories taking into account temperature, humidity and wind direction, finding small differences in the time of maximum pollen concentration with temperature and wind direction.

This study was carried out in two different urban environments. Exposure to grass pollen in urban environments is particular important because some air pollutants seems to correlate with the daily patterns of pollen concentrations (Ørby et al., 2015). Puc (2012) also saw strong correlation between intra-hourly pollen concentrations and gas air pollutants. This is important because co-exposure of air pollutants and pollen can reduce the threshold for an allergenic response (Molfino et al., 1991; D'Amato et al., 2010). In the case of Córdoba (Spain) a previous study showed that the peaks of non-biological particles in the air throughout the day are related to activities carried out by human beings in the city occurring in the morning and late in the evenings (commercial and working hours), which are probably related with resuspension process of particles (Cariñanos et al., 1999). Many of these particles originating from traffic pollution. During these hours sensitive individuals must exercise precautions. Simoleit et al. (2015) also comment that the combination between pollution and pollen load in the air represent a special health threat for urban population as pollen are considered to be more allergenic in a polluted atmosphere (D´Amato et al, 2010; Schiavoni et al 2017). Combined with the current study indicating that a high proportion of days where pollen peaks at these times, susceptible induvial may be of increased risk and must exercise precautions. The combined effects of air pollutants and aeroallergens is an important area, in particular in the urban zone, and that there need to be a focus on short-term exposure of both air pollutants and aeroallergens.

Although the two sites can be assumed to have differences in the composition of species, both sites had three clusters with some similarity in the daily pattern: Cluster type 1: late afternoon peaks, Cluster type 2: partly or entirely dominated by night time/early morning conditions, and Cluster type 3: a double peak. This result is partly the consequence of the method, determining the most distinctively different patterns. However, even with great differences in species composition, meteorology and dominating local wind patterns and patterns objectively analysed through statistical clustering, both sites showed a uniform peak in the afternoon or evening as the most frequent pattern. For Denmark, the evening peak was also seen as the dominating peak in the main season in the city of Aarhus (Peel et al, 2014). This indicates that the advise given for allergen-avoidance should emphasise that peak concentrations may occur at all times of day, but the most frequent peak, dominating the seasonal peak, is in the early evening.

Conclusions

Here we propose the use of clustering methodology to study intra-hourly variations of airborne pollen. Differences in hourly-patterns recorded at southern Europe (Spain) and northern Europe (Denmark) could not directly be related with differences in meteorological conditions at either location.

The studies carried out in both cities show strong variation in the diurnal pattern of grass pollen in the air, with approximately 70% of days (without rain and daily pollen concentrations above 20 pollen grains m^{-3}) fitting 3 statistically (although not significant) determined clusters of patterns, with peaks at either both morning, midday, evening or night. For both sites however, one late afternoon (Córdoba) or early evening peak (Copenhagen) is the most frequent distinctive pattern.

The peak can occur at all hours of the day, most likely depending on flowering patterns of the dominant grass species and a complex effect of meteorological parameters. In view of the results average curves are not satisfactory for describing the diurnal pattern of grass pollen as they mask the day to day variation and long term season effects.

Acknowledgments

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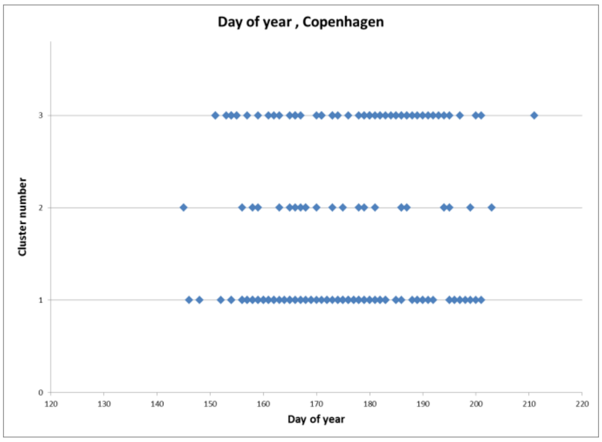
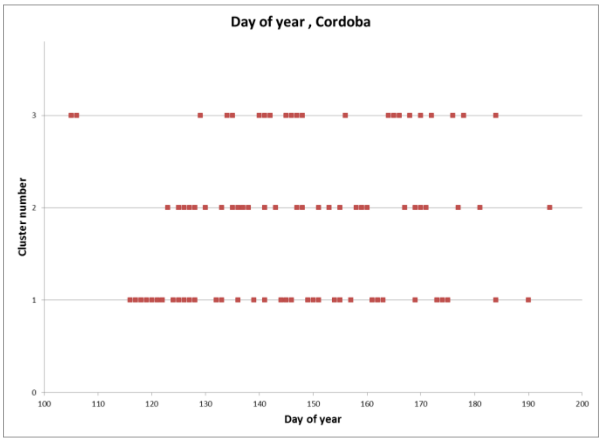
Appendix 1





Appendix 1 Day of year for the diurnal profiles in each of the three clusters.

Manuscript IV

The effect of seasonal priming on birch and grass allergen specific inhalation challenges among persons with allergic rhinitis

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Abstract

Background: Seasonal exposure to pollen may induce a priming effect on the bronchial response. Priming has primarily been observed asthmatics, but may also be seen among persons with allergic rhinitis.

Objective: We examined the effect of seasonal priming on bronchial responsiveness among persons with allergic rhinitis and no or mild asthma. Secondary objectives were to explore how participants' baseline characteristics affected severity of bronchoconstriction, and to evaluate the application of a novel non-linear regression model to the log-dose-response curves.

Methods: In a cross-over design performed in a human exposure chamber thirty-six participants underwent specific inhalation challenges with either grass or birch allergen, outside and at the end of the season. The differences in bronchial response were evaluated by comparing PD_{20} estimated by applying a non-linear regression model.

Results: Twelve of the 19 grass pollen exposed participants had a lower PD_{20} at the end of the season compared to outside season, however no statistically significant effects of the seasonal pollen exposure were found on neither shape or magnitude of the modelled dose-response curves, nor on PD_{20} for either of the allergens. Among baseline characteristics only the size of skin prick test for grass allergen was significantly positively associated to PD_{20}. The model depicted a good fit to the data.

Conclusions & Clinical Relevance: We found a tendency to lower PD_{20} by the end of the season among subjects with allergic rhinitis and no or mild asthma; however only for grass. Modelled dose-response profiles showed a good fit to data. Priming may depend on individual differences in severity as well as pattern of response, potentially only affecting certain endotypes.

Abbreviations

Forced expiratory volume in one second (FEV_1), Dose eliciting a 20% decrement in FEV_1 (PD_{20}), Specific inhalation challenges (SIC), Non-linear four-parameter logistic model (fpl), skin prick test (SPT).

Introduction

The impact of pollen on quality of life for allergic individuals as well as on the socioeconomic costs for society, depend strongly on the severity of symptoms. One of the factors affecting severity of symptoms is "the priming effect", a mechanism where repeated exposures to an antigen induces increased responses at similar exposure levels. For pollen allergy, the priming effect was first demonstrated for nasal symptoms (1). More recent studies on the priming effect has included both experimental challenges of repeated doses (e.g. (2;3) and effect of the natural season (4;5), exploring the effect on for example symptom scores (6), nasal cells, and bronchial response (7). The majority of studies on the bronchial response are performed on persons suffering from asthma. In the current study we looked to examine the bronchial response in participants with allergic rhinitis, with no or only mild bronchial symptoms during the pollen season. According to the theory of "one airway, one disease" and the "united airway" (8;9), these patients could also be expected to have increased bronchial response due to priming, which was not been explored in previous studies.

The majority of studies on bronchial response to repeated allergen exposures apply a measure of the allergen dose eliciting a drop of e.g. 20% in FEV_1, the PD_{20}, as a proxy for the degree of responsiveness. PD_{20} is estimated by linear interpolation between the two doses eliciting a drop below and above 20% on a logarithmic dose-response curve, although the response pattern is rarely linear. In this study we wanted to examine the use of a non-linear regression model, to fit the log-dose-response curve and apply this in the estimate of the PD_{20} value.

Objective

The objective of this human exposure study was to evaluate the priming effect of natural seasonal exposure on the bronchial response in allergic rhinitis participants with none or mild asthmatic symptoms. We hypothesise bronchoconstriction during specific allergen challenge is larger at the end of the natural pollen season, compared to outside pollen season. The effect on the severity of bronchoconstriction is evaluated by applying a non-linear regression model to estimate PD_{20} from log-dose-response curves. Furthermore we wanted to assess how baseline characteristics of the participants affected the size of PD_{20}.

Materials and methods

Study participants

Potential study participants with self-reported allergic rhinitis were recruited at Aarhus University, and skin prick testing (SPT) was performed to confirm sensitisation. SPT was performed for grass, birch, artemisia, horse, dog, cat, house dust mites (HDM; Dermatophagoides pteronyssinus and Dermatophagoides farina) and fungal spores (Cladosporium herbarum and Alternaria alternaria). Ninety-two persons were initially tested, and thirty-six participants with a positive SPT > 3mm to grass or birch were recruited. , Of these 17 underwent specific inhalation challenges (SIC) with birch allergen, and the remaining 19 participants underwent SIC with grass allergen, table 1. Twenty of the participants reported respiratory symptoms (cough, wheeze, or chest tightness) more than a few times a year.

	Grass	Birch
N participants	19	17
Gender	F 10 M 9	F 10 M 7
Weight	F 68 (13) M 77 (8)	F 70 (14) M 86 (16)
Height	F 169 (5) M 183 (8)	F 169 (6) M 179 (6)
Age	24.2 (2.7)	24.4 (2.3)
Wheal size , SPT mm*	9.4 (3.2)	6.5 (2.5)
Number of positive SPT (min. / max.)	3.1 (1/6)	5.9 (4/8)
% Predicted, Baseline FEV_1	F 99 (14) M 86 (12)	F 97 (12) M 96 (12)

Table 1 Study participant characteristics, mean (std. dev.). F= female, M=male. * Wheal size of SPT for the allergen applied in the SIC (grass or birch).

Ten participants reported mild asthma; however only 7 of them recorded symptoms more than a few times a year. None of the 36 participant used asthma medication.

The participants provided information about their potential exposure to tobacco smoke, as well as about recent infections and use of medication. None of the participants used asthma medication, and none had used antihistamines within 72 hours prior to the SIC. All participants had an initial FEV_1 higher than 70% of predicted, and underwent methacholine bronchial challenge test with a maximum cumulated dose of 4.51 mg.
The study is conducted in compliance with the Helsinki Declaration. The protocol, enrolment procedure and written consent forms were approved by the Scientific Ethics Committee for Central Denmark Region (M-20090215).

Study design

The study was conducted as a cross-over design and performed during four study days. During the first two days, the participants underwent SPT, symptom history, medical examinations and methacholine challenge test. On the third and fourth day, SIC were performed in a human exposure chamber, either outside or at the end of season for birch or grass pollen. For details see Figure 1. Of the 19 participants who underwent SIC with grass allergen, 13 were included in two study rounds outside the grass pollen season. Both rounds of SIC were included in the analysis. All ”out of season” SICs were performed outside the official grass or birch pollen season, defined by the registrations from nearest operational pollen monitoring station in Viborg 60 km away (10-12). We did not have any information on allergies to other tree pollens. However, no participants reported any symptoms related to these pollen types and therefore no relevant allergy from these on study days was anticipated.

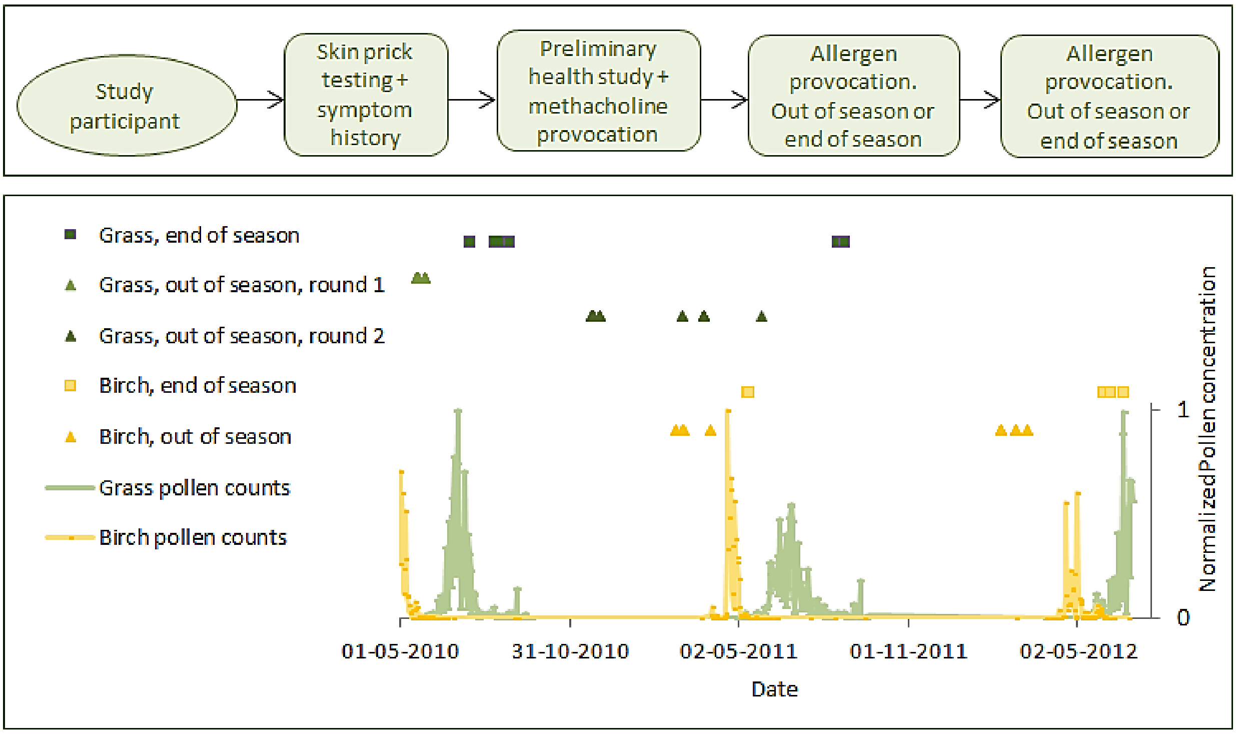

Figure 1 Study design (above) and timeline (below), depicting the SIC´s and the daily pollen counts measured at the operational trap in the city of Viborg (below), normalized across the three years and two pollen species.

Exposure chamber

The SIC were conducted in a 79 m^2 human exposure chamber (13;14). One to 4 participants and investigators were situated in the chamber.. Though, the 13 initial grass SIC´s out of season, were performed in a smaller 33 m^2 chamber in conjunction to the large chamber, with participants seated in the large chamber between each SIC step.Participants were instructed to exhale in an extraction device to limit exhalation of allergens into the air of the chamber. Additionally ventilation was installed at the exposure area. Airflow, temperature, humidity and CO_2 were measured every 60 sec. The chambers were provided with filtered air at 22.5°C (std. 0.5°C) and humidity of 42.0 % (std. 3.0%).

Specific inhalation challenges

We used allergen extracts from ALK-Abelló (Hørsholm, DK) containing 100,000 SQ-U/ml of Phleum pratense or Betula verrucosa. Solutions resulting in doses from 1,4 – 5,600 SQ-U were administered with a Spira Dosimeter nebulizer and FEV_1 was measured with a handheld MicroDL spirometer connected to a computer, where all data were logged. SIC with aerosolized allergen were performed according to the procedure outlined in Figure 2. FEV_1 was measured 15 minutes after each inhalation as best of three consecutive measurements. Baseline FEV_1 was measured after inhalation of diluent.

		Baseline	Level 1 Dilution 100 SQU			Level 2 Dilution 1,000 SQU			Level 3 Dilution 10,000 SQU			Level 4 Dilution 100,000 SQU			
Step	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Conc.	*	Diluent	2×100	4×100	8×100	2×1,000	4×1,000	8×1,000	2×10,000	4×10,000	8×10,000	2×100,000	4×100,000	8×100,000	Bricanyl
Dose, SQU			1.4	2.8	5.6	14	28	56	140	280	560	1400	2800	5600	

Procedure based on % drop in FEV1, based on baseline measurement after diluent (step 2)	
< 5 %	Next level – skip two steps., If < 5 % drop at level 12, no response is assumed and the provocation ended.
> 5 – 15 %	Next step.
>15 – 20 %	Repeat same step, maximum 3 times. If still < 20 %, next step is administered.
> 20 % or unacceptaple discomfort.	Disrupt provocation and proceed to administer bronchodilator (Bricanyl).

Figure 2 Protocol for the SICs. The administered dose is dependent on the magnitude of the drop in FEV_1 induced by the previous dose, resulting in either one *step* or one *level* increase in dose.

After the SIC, participants measured their FEV_1 every 15 min. for the first hour and thereafter hourly in the laboratory and later at home until bed time for late phase responses, as a safety measure.

Statistical analysis

Natural log transformation of the administered dose of allergen concentrations was applied similar to other studies (2;4;5;15;16).

Bronchial hyper responsiveness has previously been shown to depict a non-linear shape of the log-dose-response curves for normal non-asthmatic subjects (17;18). We therefore fitted a non-linear regression model in the shape of a four-parameter logistic curve to the FEV_1 data using the log cumulative dose as independent variable:

$$FEV1(x) = a + \frac{b - a}{1 + exp\frac{c - x}{d}}$$

where x is the log cumulative dose, a is baseline FEV_1, b is FEV_1 at maximal log cumulative dose, c is the log cumulative dose corresponding to half the decrease in FEV_1 (the inflection point), and d is a scaling parameter. The repeated measurements for each subject were taken into account by including random subject effects, for each of the four parameters describing the FEV_1-curve. Model validation was performed by inspecting plots of observed and fitted FEV_1-values against cumulative dose and residual plots.

Allergen responsiveness was evaluated as the natural logarithm to the cumulative dose of allergen theoretically eliciting a 20% drop in baseline FEV_1 (PD_{20}). PD_{20} was estimated from the logistic model which means that the information contained in all the FEV_1 observations was used and not only the FEV_1 responses to the last two doses administered, as in previous studies. The non-linear regression model allows for estimating differences in PD_{20} corresponding to different exposures.

The association between PD_{20} and baseline characteristics of the study population was investigated using a linear regression model with PD_{20} on a logarithmic scale. Groups were compared using post-hoc t-tests based on non-linear mixed effects regression analysis.

All results were obtained using the nlme package in the R Programming Environment for Data Analysis and Graphics, Version 3.2.3 (19). Statistical significance was set at p = 0.05.

Results

Pollen seasons

The natural seasonal exposure during the study period was compared to the 30-year average seasons in both progress and magnitude, and slight differences were detected. The grass pollen season in 2010 was slightly shorter and more intense than normal. In 2011, the birch pollen season was slightly lower, possibly due to western winds from areas with less birch trees during the main season, and ended slightly earlier than normal. In 2012 a gap in measurements resulted in the peak of the birch pollen season not being recorded at the nearest monitoring station in Viborg, however the counts from the station in Copenhagen, indicated a normal season, although ending slightly abrupt due to cold weather in May. The yearly total pollen count for Viborg monitoring station for grass pollen was 2278 in 2010 and 2097 in 2011. The norm is only available for Copenhagen, where similar counts are measured, and the current norm measured to be 2200 pollen. For birch pollen, the yearly total was 3585 in 2011 and not listed in 2012. This was lower than for Copenhagen, 6037 pollen, however, a nearby birch forest is affecting the Copenhagen counts, leading to generally much higher counts at this site.
For better visualization of seasonal progression, normalised pollen levels are shown in Figure 1.

Airway responsiveness to methacholine

The methacholine PD_{20} estimate for all participants was 4.4 (95%-CI: 2.8; 6.8) mg methacholine bromide. No systematic errors in the residuals were seen. Individual methacholine PD_{20} estimates were modelled for all SICs.

SIC for grass and birch allergen

There were no significant differences in baseline FEV_1 between SICs outside season and at the end of season (birch, p= 0.98; grass, p= 0.91). 82% and 74% of SIC´s at end of season dropped more than 20% in FEV_1 for birch or grass respectively; the numbers were 82 % and 78 % for birch and grass outside the season.

The four-parameter logistic model was run on all individual SIC´s showing a good fit to the measured data (Figure 3). The validity of model assumptions was checked by inspecting plots of residuals against fitted values and QQ-plots of residuals. No systematic errors in the residuals were seen. Individual PD_{20} estimates were modelled for all SIC´s, assuming both 1) no difference between exposure times (out of season and seasonal), and 2) allowing for a difference between exposure times.

Individual PD_{20} estimates for "out of season" and "season" SIC´s are shown in Figure 4. For grass, 12 of the 19 participants required more allergen to drop 20% in FEV_1 outside the season. For birch this was true for 9 of the 17 participants, however, no significant effect of season was found (birch, p= 0.30; grass, p= 0.39).

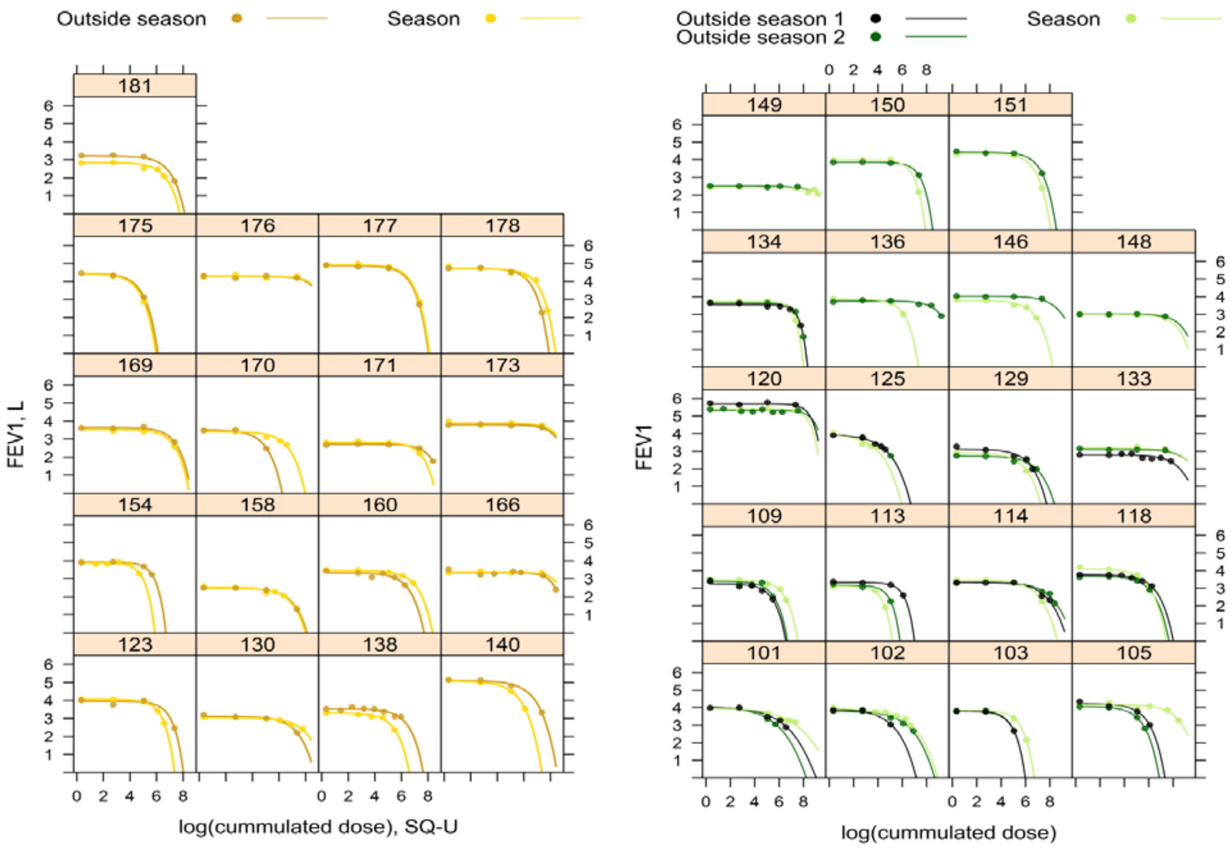

Figure 3 Individual modelled dose-response profiles for the17 participants challenged with birch allergen (left) and the 19 challenged with grass allergen (right). Numbers indicate the individual ID´s of participants. Thirteen of the grass participants were challenged twice outside season.

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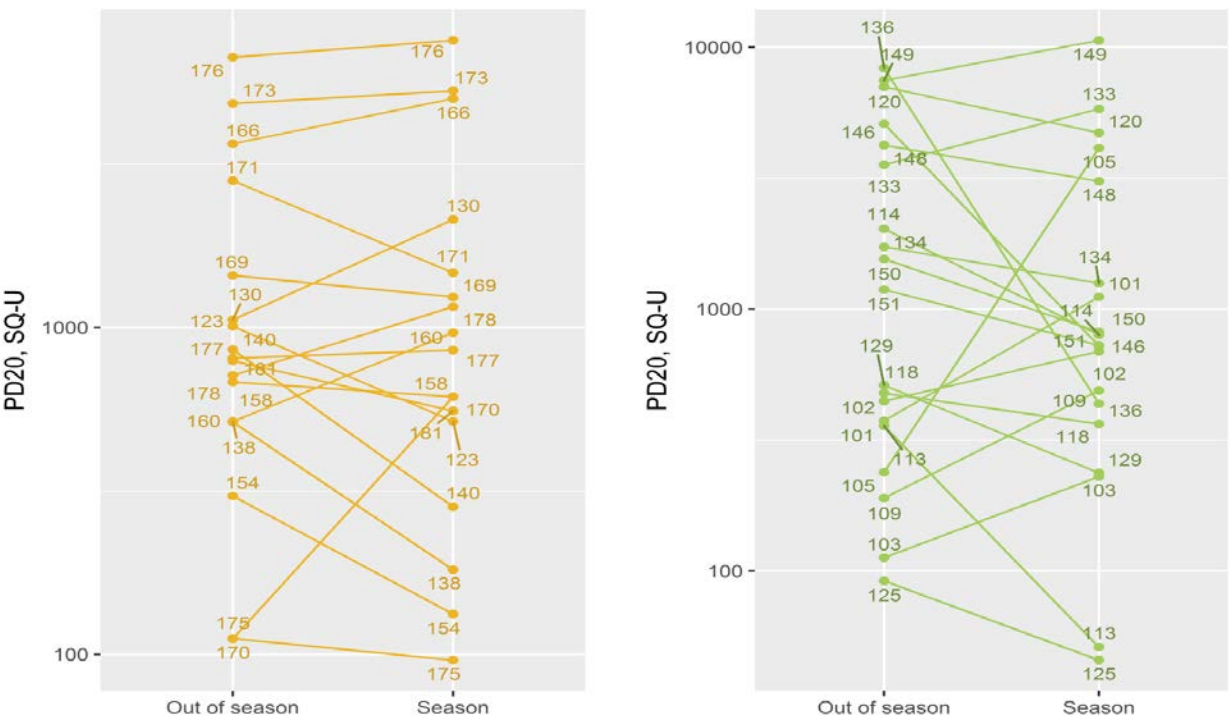

Figure 4 Individual PD_{20} estimates for challenges "Out of season" and at the end of "Season". Estimates are shown for the 17 participants challenged with birch (left) and the 19 challenged with grass allergen (right). Numbers indicate the individual ID´s of participants.

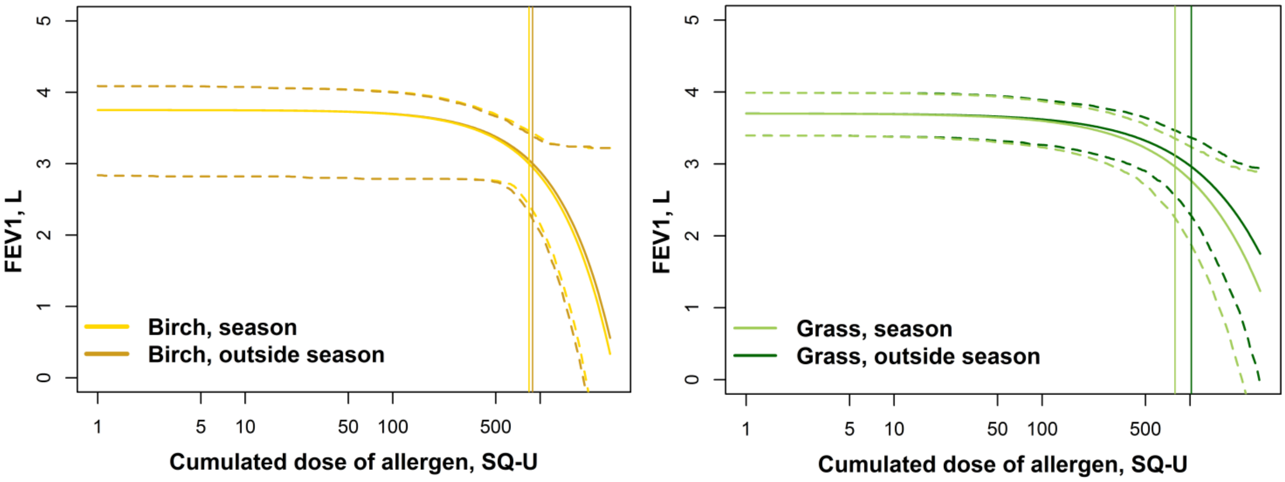

Figure 5 Modelled log-dose-response curves with 95% confidence intervals. The vertical lines indicate the modelled PD_{20} estimates.

The PD_{20} estimates (95%-CI) based on all SIC´s for birch were 889 (494; 1599) SQ-U outside season and 840 (464; 1519) SQ-U at the end of the season. For grass, PD_{20} estimates are 1020 (512; 2031) SQ-U outside season and 792 (400; 1564) SQ-U at the end of the season. The PD_{20} estimate for grass is associated with a 24% (-48; 59%) smaller dose at the end of the season for grass participants, and a 6 % (-39; 36%) smaller dose by the end of season for birch participants. However, none of the differences between season and outside the season were statistical significant (birch, p=0.77; grass, p= 0.45). The modelled FEV_1 / log cumulated dose of allergen curves are shown in Figure 5 with 95% CI-intervals, and indications of the PD_{20} estimates. No significant differences in the shape or magnitude of the curves were seen between exposure times.

Associations between baseline characteristics and PD_{20}

Table 2 and Figure 6 present the associations between four baseline characteristics of the participants and allergen PD_{20}.

	% change in PD_{20}	**P**
Methacholine	**% change in PD_{20} allergen per 10 % change in PD_{20} methacholine**	
Birch	3.75 (-1.15 ; 8.88) %	0.158
Grass	0.77 (-4.21 ; 6.02) %	0.770
Skin prick test	**% change in PD_{20} per mm change in SPT-wheal**	
Birch	- 17.95 (-33.47 ; 1.18) %	0.085
Grass	*- 25.78 (-36.64 ; -13.10) %*	*0.002**
Number of pos. SPT	**% change in PD_{20} per pos. SPT**	
Birch	-19.39 (-45.70 ; 19.67) %	0.304
Grass	27.77 (-11.32 ; 84.21) %	0.208
Baseline FEV_1	**% change in PD_{20} / (l/sec) change in Baseline FEV1**	
Birch	-17.60 (-59.99 ; 70.80) %	0.608
Grass	5.05 (-58.83 ; 172.82) %	0.920

Table 2 Associations between baseline characteristics for the two participants groups (birch and grass), and Allergen-PD_{20}, Methacholine-PD_{20}, size of SPT, number of positive SPT and baseline FEV_1.

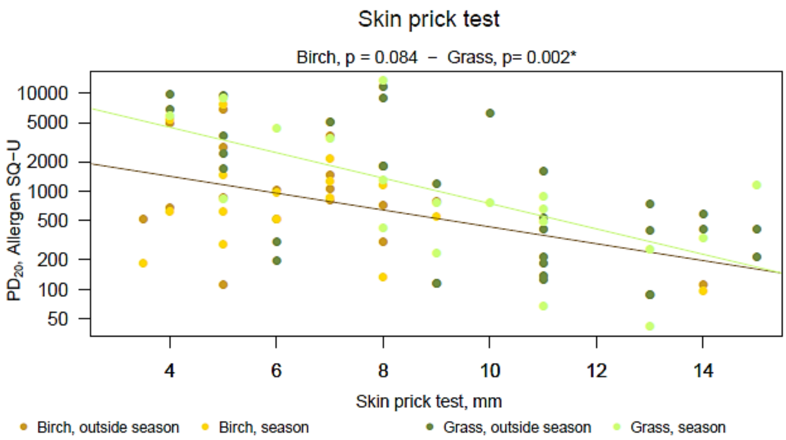

Figure 6 Association between SPT and Allergen-PD_{20} for all SIC´s.

As no effect of season was seen on the PD_{20} estimates, the analysis was performed on a combined dataset. Size of the SPT for grass allergic participants showed a clear and significant association with PD_{20}. On average, 1mm larger reaction in skin prick test was associated with a 26 % lower PD_{20}. Otherwise no significant association between baseline characteristics and PD_{20} was seen.

Discussion

In this study we found that the majority of the participants had a positive SIC´s with a reduction of more than 20% in FEV_1, although only 7 reported more than mild asthmatic symptoms a few times a year. This is consistent with the theory of the "United airway", and our assumption of an allergic bronchial response in rhinitis participants. The modelled individual PD_{20} estimates were lower by the end of the season for 12 of the 19 grass participants, and the overall PD_{20} estimate was associated with a 24% (-48; 59%) decline in dose. For birch, almost no differences were seen, as 9 of the 17 participants had a lower PD_{20} by the end of season, and the overall PD_{20} estimate was associated with a 6 % (-39; 36%) smaller dose. Although none of the differences were statistical significant, the results for the grass participants indicate a lower tolerance by the end of the season, and a potential priming effect of the seasonal exposure.

The differences in findings between SIC´s with birch and grass allergen could be due to differences in pattern and magnitude of natural pollen exposure in the season between the two groups.
Differences in the effect of pre-priming induced by e.g. homologous pollen types (20), could also serve as an explanation for the difference between the response in grass and birch pollen SIC´s. .
Homologous pollen types his is especially an issue for those suffering from birch allergies, since many of the early spring flowering trees have homologous allergens and often induce cross-reactivity. In this study we did not have SPT or IgE results for any of these allergens. Although none of the participants report symptoms related to these tree pollen, pre-priming could still occur if sensitization is present, since exposure to low doses of allergen, not eliciting symptoms, may still cause bronchial inflammation and increase responsiveness (3;21)

The natural priming exposure of birch pollen may also have been blunted in 2012, when concentrations fell slightly more abruptly than normal, due to cold weather. This resulted in difficulties in planning in relation to the pollen season, and two of the "end of season SICs" with birch allergen in 2012 were not performed until three weeks after the season. The effect of priming by repeated exposures has previously been seen to be almost vanished 3- 6 weeks after the exposures (3), and therefore we cannot be sure that the two late challenges were affected by seasonal priming. Another issue related to the timing were one out-of-season SIC performed only one day before the season started, and it is likely that this person is at risk of having been pre-primed by low pre-season concentrations.
Two of the participants challenged with grass allergen, ID 102 and ID 133, also had a positive SPT to birch, and may have been pre-primed by the seasonal exposure to birch. The lowest PD_{20} for the out-of season round one, maybe due to this pre-priming by the birch season. Excluding these SIC´s did, however, not alter the results.

Both rate and degree of symptoms could be affected by non-specific pre-priming in participants co-sensitized to perennial allergies (22;23). Fourteen birch participants and 7 grass participants in this study were also sensitized to HDM.
Only half of the participants with positive SPT to HDM experienced symptoms from this, however, sensitization alone could still affect the pre-priming. An assessment of the differences in individual PD_{20}-estimates for grass participants out-of-season challenges show a 3-fold increase of the PD_{20}-estimates for the 7 participants with HDM sensitization, compared to the 12 without ($p=0.06$),as well as a 15 times larger difference in PD_{20} between season and out-of-season. This strongly indicates that there was no separate priming effect of this sensitization on the bronchial response. Even so, future studies should consider including only the allergen of interest.

The applied protocol was developed to consider prevention of exhaustion, and therefore limiting the steps of the challenges. This resulted in 8 SIC´s completed without the maximum dose administered or a 20% drop in FEV_1 reached. Although the majority of participants experienced a 20% or greater drop in FEV_1during the SIC´s and many of the remaining were non-respondent at all challenges, some may also have had a response if the maximum dose had been administered. We recommend In future studies to provide the maximum dose to all participants a high enough to elicit a response in all sensitized subjects (24).

The model demonstrated a good fit to data, and a suitable method for producing PD_{20} estimates and modelling log dose-response curves. This method allows for all information on dose-response pattern to be included in the calculation of the PD_{20}-estimate, and not solely on the individual response to the last two doses administered, as in previous studies. Although individual differences were apparent, the modelled dose-response curve based on all SIC´s indicated similar response patterns for both allergens and exposure times. To the author's knowledge, this has not previously been shown.

Previous studies examining the effect of seasonal exposure on the bronchial response of allergen are heterogeneous in methods, measures of effects as well as in results. Overall, they appear to indicate that the priming effect could affect the allergen PD_{20}, although not for all patients. Dente et al (5) found a significant 3 fold decrease in PD_{20} for asthmatics in the grass pollen season compared to outside season, but only for those having a dual response, i.e. both EAR (Early Airway response) and LAR (Late Airway Response). Paggiaro et al (16) also found 3-fold decreased PD_{15} during the grass pollen season in asthmatic subjects, compared to outside the season, but only for those who shifted from an EAR+LAR response outside the season, to an EAR response within the season. No consistent measurements of LAR were conducted in the current study, where focus was on EAR and analysis of LAR was therefore not possible. From the theory of "one airway, one disease" and the "united airway" (8;9), it was our hypothesis, that participants with allergic rhinitis symptoms, would also show an increased bronchial response to allergeb due to priming. The priming effect on the bronchial response is not well established for this group, and even though we saw a tendency to a priming effect among grass sensitised no significant results were seen.

The priming effect, expressed in terms of increased non-specific bronchial hyper responsiveness following both seasonal and repeated administered allergen for subgroups of study populations, has also been indicated in previous studies. De Bruin-Weller (2) found a significant priming effect, but only for those having high non-specific bronchial hyper-responsiveness. Ihre and Zetterstöm (3) found a priming effect in for the subgroup with a high IgE level, and Dente et al (5) found increased bronchial response to methacholine, for those with LAR outside the pollen season. All these studies were however performed on asthmatic subjects. Some studies do however find a general increased non-specific bronchial hyper responsiveness during the season in their study population, and also in mixed groups including non-asthmatics. A study by Crimi et al (4) found a significant increase in both EAR and LAR following natural seasonal exposure for all participating birch atopic patients, and this suggests a correlation with increasing IgE as a part of the mechanism; Walker et al (25) found a significant decrease in methacholine PD_{20} during the pollen season for all tested patients. Madonini et al (26) found significantly lower carbachol-PD_{20} in season that outside season in 27 grass atopic subjects without asthma, and generally a larger priming effect in those who had a positive response outside season. All studies were performed on participants with no HDM sensitization, and therefore no risk of pre-priming.

Size of SPT for grass sensitisation was the only baseline characteristic associated to $PD_{20.}$ We did not find a similar association for PD_{20} and birch SPT and this may be due to the smaller wheal sizes for this allergen, and therefore less variability in measurements. These findings are consistent with previous studies (16;27).
We expected to also find an association between allergen PD_{20} and baseline unspecific bronchial responsiveness to methacholine. A study by De Bruin-Weller et al (2) found significantly higher non-specific bronchial responsiveness following allergen exposure, and a significant correlation of this with baseline non-specific bronchial responsiveness, but only in the patients with high baseline histamine PD_{20}. This could indicate that the impact of the effect may be blunted for those who are very responsive. Another study by Barnig et al (28) also found an association between grass allergen PD_{20} and methacholine reactivity. The lack of such a finding in our study may be due to the fact that only 44% of our participants were in fact hyper responsive with a 20% or greater drop in FEV_1 after bronchial provocation test with methacholine.

The current study forms a strong basis for a well-established PD_{20} estimate representative for those with allergic rhinitis and only mild or no allergic asthma, a very large and previously not frequently studied patient group. The study confirms that the priming effect is not an important factor for the magnitude of the allergic reaction in all patients. However, the study was not powered to analyse the impact of different endotypes and did not systematically evaluate LAR. To better examine the priming effect of seasonal exposure, future studies should include analysis focusing on the impact of IgE-levels, response pattern, and the level of non-specific bronchial responsiveness.

Conclusion

In conclusion we found a tendency to lower PD_{20} by the end of the season among subjects with allergic rhinitis and no or mild asthma, however only for grass. A significant association between allergen PD_{20} and baseline characteristics were found for grass SPT. Modelled dose-response profiles showed a good fit to data. Priming may depend on individual differences in severity as well as pattern of response, potentially only affecting certain endotypes, which will be important to study further in the future.

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Contributors

PVØ, corresponding author, has contributed to the experimental study, the analysis, full-text screening, drafting the article and final approval of the version to be published. JB, TS and VS have contributed to the experimental study, revising the article critically for important intellectual content and final approval of the version to be published. PR has contributed to the experimental study, revising the article critically for important technical content and final approval of the version to be published. OH has contributed to revising the article critically for important intellectual content and final approval of the version to be published. BB has contributed to the statistical analysis, revising the article critically for important intellectual content and final approval of the version to be published. PVØ and VS are responsible for the overall content as guarantors.

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Manuscript V

An assessment of the potential for co-exposure to allergenic pollen and air pollution in Copenhagen, Denmark

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An assessment of the potential for co-exposure to allergenic pollen and air pollution in Copenhagen, Denmark

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Co-exposure to air pollutants and pollen allergens can aggravate the allergic reaction and reduce the threshold at which susceptible individuals are affected. Here we assessed which air pollutants may be of particular relevance when investigating co-exposure with pollen. We examined the yearly variation and diurnal patterns of pollen and air pollution on days with peak pollen concentrations and non-peak days separately. The analysis was performed for measurements of grass and birch pollen, sulfur dioxide, ozone, nitrogen dioxide and particulate matter. Results indicated that high ozone concentrations coincide both seasonally and diurnally with high pollen concentrations, and that ozone concentrations were higher on peak pollen days, potentially leading to clinically relevant simultaneous co-exposure. For nitrogen dioxide and sulfur dioxide no periods with peaks simultaneously with peaks in pollen

Abbreviation: IgE, Immunoglobulin, antibody related to the allergic response; FEV1, Forced expiratory Volume. The maximum volume of air a person can exhale within 1 second.

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loads was identified, and concentrations were below potential thresholds for adjuvant effects to the allergic reaction. For particulate matter no simultaneous peaks in diurnal or seasonal concentrations were identified, however concentrations were higher on peak pollen days compared with non-peak days. When considering co-exposure effects from pollen and pollutants, ozone appears to be the most relevant pollutant to further examine for effects of simultaneous co-exposures.

1. Introduction

Individuals exposed to allergenic pollen are at risk of developing allergic diseases such as rhinitis, conjunctivitis, asthma or combinations thereof (Cecchi, 2013). This may have a negative impact on quality of life by affecting learning, sleeping, social interactions, and numerous other daily activities, resulting in considerable socio-economic costs (Petersen et al., 2008; Pawankar et al., 2011). Co-exposure to air pollutants and pollen allergens can furthermore elicit or exacerbate a number of conditions in susceptible individuals, including allergic airway diseases (Emberlin, 1998), and potentially lower the exposure thresholds above which health effects are evinced (Molfino et al., 1991).

While the monitoring of air pollutants has developed considerably over recent decades through the introduction of on-line detection and chemistry-transport models (Hertel et al., 2007), a similar development in the monitoring of pollen and other airborne allergens has been less rapid. Whilst real-time online methods are available for key air pollutants (e.g. ozone, NOx, particles), the current core pollen detection method relies on the capture of pollen grains using a low volume air sampler, followed by manual visual detection using a microscope – a labor intensive methodology based on a sampler developed more than 60 years ago (Hirst, 1952). This contrast between air pollution and airborne allergens is also seen in relation to regulation at both national and international level – for example in Europe the air quality directive requires monitoring of a variety of air pollutants but not of pollen, since in this context pollen has not been defined as an air pollutant. For airborne allergenic pollen, the only exposure thresholds commonly applied are related to daily mean concentrations, whilst for air pollutants limit values at multiple time scales from hourly to annual values have been established. The diurnal variation in pollen concentrations can be large and peak values over the day may affect the allergic reaction, making measurements at higher temporal resolution useful in assessment of health effects.

Methods for assessing air allergen content have come to prominence in recent years (Buters et al., 2012), however, pollen grain and allergen measurements are not always well correlated (Rodríguez-Rajo et al., 2011; Galán et al., 2013). To some degree they complement one-another, meaning that future monitoring activities should properly take both of these into account. This however requires changes in the current monitoring program. Future efforts within development of technologies for air pollution monitoring should consider including pollen with a particular focus on allergens. The advantage of focusing on allergens is twofold: firstly it is the allergens that cause the human reaction, the pollen grain is merely the vector by which it is transferred to the exposure target. Secondly, the detection of allergen will automatically take into account the observed alterations of allergens by chemical reactions. This will make co-exposure assessments much more straightforward and provide a direct observation of the causal agent (the allergen) instead of a proxy (the pollen grain). Developments of new cheap and lightweight technologies are crucial for advancing the monitoring of pollen and allergens in relation to human health.

Outdoor concentrations of pollen and air pollution measured at urban background monitoring stations are often used as an estimate for population exposure (Hertel et al., 2013, 2001; Momas et al., 2003; Anderson et al., 1998; Mücke et al., 2014). This is especially the case when health effects related to short-term air pollution exposure is studied, whereas long-term exposure is often obtained from modelling (Hertel et al., 2013). It has been argued that the location of urban background monitoring stations of pollen at roof level are chosen in order to better represent the regional rather than the local situation (de Weger et al., 2013). Background concentrations are not directly equivalent to exposures, which are innately personal and vary from individual to individual. In health assessment studies related to air pollution, urban background is generally considered a better scale to assess exposure compared with street pollutant levels e.g. because people spend most of the time indoors and buildings are usually ventilated to the backyards rather than to the trafficked street. Considering pollen, only limited amounts are entering the buildings. Dose–response relationships have been determined for air pollutants and various health endpoints using urban background as exposure proxy (see e.g. studies like (Andersen et al., 2009)). It has been shown that background pollen concentrations correlate well with those made at human breathing height (Rantio-Lehtimäki et al., 1991; Alcazar et al., 1999; Peel et al., 2013b). Furthermore background concentrations have been shown to correlate well with average daytime personal exposure and short-term pollen grain dose measurements (Riediker et al., 2000; Peel et al., 2013a), albeit with quantitative differences that can be substantial. Outdoor background concentrations of pollen and chemical air pollutants can therefore be expected to be good qualitative proxies of exposure.

Concentrations of all atmospheric agents are influenced in different ways by numerous factors, e.g. patterns in anthropogenic and natural emissions, prevailing wind direction and wind speed. Both meteorology and emissions tend to have typical patterns of annual and diurnal variation. It is therefore possible that meteorology and emissions could show a tendency to produce concurrently high concentrations of specific air pollutants and pollen taxa, which may result in exacerbated health effects.

In the current study we aim to analyse patterns in pollutant and selected pollen concentrations for which co-exposure may be a particular risk with respect to adjuvant health effects in Copenhagen, Denmark. This is pursued in order to establish if there are typical patterns of concurrently high concentrations. The results might help to improve the guidance given to the allergic and asthmatic public. We pursue this aim by:

(1) Comparing the yearly pattern of the concentrations of pollutants with the timing of pollen seasons.
(2) Comparing the average diurnal pattern of concentrations of pollen and pollutants on days with (a) high pollen concentrations and for (b) the remaining part of the pollen season.

The analysis is based on urban background pollutant measurements from the Danish Air Quality Monitoring Program in combination with atmospheric pollen concentrations from the routine monitoring station in Copenhagen, for the period 1997–2012. The analysis includes grass and birch pollen, the two principle allergenic pollen taxa in Northern Europe, and the air pollutants sulfur dioxide (SO_2), ozone (O_3), nitrogen dioxide (NO_2) and coarse and fine particulate matter ($PM_{2.5}$ and PM_{10}, i.e. particles with aerodynamic diameters below 2.5 and 10 micro-meters respectively).

2. Materials and methods

2.1. Site description

All data were obtained through monitoring activities in Copenhagen, the capital of Denmark.

Atmospheric pollen concentrations were collected at the Copenhagen pollen monitoring station using a Burkard 7 day recording volumetric spore trap (Hirst, 1952) situated 15 m above ground level on the roof of the Danish Meteorological Institute (55°43 N, 12°34 E). The Copenhagen pollen monitoring station is one of the two permanent stations in the Danish pollen monitoring network, and is typically in continuous operation from January to October. The other station is located in Viborg.

Air pollution data were obtained from a single background monitoring station situated at the H.C. Ørsted Institute (HCØ), and from two roadside stations, on H.C. Andersens Boulevard (HCAB) and

Jagtvej. All three stations are part of the Danish Air Quality Monitoring Program (Hertel et al., 2007; Ellermann et al., 2013). The HCØ station is situated on the roof of the six-storey high H.C. Ørsted Institute at University of Copenhagen, in the vicinity of a large park and several major roads. Jagtvej is one of the main routes into central Copenhagen and has a daily traffic flow of around 17,600 vehicles (The Technical and Environmental Administration/Teknik og Miljøforvaltningen, 2013). At the site of the monitoring station, Jagtvej is a 20 m wide street and delimited on either side by three-five story residential buildings. H.C. Andersens Boulevard is a six lane street in central Copenhagen, with a traffic flow of around 50,500 vehicles per day (The Technical and Environmental Administration/Teknik og Miljøforvaltningen, 2013). At the site of the monitoring station HCAB is delimited to the East by five-six storey buildings and to the West by the amusement park Tivoli.

Precipitation data were obtained from Kastrup airport weather station (WMO station ID 06180), located about 8 km from Copenhagen city centre, via the UK Met Office Global Weather Data collection (UK Meteorological Office, 2013). The locations of the pollen and pollutant monitoring stations and the weather station used in this study are shown in Fig. 1.

2.2. Atmospheric pollen concentrations

Pollen concentration data were obtained with a Burkard 7 day recording volumetric spore trap, a type of impaction device. Air was aspirated through a 2 mm wide slit, and samples were collected

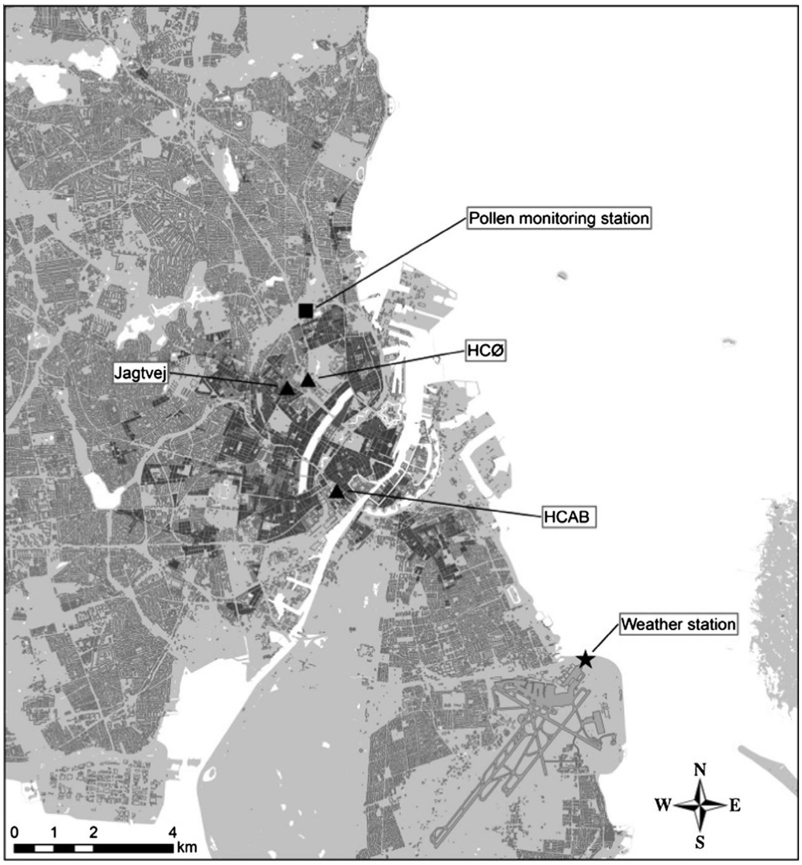

Fig. 1. Map showing the locations of the Copenhagen pollen monitoring station, the Kastrup weather station, and the HCA Boulevard (HCAB), HC Ørsted Instituttet (HCØ) and Jagtvej air quality monitoring stations.

either on silicone coated Melinex tape with a 7-day drum, or on silicone coated standard microscope slides collecting for 24 h, since the method changed from weekly to daily sampling during the 15 year study period. Both the tape and the microscope slides moved past the slit at a rate of 2 mm per hour. Each microscope slide was exposed for 24 h. The 7-day drum was exposed for 7 days, after which the tape was cut into 7 pieces, each corresponding to a 24-h period, which mounted on individual slides. Bi-hourly concentrations were obtained by counting the number of pollen grains deposited along each of 12 transverse transects on each daily slide using the method of Käpylä and Penttinen (1981). Counting was done using a light microscope, with counts then converted into concentrations in grains m^{-3}. In this way a total of 8–10% of each daily slide was assayed, depending on the microscope model used, which varied over the 15 year period covered by this study.

Atmospheric pollen concentrations from all grass and birch pollen seasons within the period 1997–2012 were used in this study. For each taxa and each year, the pollen season was defined as the period between the days on which the cumulative number of pollen grains sampled was between 2.5% and 97.5% of the yearly total, as per the method of (Goldberg et al., 1988). For each taxon, atmospheric pollen concentrations were assigned to one of two groups depending on whether daily average concentrations were above or equal to (peak) or below (non-peak) a taxa-specific threshold of 50 grains m^{-3} for grass pollen, and 100 grains m^{-3} for birch pollen. These taxa-specific thresholds correspond to the threshold concentrations that are used by the Danish pollen warning service to classify a daily average concentration as "high", and are typically considered to be the daily average concentrations above which all subjects with grass respectively birch pollen allergy are expected to react (de Weger et al., 2013; Davies and Smith, 1973).

Atmospheric pollen concentrations were coupled with information on daily cumulative precipitation because of the effect of this on ambient pollen concentrations. Precipitation is known to remove pollen grains and other particles from the atmosphere via washout, e.g. McDonald (1962) reports that 1 mm of rain with a drop diameter of 1 mm will remove 72% of Juniper pollen, which at 26 μm in diameter is within the size range of both birch and grass pollen according to Morrow, Brown and Irving (1973). Precipitation can potentially also have a negative effect on pollen emission (Subba Reddi et al., 1988). Although local showers will only affect nearby air masses and vegetation, and pollen loaded air masses can arrive from remote areas, precipitation can have a distorting effect on the diurnal pattern of pollen. Days with a total amount of accumulated precipitation above 0.5 mm were therefore omitted from the present analysis, as well as days with missing precipitation data. For grass pollen, 572 out of the 1439 days were excluded due to precipitation or missing data on this. This corresponds to 40% of the days during the grass seasons in the period with data for atmospheric grass pollen concentrations. For birch pollen 124 out of 422 days were excluded, corresponding to 30% of the days during the birch seasons for which pollen concentrations were obtained.

2.3. Pollutant data

For the gasses SO_2, O_3, and NO_2, half-hourly data were available for the roadside stations and hourly data for the urban background station (Ellermann et al., 2013). Missing data for periods of less than three hours were estimated using linear interpolation. Days with gaps of more than three consecutive hours were eliminated from the analysis and days with rain were excluded from the analysis of diurnal patterns and mean concentrations. For particulate matter (PM), half-hour data was available from ambient particulate monitoring using TEOM monitors (Hertel et al., 2008). These data are associated with a negative bias (typical 20–30%) since the TEOM monitor heats sampled air up to 50 °C, leading to the potential evaporation of particle constituents like volatile organic compounds, ammonium nitrate and water and thereby leading to underestimation of concentrations. This bias may vary somewhat throughout the day. However, the daily variation of the bias will only have little impact on the average daily variation in PM since the bias is relatively small (20–30%). TEOM data were therefore used for calculating the diurnal variation in PM without correction for the bias.

Daily PM measurements were obtained using either β-ray methods or filter pack and an SM200 particle monitor sampler (Ellermann et al., 2013) are used in the quantitative elements of the analysis. The availability of data is shown in Table 1 for each of the pollutants.

Table 1

Data on pollutants and pollen from the four stations, HCØ, Jagtvej, HCAB and the Copenhagen pollen monitoring station. Time period and number of measurements included in the analysis is shown. Results of the statistical test (the Wilcoxon rank-sum test) are listed for days with peak pollen counts and for non-peak days. Results are also shown for statistical difference between the two groups, and for correlation (r_s) between pollen and pollutants on bi-hourly and daily data. Bi-hourly PM measurements were not available for Jagtvej. Gasses are in ppb, PM in μg m^{-3}, and pollen in grains/m^3. The higher $PM_{2.5}$ than PM_{10} concentrations measured at HCØ can be explained by data not being measured at identical periods. PM data is shown for SM200 measurements only as this is the more accurate method for estimation of the magnitude of the daily averages.

		HCØ				Jagtvej			HCAB		Pollen monitoring station	
		NO_2	O_3	PM_{10}	$PM_{2.5}$	NO_2	O_3	PM_{10}	SO_2	$PM_{2.5}$	Grass	Birch
Grass season												
Peak days	Mean (range)	9.4 (3.0–23.2)	36.5 (23.8–8.6)	25.3 (9.6–51.6)	26.6 (2.5–86.4)	23.4 (4.5–56.9)	26.5 (11.0–49.5)	31.9 (7.8–64.8)	1.01 (−0.05–7.43)	20.4 (6.7–48.7)	92.1 (50–285)	
	n, sample size[a]	147	102	107	31	173	159	132	135	63	175	
	r_s (p), bi-hourly	*$-1.2*10^{-4}$ (0.996)*	0.246 (<0.001)	0.244 (<0.001)	0.078 (0.035)	0.096 (<0.001)	0.168 (<0.001)	–	0.066 (0.008)	0.115 (<0.001)		
	r_s (p), daily	*−0.048 (0.568)*	0.291 (0.003)	0.212 (0.028)	*0.341 (0.061)*	*−0.058 (0.449)*	0.272 (<0.001)	*0.151 (0.084)*	*0.137 (0.113)*	*0.183 (0.152)*		
Non peak days	Mean (range)	9.2 (1.9–26.6)	32.3 (13.2–49.5)	22.4 (2.1–56.9)	25.8 (0–124.9)	21.8 (3.8–53.3)	21.6 (6.7–40.2)	28.7 (0–71.6)	1.08 (−0.18–5.24)	16.4 (2.5–36.1)	14.1 (1–49)	
	n, sample size[a]	621	352	402	158	665	583	486	431	233	692	
	r_s (p), bi-hourly	−0.116 (<0.001)	0.242 (<0.001)	0.043 (0.014)	0.070 (<0.001)	−0.063 (<0.001)	0.204 (<0.001)	–	*−0.017 (0.230)*	*−0.015 (0.341)*		
	r_s (p), daily	−0.214 (<0.001)	0.263 (<0.001)	−0.126 (0.011)	*−0.001 (0.988)*	−0.212 (<0.001)	0.318 (<0.001)	−0.134 (0.003)	−0.206 (<0.001)	*0.003 (0.967)*		
Wilcoxon test		*p = 0.442 z = 0.768*	*p* < 0.001 *z* = 5.130	*p* < 0.001 *z* = 3.385	*p = 0.315 z = 1.004*	*p = 0.186 z = 1.322*	*p* < 0.001 *z* = 7.601	*p* = 0.012 *z* = 2.512	*p = 0.704 z = −0.380*	*p* < 0.001 *z* = 3.563	*p* < 0.001 *z* = 20.515	
Birch season												
Peak days	Mean (range)	12.3 (2.6–25.7)	39.7 (23.1–62.9)	31.0 (10.9–63.4)	29.2 (1.3–121.1)	29.4 (4.9–54.7)	24.7 (5.5–49.3)	42.9 (13.5–77.4)	1.44 (−0.04–5.52)	24.2 (4.2–50.6)		544.0 (103–3974)
	n, sample size[a]	137	89	96	33	142	119	102	107	55		143
	r_s (*p*), bi-hourly	−0.053 (0.032)	0.340 (<0.001)	*−0.026 (0.459)*	0.120 (0.005)	0.084 (<0.001)	0.173 (<0.001)	–	0.138 (<0.001)	*0.071 (0.061)*		
	r_s (*p*), daily	*0.028 (0.745)*	0.261 (0.013)	*0.165 (0.109)*	*0.045 (0.804)*	*0.081 (0.339)*	*0.089 (0.335)*	*0.053 (0.560)*	*0.104 (0.287)*	*0.055 (0.690)*		
Non peak days	Mean (range)	11.1 (2.9–27.7)	36.9 (23.5–50.4)	22.2 (6.0–64.8)	20.1 (2.7–67.2)	26.8 (6.0–45.0)	21.9 (6.5–40.5)	36.2 (9.7–93.4)	1.22 (−0.05–4.28)	19.1 (8.3–38.9)		38.6 (1–98)
	n, sample size[a]	154	74	80	34	148	129	91	81	47		155

r_s (p), bi-hourly	−0.048 (0.041)	0.098 (0.004)	*0.031 (0.395)*	*0.082 (0.119)*	*0.037 (0.116)*	0.160 (<0.001)	–	0.066 (0.039)	*−0.039 (0.289)*		
r_s (p), daily	*0.070 (0.390)*	*−0.201 (0.08)*	*0.139 (0.219)*	*0.187 (0.290)*	*0.019 (0.821)*	*0.169 (0.056)*	*−0.048 (0.653)*	*0.178 (0.111)*	0.350 (0.016)		
Wilcoxon test	*$p = 0.072$ $z = 1.800$*	*$p = 0.063$ $z = -1.855$*	$p < 0.001$ $z = -5.323$	*$p = 0.063$ $z = 1.863$*	$p = 0.019$ $z = 2.330$	$p = 0.020$ $z = 2.321$	$p < 0.001$ $z = -3.462$	*$p = 0.109$ $z = -1.605$*	$p = 0.006$ $z = -2.726$		$p < 0.001$ $z = 14.912$
Time period	1997–2012	07/2003–2012	05/2002–2012	03/2008–2012	1997–2012	1997–01/2011	07/2000–2012	03/2001–2012	04/2007–2012	1997–2012	1997–2012

[a] Number of days with less than 0.5 mm rain and maximum 3-h gaps within a day.

For the hourly or half-hourly data all time stamps correspond to the start of the averaging period, and are given in Central European Time (UTC+1).

Air pollution data corresponding to days within the pollen seasons were selected for analysis and divided between the following 4 groups of days with:

(1) peak birch pollen concentrations,
(2) non-peak birch pollen concentrations,
(3) peak grass pollen concentrations and
(4) non-peak grass pollen concentrations.

Air pollutant measurements on days in the pollen season with missing pollen concentrations were not included in further analysis.

2.4. Statistical analysis

Daily mean SO_2, O_3, and NO_2 values were calculated for both background and roadside data. Daily data for these species as well as daily average for roadside and background PM_{10} and $PM_{2.5}$ measurements made with the SM200 sampler were then divided between groups 1–4 defined above. The Wilcoxon rank-sum test was used to test for differences between the distributions of groups 1 and 2 (peak and non-peak birch pollen days respectively), and groups 3 and 4 (peak and non-peak grass pollen days respectively). Daily mean birch and grass pollen concentrations were similarly computed, and the Wilcoxon rank-sum test used to compare data corresponding to peak and non-peak days for each taxa.

The correlation between pollen count and the corresponding pollutant concentration measurements were also calculated for bi-hourly as well as daily concentrations.

Results were considered significant at the 95% level. The Wilcoxon rank-sum tests and the statistical correlations were conducted using MATLAB (MATLAB, 2008), whilst all other analyses was carried out using the Microsoft programs Excel 2010 and Access 2010.

3. Results

3.1. Annual variation

Fig. 2 shows the typical annual variation of pollutant concentrations, and the typical extent of the birch and grass pollen seasons. As expected, a tendency for O_3 concentrations to be higher at HCØ than at the Jagtvej roadside station was observed at all times of year, due to the reciprocal relationship between O_3 and NO (see the Discussion paragraph). A similar pattern of annual variation was seen at both stations, with concentrations reaching their maximum values between late April and early June, and remaining high throughout the birch and the majority of the grass pollen season, whilst the lowest concentrations were seen during the winter. For both NO_2 and SO_2 little annual variation was seen, although there appeared to be a tendency for slightly lower average concentrations in the summer during the grass pollen season. NO_2 concentrations at the Jagtvej station were at their highest during the birch pollen season.

The yearly patterns for PM_{10} and $PM_{2.5}$ (Fig. 2a) indicated that there was little annual variation for either PM_{10} or $PM_{2.5,}$ although there appeared to be a tendency for PM_{10} levels to be lower during the summer, coinciding with the grass pollen season. PM_{10} concentrations were higher at roadside than at background level at all times of year, as well as higher than $PM_{2.5}$ roadside concentrations.

3.2. Peak/non-peak relationships and diurnal variation

For each pollutant species and pollen taxa the concentration range and mean, sample size, and period for which data were available are presented for each of the four groups defined in Section 2.3 (peak- and non-peak days for the two pollen taxa) in Table 1. The results of the bi-hourly and daily

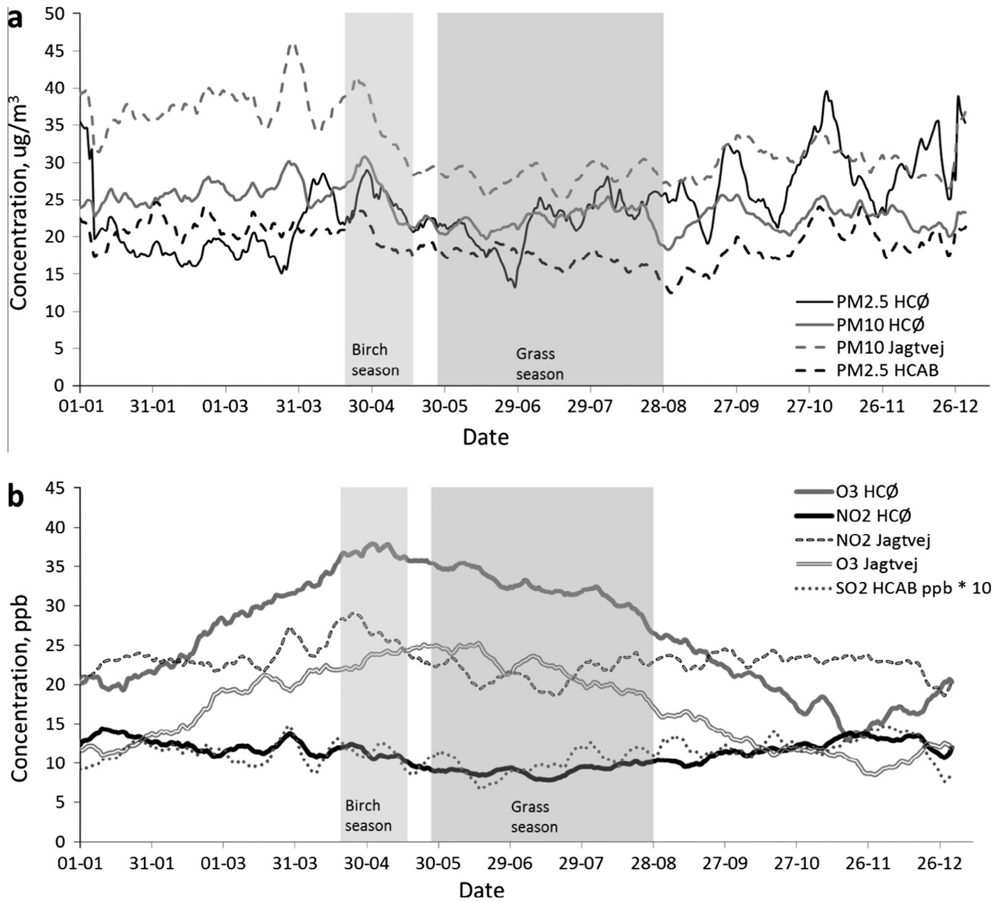

Fig. 2. Yearly plots were constructed by averaging daily mean concentrations by day of the year, and then calculating an 11-day running mean for 1997–2012 for PM (a) and the gasses O_3, NO_2 and SO_2 (b) for both roadside (Jagtvej or HCAB) and urban background (HCØ). Typical birch and grass pollen seasons based on the period 1985–2009 are shaded grey (Sommer and Rasmussen, 2012).

correlations between pollen and pollutant concentrations, as well as the Wilcoxon rank-sum test, used to test for differences between daily average pollutant concentrations corresponding to peak and non-peak days of each pollen taxon, are also presented.

3.2.1. Pollen

The percentages of peak pollen days during the respective pollen seasons were 20% for grass and 48% for birch. Figs. 3a and b show the average diurnal patterns for both peak and non-peak days for birch and grass pollen respectively. Both pollen taxa showed similar patterns on peak and non-peak days. However, average bi-hourly concentrations on peak days were 5–9 times higher for grass and 11–17 times higher for birch compared with conditions on non-peak days. The maximum birch pollen concentrations were reached in the afternoon at around 15:00, and the lowest concentrations were observed in the early morning at around 05:00. Grass pollen concentrations were highest in the early evening at around 18:00, and the lowest concentrations were seen at around 05:00 in the early morning, as for birch.

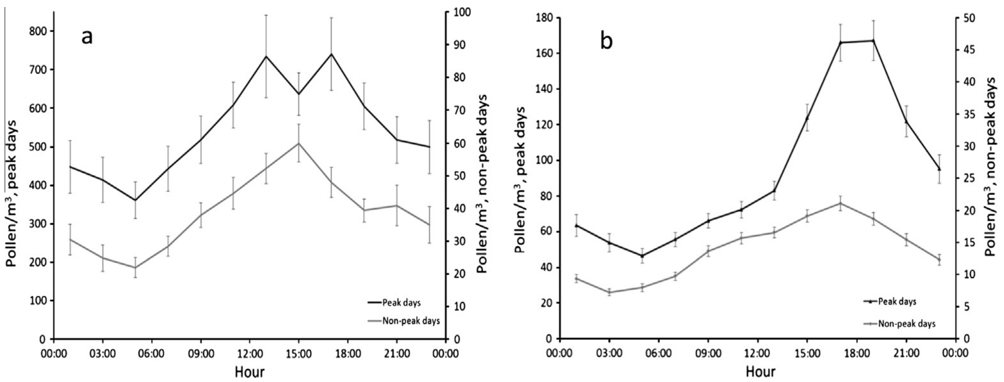

Fig. 3. Diurnal charts of the average patterns of (a) birch pollen (the two-peaked pattern on peak-days is a result of three bi-hourly measurements with concentrations of above 7000 grains m^{-3} out of the 1716 bi-hourly measurements in this group) and (b) grass pollen, with standard error of the means.

3.2.2. Pollutants

Figs. 4a and b show the average diurnal $PM_{2.5}$ and PM_{10} profiles for each of the four groups (peak birch, non-peak birch, peak grass and non-peak grass) at HCAB and HCØ based on TEOM data. Both PM_{10} and $PM_{2.5}$ concentration profiles were similar for all four groups of HCAB measurements, which showed an early morning peak, and for all four groups of HCØ measurements, which showed largely invariant profiles. Table 1 shows the results for the SM200 PM measurements for the four groups. PM_{10} concentrations were significantly higher on peak than on non-peak days during both grass and birch seasons for both HCØ and Jagtvej. $PM_{2.5}$ concentrations showed significantly higher levels on peak than on non-peak days for both taxa at HCAB, however not for HCØ. These relative magnitude differences are also evident in the diurnal profiles seen in Fig. 4a and b.

Correlations between PM and pollen concentrations were either very weak or non-significant with the exception of PM_{10} for peak grass pollen days at HCØ and $PM_{2.5}$ at HCAB for peak birch days (see Table 1). In general the correlations are mainly non-significant for the birch season, where more significant correlations are seen during the grass pollen season.

Figs. 4c–f show average diurnal gaseous pollutant variation for each of the four groups. For each pollutant species, diurnal patterns were qualitatively similar for each group. Fig. 4c shows the average diurnal patterns of O_3 and NO_2 at HCØ, the urban background station. The two gasses showed a clearly inverse relationship, with the highest NO_2 and lowest O_3 concentrations occurring in the early hours of the morning, and the lowest NO_2 and highest O_3 concentrations between the early afternoon and early evening. Analogous patterns were seen for both pollutants at the Jagtvej station, although O_3 concentrations were in general a little lower and NO_2 concentrations a little higher compared with HCØ (Fig. 4d and e).

Concentrations of O_3 at both HCØ and Jagtvej were higher on peak than on non-peak pollen days for both grass and birch pollen, however at HCØ this difference was only statistical significant for grass pollen (Fig 4c and e, Table 1). Correlations between O_3 and pollen concentrations during the grass pollen season were all relatively high and statistically significant. This agrees with what is seen in the respective diurnal patterns (Figs. 3b, 4c and e). For the birch season no consistent pattern is seen. The correlations are relatively high and statistically significant for HCØ on peak days. Correlations for the bi-hourly concentrations are all statistically significant with varying strength, whereas the correlations for the daily concentrations are not statistically significant with the exception of peak days at HCØ.

The highest O_3 concentrations were observed in spring on peak birch days, consistent with the overall peak in the yearly pattern of O_3 (Fig. 2b).

Daily average NO_2, concentrations tended to be greater on peak than on non-peak days for both pollen taxa at both monitoring stations, however this difference was only statistical significant for

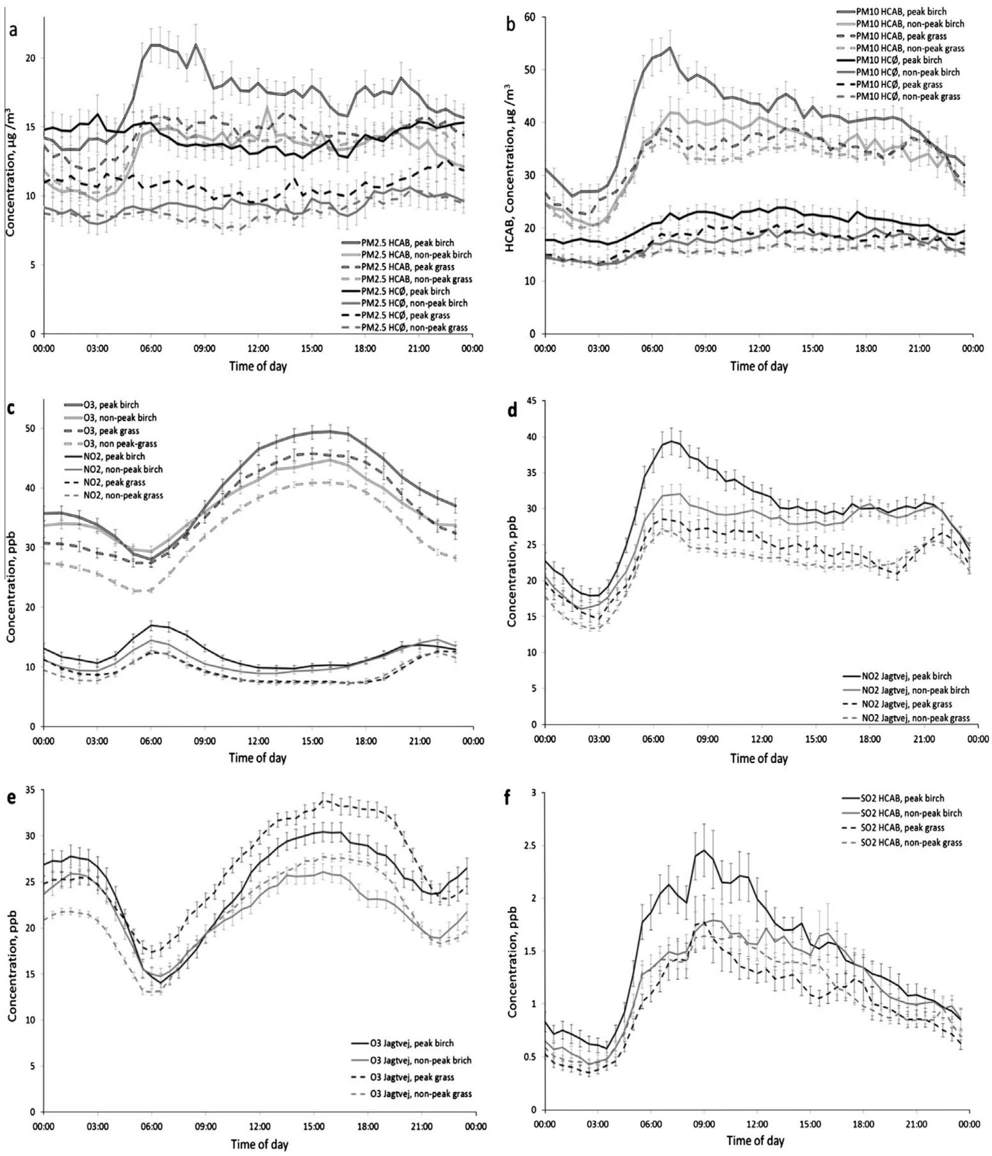

Fig. 4. Diurnal charts of the average patterns of pollutants with standard error of the means; (a) $PM_{2.5}$ at HCAB and HCØ, (b) PM_{10} at HCAB and HCØ, (c) O_3 and NO_2 at HCØ background station, (d) NO_2 at Jagtvej roadside station, (e) O_3 at Jagtvej roadside station, (f) SO_2 at HCAB roadside station. The PM profiles are based on the following number of days with half-hour TEOM measurements; 564 for HCAB PM_{10}, 462 for HCØ PM_{10}, 524 for HCAB $PM_{2.5}$ and 328 for HCØ, $PM_{2.5}$, from the period 2001 to 2012. Number of days included in the profile for all variables can be seen in Table 1.

birch pollen at Jagtvej (Table 1). The largest quantitative difference between average peak and non-peak diurnal concentration profiles was in general seen for the hours with the maximum concentrations. Correlations between NO_2 and pollen concentrations were for the majority of the significant cases negative. This agrees with the respective diurnal patterns (Figs. 3a, 4c and d). For the daily concentrations the only significant correlations were for non-peak grass days. In general all bi-hourly correlations were small or insignificant.

Fig. 4f shows SO_2 data from the HCAB roadside station for peak and non-peak days for both pollen taxa. Average concentrations showed similar diurnal variation for all four groups, with concentrations reaching a maximum around 09:00. During the grass pollen season SO_2 concentrations in morning hours were greater on peak than on non-peak. However, daily average SO_2 concentrations did not differ significantly between peak and non-peak days for either pollen taxa (Table 1). The correlations between SO_2 and pollen concentrations are in general statistically significant for bi-hourly data and non-significant for daily data, except for non-peak grass days where this pattern is reversed.

4. Discussion

This study indicates that high ozone concentrations coincide both seasonally and diurnally with high pollen concentrations, and that ozone concentrations were higher on peak pollen days. For nitrogen dioxide and sulfur dioxide no coincidence in concentration peaks with pollen was found. For particulate matter no coincidence of diurnal or seasonal concentration peaks were found, however concentrations were higher on peak pollen days than on non-peak days.

Pollen concentrations within cities are influenced by urban planning decisions, in particular choice of ornamental plants and maintenance of green spaces (Skjøth et al., 2013). Vegetation within a city typically occurs in relatively small discrete patches, which can lead to large temporal and spatial gradients in pollen concentrations (Skjøth et al., 2013; Emberlin and Norris-Hill, 1991), and potentially also in exposure. Similar gradients are seen for chemical air pollutants in urban environments (Hertel et al., 2008). There is thus a potential for developing new abatement strategies to reduce co-exposure and the potential resulting health effects of both pollen and chemical air pollutants and for setting up requirements on routine monitoring to carefully follow concentrations and trends.

4.1. Co-exposure to pollen and O_3

Several experimental studies have focused on co-exposure to O_3 and allergens (Molfino et al., 1991; Peden et al., 1995; Jörres et al., 1996; Vagaggini et al., 2002). After only one hour of exposure to 120 ppb of O_3, Molfino et al. (1991) found that only half the allergen (ragweed and grass pollen) dose was needed in order to provoke a response similar to that observed following exposure to clean air (20% drop in FEV1). The O_3 concentrations observed in this study in Copenhagen are lower than these, but their effect can nonetheless be expected to be of clinical relevance since it has been suggested that there is no lower limit for the effects of O_3 on the respiratory system (Van Bree et al., 1995).

The peak O_3 concentrations measured in this study may result in potentially clinically relevant simultaneous co-exposure. The yearly pattern of O_3 in Fig 2b shows the highest values in late April and early May, coinciding with the birch pollen season, and remains high also during the grass pollen season. The spring time peak could be due to a combination of a high amount of available sunlight and intrusions of stratospheric ozone, which often occurs during the spring (Monks, 2000). Most of the observed of O_3 in the troposphere is formed through photochemical chain reactions involving nitrogen oxides and hydrocarbins (Guerreiro et al., 2013; Seinfeld and Pandis, 2012). In urban streets O_3 is titrated in the reaction with NO from local traffic emissions, leading to a reciprocal relationship between O_3 and NO_2 (Palmgren et al., 1996). The effect is seen in Fig 4c, where O_3 and NO_2 show reciprocal diurnal variation. Other sources that can lead to high O_3 concentrations are e.g. formation of ozone due to emissions of NOx and VOĆs, and transport of O_3 from southern Europe (Bloss, 2009).

Daily average O_3 concentrations at Jagtvej were significantly higher on peak than non-peak days during both pollen seasons, indicating higher co-exposure on these days. The same is seen for O_3 measured at HCØ for the grass pollen season, however for the birch pollen season, the difference is significant at the 95% level only for the period 07:00–22:00 ($z = -2.675$, $p = 0.008$), the part of the day when individuals are more likely to be outdoors, and not for the overall daily average (Table 1) most likely due to very similar concentrations for the two groups during night. Daily grass pollen concentrations are statistically significantly correlated with O_3, whilst birch concentrations are not (Table 1). One factor that contributes to this difference could be the tendency for extreme birch pollen concentrations of several thousand grains m^{-3}, not seen for grass pollen which does not exceed a few hundred grains

m^{-3}. This difference is illustrated by the greater skewness and kurtosis of the distribution of daily birch pollen concentrations (skewness/kurtosis are 3.9/19.6 for birch, and 2.4/7.1 for grass). The corresponding values for the distributions of O_3 during both birch and grass pollen seasons are very low (0.63/0.98 and 0.15/0.21 respectively). As weather conditions become more favourable to high concentrations of both pollen and ozone, we would expect birch concentrations to increase exponentially relative to O_3 concentrations, having a negative effect on the correlation between the two. This effect would be less pronounced during the grass pollen season, since the distribution of daily average grass pollen concentrations is less strongly skewed.

The long, slow decline in both daily O_3 and grass pollen concentrations at the tail end of the grass pollen season is another potential contributing factor. The grass pollen season typically peaks in June, with daily average concentrations gradually dropping off during July and August. As can be seen in Fig. 2b, daily average O_3 concentrations show a gradual decline throughout the entire grass pollen season. The similarity in these trends during the tail of the grass pollen season would be expected to have a positive effect on the strength of correlation between daily grass pollen and O_3 concentrations.

High pollen concentrations have often been linked to sunny warm days on which conditions are conducive to flowering and the emission of pollen (Smart et al., 1979; Khwarahm et al., 2014; Galán et al., 1995), and strong solar radiation is a primary factor for formation of ozone in the boundary layer. Therefore the coincidence of peak pollen days and high O_3 may be linked to the effect of sunlight on both variables.

The diurnal peaks of O_3 and pollen also coincide (Fig. 3a and b, and Fig. 4c and e), indicating that simultaneous co-exposure to these two compounds may be particularly relevant. This is also reflected in the statistical significant correlations between pollen and O_3 for bi-hourly data (Table 1). The correlation is strongest on peak birch pollen days at HCØ, indicating the largest risk of co-exposure. Diurnal variation of pollen concentrations is mediated by factors effecting the emission and/or the dispersal of pollen. Birch pollen is released at a greater height than grass pollen, and may as a consequence be more prone to undergo long distance transport, bringing remote sources into play. This could have an impact on diurnal birch pollen concentration. Episodes of long distance transport have been documented a number of times for birch pollen for the Copenhagen area (Skjoth et al., 2007, 2008; Mahura et al., 2007) as well as for the London area (Skjoth et al., 2009), but to our knowledge never for grass. Variation in the diurnal pattern of grass pollen concentrations could be linked to differences in time of emission between different grass species (Reddi and Reddi, 1985). There has also been speculation that both transport from non-urban areas and the collapse of the boundary layer when convection ceases may lead to large late-evening grass pollen concentrations in urban areas (Norris-Hill and Emberlin, 1991). One should keep in mind that the pollen concentration profiles presented in this study are averages. Diurnal pollen concentration patterns can vary substantially from day to day, and have been observed to exhibit systematic seasonal variation (Peel et al., 2014).

The O_3 levels in this study measured in Copenhagen, Denmark tend to be low, but many countries in South Europe experience problems with O_3, with more than 25 days per year exceeding the EU target value for human health (maximum daily 8-h mean > 120 $\mu g\, m^{-3}$) (Guerreiro et al., 2013). The potential health effects of simultaneous co-exposure to pollen and O_3 may therefore be aggravated in more southern European countries.

4.2. Co-exposure to pollen and other pollutants

NO_2 have been shown to act as an adjuvant to allergic reactions, however a review by Hesterberg et al. (2009) of the short term effects of NO_2 exposure found that there were no direct augmentation of allergic response after co-exposure to NO_2 levels below 200 ppb. In this study, the highest ½ hour NO_2 concentration was 143 ppb. In addition to this, NO_2 levels were found to be at their seasonal lowest during the grass pollen season, as well as their lowest diurnal levels during the peak pollen hours of the day, for both birch and grass pollen. This might be explained by the previously mentioned inverse relationship between O_3 and NO_2 which results in the depletion of NO_2 through a photochemical reaction, and the coupling to peak pollen days through the effect of sunlight on both. NO_2 concentrations are higher on birch peak than non-peak days at Jagtvej (Table 1). No apparent explanation is seen for this.

Although it appears that there is low risk of co-exposure effects from simultaneous high levels of pollen and NO_2, the NO_2 concentrations can be important as the chemical reaction between pollen and NO_2 has been suggested to alter the amount of allergens within the pollen grains (Franze et al., 2005). This effect, however, will likely take place over different timescales than those of relevance to simultaneous co-exposure.

SO_2 has been shown to have a synergistic effect with the allergic reaction and other pollutants, e.g. SO_2 (200 ppb) and NO_2 (400 ppb) in combination have been shown to decrease the allergen dose (house dust mite) required to produce a 20% drop in FEV1 by 60% (Devalia et al., 1996). However, high SO_2 concentrations have also been shown to reduce the allergen release from pollen (Behrendt et al., 1997; Santra et al., 1991). SO_2 levels in outdoor air are however far below these levels, and the EU hourly threshold for protection of human health of 124 ppb is rarely exceeded in Europe (Guerreiro et al., 2013). In this study we find very low SO_2 concentrations, with a diurnal pattern of maximum concentrations in the morning hours. This is supported by the weak correlations with bi-hourly pollen concentrations (Table 1). This low magnitude however leads to potential inaccuracy on the detection of the diurnal variation due to the limitations of the measurement method at these concentrations. From this it does not appear likely that ambient SO_2 constitutes a risk factor when assessing co-exposure to pollen allergens.

PM has been shown to adhere to the surface of pollen grains and induce the release of allergenic material (Behrendt et al., 1997), thereby becoming a carrier of allergenic particles. In the same manner, pollen grains have been shown to be carriers of particulates that bind to the pollen (Shahali et al., 2009). This could be one of the reasons why PM has been shown to increase the risk of hospitalization for asthma, with a greater risk for those also suffering from allergic rhinitis (Tecer et al., 2008), however only during pollen seasons. For PM, diesel exhaust particles in particular have been associated with increased allergic reactions. They can, by various mechanisms, increase IgE[1] production and thereby induce stronger allergic reactions (Nel et al., 1998), and have an additive effect by enhancing the airway inflammation induced by ozone (Bosson et al., 2008).

In this study $PM_{2.5}$ concentrations appear to be higher on peak pollen days than on non-peak days. This is seen as the higher diurnal profiles during the birch pollen season at both HCAB and HCØ and during the grass pollen season at HCØ (Fig. 4a + b), and as the statistically significant differences in $PM_{2.5}$ concentrations between peak and non-peak days at HCAB (Wilcoxon test, Table 1). It is however not seen in the background measurements from HCØ, indicating that the particles may originate from the traffic in the canyon, although no apparent explanation for the correlation with peak pollen concentrations can be seen. Also no explanation was found for the statistically significantly correlation between $PM_{2.5}$ and birch pollen concentrations on non-peak days at HCAB.

For HCØ, daily mean PM_{10} concentrations were higher on peak than on non-peak days for both grass and birch pollen, whilst at the roadside station this was only the case for the birch season. The diurnal peak, when present, occurs however during the morning hours and does not coincide with the pollen peak of either examined taxa, evidencing that they originate from different emission sources. The measurements of PM_{10} and $PM_{2.5}$ could include fragments of pollen grains however the authors have found no evidence in literature supporting this. Birch and grass pollen grains are typically in the size range of 20–40 μm (Morrow Brown and Irving, 1973), and therefore larger than the particles included in PM_{10} measurements. Also, the amount of birch allergen found in the fraction above 10 μm was 93% (Buters et al., 2010), indicating that very little of the allergen is present in the PM_{10} fraction. It is therefore not likely that the elevated PM_{10} and $PM_{2.5}$ concentrations are due to the appearance of fragments from pollen in the air. Other biogenic particles may however contribute to the PM, e.g. VOĆs. In background areas a typical fraction of about 30% will arise from carbon containing species and a dominating part of this from biogenic emissions. In the urban environment this fraction is smaller due to the vicinity of anthropogenic sources.

The significantly higher PM levels on peak days during the birch pollen season may be related to ammonia emissions, which show their annual peak in Denmark at around the time of the birch pollen season. Ammonia makes an important contribution to secondary atmospheric particles and is a

[1] Immunoglobin E, antibody associated with allergic reaction.

significant component of both PM_{10} and $PM_{2.5}$. Ammonia emission rates increase with temperature (Skjøth and Geels, 2013), and thus would be expected to be high on the warm, sunny days that promote birch pollen emission.

During the grass pollen season, the fungal spores Cladosporium and Ganoderma could be contributing to the PM concentrations. Both spores are found in the size range of PM_{10} and can be present in the air during the grass pollen season. Conditions favoring dispersion of pollen, may also favor dispersion of these. This might explain the statistically significant and relatively strong correlations between PM_{10} and grass pollen concentrations on peak grass pollen days (Table 1).

The co-variation of particle concentrations and pollen therefore appear to be related to common causes that support high concentrations.

4.3. Clinical relevance of simultaneous co-exposure

The initial symptoms of an allergic reaction to pollen usually occur within 15 min of exposure. However, a secondary late-phase reaction can occur 4–6 h after the first symptoms, and it has been shown that those suffering from such a reaction also will experience a stronger initial response (Machado et al., 1986). This study focuses on simultaneous co-exposure to pollen and pollutants, but it is also possible that exposure to air pollutants before and during the late phase response to allergen primes the airways for a more severe late-phase response. Such delayed changes in allergen responses after changes in air pollutant levels might be explained by inflammation and mucosal irritation in the airways occurring after pollutant exposures that has the potential to increase reactivity to allergens (Jörres et al., 1996; Molfino et al., 1991). A period with high pollen concentrations could on the other hand also induce an inflammation in the airways, making them more responsive to later pollution exposure. High concentrations of air pollutants may therefore be of relevance if they occur several hours or days after, or prior to, high pollen concentrations.

There has also been speculation that pollutants from traffic do not only corrode the outer wall of the pollen grain and thereby increase the bioavailability of the allergen, but also alter the allergen. In a study from Tehran the amount of the major allergen of Arizona cypress pollen decreased in exposed pollen, and a new protein, also shown to be allergenic, became dominant (Shahali et al., 2009). The implication of this could e.g. be that the standard allergy tests do not test for the altered allergen, with allergy sufferers therefore experiencing symptoms without a positive result on the allergy test.

If the allergens produced by two pollen taxa are similar, an individual sensitised to one of these taxa may also react to the other. This is known as a cross-reaction (Brostoff and Gamlin, 1996). Individuals allergic to birch pollen also commonly react to alder and hazel pollen, two fellow genera of the Betulaceae family (Corden et al., 2000). In Denmark, alder and hazel both flower early in the year, and their pollen seasons typically only overlap very little with that of birch (Sommer and Rasmussen, 2011, 2012; Brændholt and Rasmussen, 2013). The flowering of alder and hazel is usually in February–March where the ozone concentrations are considerable lower (Fig 2b), which will result in less co-exposure. This stands in contrast to Southern Europe, where ozone concentrations in general are higher than in Denmark and where the flowering of these trees is more simultaneous (Rizzi-Longo et al., 2007). This means that whilst alder and hazel may induce priming in a subset of birch-allergic individuals in Denmark, thereby altering their allergic response pattern during the birch pollen season, we would not expect any direct modification of the processes discussed in this geographical region due to cross-reactivity.

5. Conclusions

The aim of this study was to perform an examination of the potential for simultaneous co-exposure to pollen and pollutants in Copenhagen. The results showed that peak O_3 levels coincide both seasonally and diurnally with peak pollen concentrations, resulting in potentially clinically relevant simultaneous co-exposure. O_3 concentrations were furthermore found to be higher on days with peak birch or grass pollen concentrations, than on the remaining parts of the pollen seasons. For NO_2 and SO_2 no coincidence was found between either seasonal or diurnal pollen concentration peaks, and the concentrations measured in this study were well below thresholds for adjuvant effects to the allergic reaction, leading to only a small risk of co-exposure effects from these pollutants. For PM, no seasonal or diurnal coincidence of peak concentrations with peak pollen concentrations was found, however daily average concentrations were generally found to be higher on days with peak pollen concentrations compared with on non-peak days.

This study therefore indicates that when considering co-exposure effects from pollen and pollutants in Copenhagen, O_3 appears to be the most relevant pollutant to further examine and include in pollen service forecasts, when interested in the effects of simultaneous co-exposures. However, it is unclear if late phase allergic responses or delayed reactions to high pollution concentrations could result in a relevance of lagged co-exposure, in which case further analysis into this would be of relevance.

The scope of this study is limited to the two major pollen types, Betula and Poaceae. Due to the cross-reactivity between the major allergens of other members of the Betulaceae family, future research could benefit from including these pollen types (e.g. Corylus and Alnus).

There are still many gaps in our knowledge of pollen allergy and co-exposure to air pollution and so far there have not yet been any attempts to include air pollution levels in pollen warning systems. However, existing knowledge on the adjuvant effects of air pollutants on the allergic reaction, suggests that the inclusion of both concentrations and diurnal variation of pollution in allergy warning systems would be a highly useful addition to existing air quality monitoring information.

Just as air pollution should be considered an important factor when evaluating health effects of pollen, future studies of the health effects of air pollution, should also take pollen loads and potential co-exposure effects into consideration.

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Manuscript VI

Modelled dose-response curves from allergen challenge show no effect of co-exposure to ozone

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TITLE PAGE

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Short title
Co-exposure to ozone and pollen allergen

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Modelled dose-response curves from allergen challenge show no effect of co-exposure to ozone.

Abstract

Background: Co-exposure to air pollution and pollen has been suggested to have a joint effect resulting in enhanced reactions in pollen sensitized individuals. A recent study pointed to ozone and pollen peaks coinciding. The aim of this study was to evaluate bronchoconstriction during specific inhalation challenge (SIC) for pollen allergen exposure alone and for simultaneous co-exposure to ozone among pollen sensitized participants. **Methods:** In a human exposure chamber, 36 pollen sensitised participants underwent SIC to grass or birch allergen and 120 ppb of ozone in a randomized single blinded cross-over study. A total of 85 SICs were included. In order to evaluate differences in exposures, dose-response profiles were modelled by a four-parameter non-linear mixed regression model, hereby including information from the entire profile to estimate PD_{20}. The model showed a good fit to data and a resulting estimated log-dose response profile for both grass and birch. **Results:** We found no statistical significant effect of the co-exposure to ozone on the size of the modelled PD_{20}, or on the shape and magnitude of the dose-response profiles. This study illustrates a new method to model dose-response-curves for allergen exposure.
Conclusions: Our study does not support that co-exposure to naturally occurring levels of ozone exacerbates the effect of pollen allergens.

Abbreviations

Forced expiratory volume in one second (FEV_1), Dose eliciting a 20% decrement in FEV_1 (PD_{20}), Specific inhalation challenges (SIC), Skin prick testing (SPT).

Keywords

Air pollution, dose-response curve, ozone, pollen allergy , specific inhalation challenge.

Introduction

Exposures to pollen and air pollutants commonly occur simultaneously and have for decades been linked to eliciting or exacerbating the allergic response in susceptible individuals (1).

A recent study by our group examined the annual, diurnal, and peak concentration patterns of pollen and air pollutants in Copenhagen, Denmark (2). The study indicated that pollen and ozone concentrations peaked at the same time diurnally and seasonally.

Previous studies on the effect of ozone on allergic reaction have primarily concluded that ozone appears to have an effect, but that this effect is not always obvious, especially at low exposures (3-6). Only a few studies found an effect at realistic outdoor levels, e.g. Molfino et al. (3) , who reported that after one hour of exposure to 120 ppb of ozone, only approximately half the amount allergen was needed to invoke a response similar to that followed by exposure to clean air and allergen.

A common way to assess the effect of allergen is the bronchoconstrictive response (3;5;7-10). This is estimated by the dose needed to provoke a decrease in the forced expiratory volume in one second (FEV_1) of 15 % (PD_{15}) or 20 % (PD_{20}). Previous studies estimate PD_{20} as the log-linearly interpolated value between the last two points on the dose-response curve (7;8). In this study we used a novel approach to examine the bronchoconstrictive response by use of a "best fit" model of the dose response curve. Since the shape of the FEV_1 curve related to dose is non-linear, we derived PD_{20} by using a non-linear four parameter logistic regression model.

Objective

The objective of this study is to investigate whether short term simultaneous exposure to pollen allergen and realistic levels of ozone increases the bronchoconstrictive response during specific inhalation challenge (SIC) compared to solely pollen allergen in pollen sensitised individuals with no or mild asthma. We also evaluated whether a non-linear four-parameter logistic regression model provides a good fit for the FEV_1/log-dose response curves and for modelling PD_{20}. Finally we evaluated whether any of the baseline characteristics for the participants were associated with modelled PD_{20}.

Materials and methods

Study participants

80 non smoking persons recruited among university students with self-reported allergic rhinitis underwent skin prick testing (SPT) for grass, birch, artemisia, horse, dog, cat, house dust mites (Dermatophagoides pteronyssinus and Dermatophagoides farina) and fungal spores (Cladosporium herbarum and Alternaria alternaria). Participants with a positive SPT (> 3mm) to birch and/or grass and with no or mild respiratory symptoms were included in the study. A total of 17 participants underwent SIC with birch allergen and 19 with grass allergen, Table 1. Of 36 participants, 20 reported respiratory symptoms (cough, wheeze, chest tightness) more than a few times a year. Ten participants reported mild asthma, however only 7 of those had symptoms more than a few times a year. None of the 36 participant used asthma medication.

	Grass	Birch
N participants	19	17
Gender	F 10 M 9	F 10 M 7
Weight	F 68 (13) M 77 (8)	F 70 (14) M 86 (16)
Height	F 169 (5) M 183 (8)	F 169 (6) M 179 (6)
Age	24.2 (2.7)	24.4 (2.3)
Wheal size , SPT mm*	9.4 (3.2)	6.5 (2.5)
Number of positive SPT (min. / max.)	3.1 (1/6)	5.9 (4/8)

Table 1 Study participant characteristics. Mean (std. dev.). F= female, M=male. * Wheal size of SPT for grass or birch applied in the SIC.

None of the participants had recent exposure to secondhand smoking, recent infections, or use asthma medication. None of the participants used antihistamines within 72 hours prior to the SIC. All participants had an initial FEV_1 higher than 70% of predicted, and underwent a bronchial challenge test with a maximum cumulated dose of 4.51 mg methacholine bromide.
The protocol, enrolment procedure and written consent forms were approved by the Scientific Ethics Committee for Central Denmark Region (M-20090215).

Study design

The study was performed as a randomized single blinded cross-over study. The study took place during four study days. During the first two days the participants underwent SPT, symptom history, medical examinations and unspecific bronchial methacholine challenge test. On the third and fourth day SIC were performed in a human exposure chamber, either with allergen alone or with allergen and co-exposure to 120 ppb ozone. All SIC´s were performed outside the pollen season for the pollen under investigation. For details see Figure 1. Of the 19 participants who underwent SICs with grass allergen, 13 were included in two study rounds without ozone exposur, and both SICs were included in the analysis. SICs were performed with a minimum of 14 days apart to avoid carry-over effect.
All study days were outside the pollen season (birch and grass), according to registrations from the nearest operational pollen monitoring station (11-13), with exception of one study day with grass SICs where the birch season had started. All participants this day had a negative SPT for birch allergen. We did not have any information on allergies to other trees. However no participants reported any symptoms related to these pollen types and therefore no relevant allergy from these on study days was anticipated.

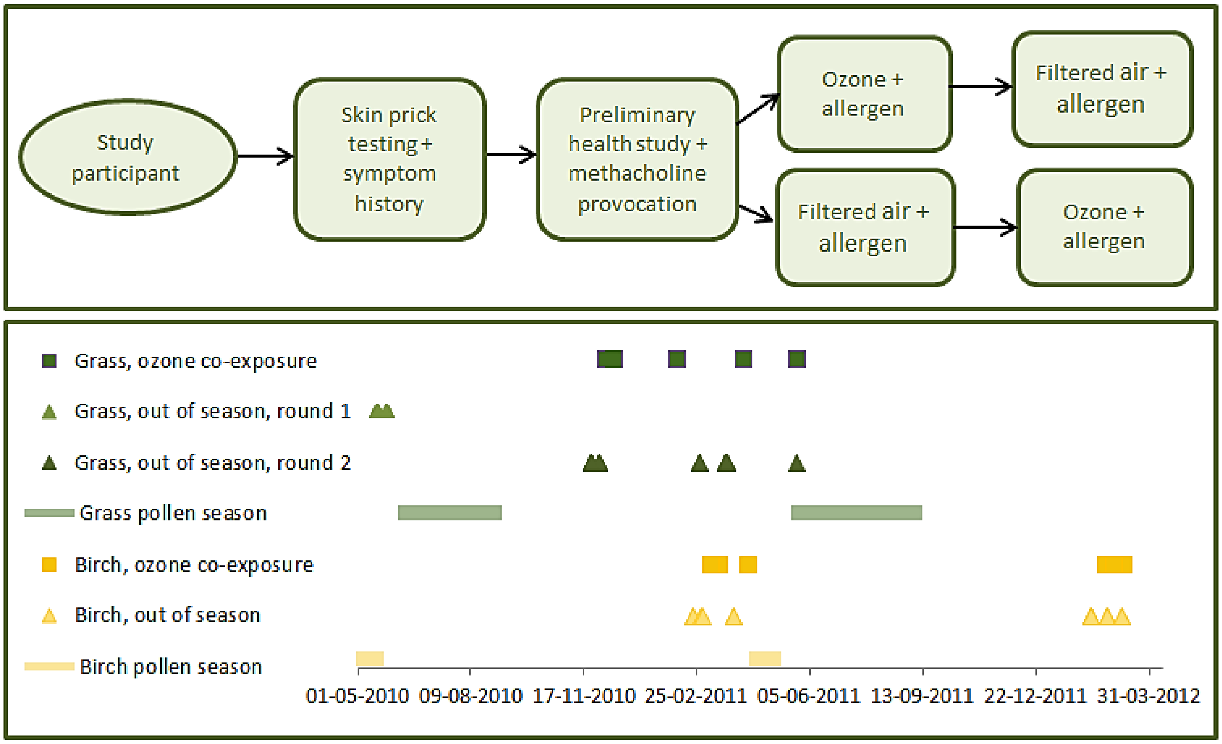

Figure 1 Study design and calendar showing grass and birch pollen season and the time of the SIC.

Exposure chamber

The SICs were conducted in a human exposure chamber system consisting of a $79m^2$ and a $32m^2$ chamber (14;15). Participants (1 to 4 at a time) were seated and the SICs performed in the large chamber only for the majority of the participants. However 13 of the 85 SICs were performed in the smaller chamber. At all exposures, participants were instructed to exhale in an extraction device to limit exhalation of allergens in the air. Additional ventilation was installed at the exposure area. Airflow, temperature, humidity, CO_2 and ozone were measured every 60 sec. The chambers were provided with filtered air at 22.3°C (std.dev 0.4°C) and humidity of 42.2 % (std.dev 1.2%).

Specific inhalation challenges

We used allergen extracts from ALK-Abelló (Hørsholm, DK) containing 100,000 SQ-U/ml of Phleum pratense or Betula verrucosa. Solutions resulting in doses from 1.4 – 5,600 SQ-U were administered with a Spira Dosimeter nebulizer and FEV_1 was measured with a handheld MicroDL spirometer connected to a computer, where all data were logged. SIC with aerosolized allergen was performed according to the procedure outlined in Figure 2. Baseline FEV_1 was defined as the best of three consecutive measurements after inhalation of diluent. FEV_1 was measured 15 minutes after each inhalation.

		Baseline	Level 1 Dilution 100 SQU			Level 2 Dilution 1,000 SQU			Level 3 Dilution 10,000 SQU			Level 4 Dilution 100,000 SQU			
Step	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Conc.	*	Diluent	2×100	4×100	8×100	2×1,000	4×1,000	8×1,000	2×10,000	4×10,000	8×10,000	2×100,000	4×100,000	8×100,000	Bricanyl
Dose, SQU			1.4	2.8	5.6	14	28	56	140	280	560	1400	2800	5600	

Procedure based on % drop in FEV1, based on baseline measurement after diluent (step 2)	
< 5 %	Next level – skip two steps., If < 5 % drop at level 12, no response is assumed and the provocation ended.
> 5 – 15 %	Next step.
>15 – 20 %	Repeat same step, maximum 3 times. If still < 20 %, next step is administered.
> 20 % or unacceptaple discomfort.	Disrupt provocation and proceed to administer bronchodilator (Bricanyl).

Figure 2 SIC protocol. The administered dose is dependent on the magnitude of the drop in FEV_1 induced by the previous dose, resulting in either one *step* or one *level* increase in dose.

After the SIC, participants measured FEV_1 every 15 min. for the first hour and thereafter hourly at the laboratory and later at home until bed time for late phase responses.

Ozone exposure

Ozone exposure levels were set to 120 ppb, based on previous studies (16-18).). The ozone was produced with an ozone generator and added to the ventilation system inlet into the large chamber. Participants were seated in the large exposure chamber for approximately one hour prior to SIC including the build-up time of the ozone concentration of around 20 minutes, to limit the detection of the odour, and keep the blinding for the participants.

Statistical analysis

Natural log transformation of the administered dose of allergen concentrations was applied as in other similar studies (5;7;9;17).
Bronchial hyper responsiveness has previously been shown to depict a non-linear shape of the log-dose-response curves for normal non-asthmatic subjects (19;20).
We therefore fitted a non-linear regression model in the shape of a four-parameter logistic curve to the FEV_1 data using the log cumulative dose as independent variable:

$$FEV1(x) = a + \frac{b-a}{1 + exp\frac{c-x}{d}}$$

where x is the log cumulative dose, a is baseline FEV_1, b is FEV_1 at maximal log cumulative dose, c is the log cumulative dose corresponding to half the decrease in FEV_1 (the inflection point), and d is a scaling parameter. The repeated measurements for each subject were taken into account by including random subject and exposure within subject effects for each of the four parameters describing the FEV_1-curve.

Model validation was performed by inspecting plots of observed and fitted FEV_1-values against cumulative dose and residual plots.

Allergen responsiveness was evaluated as the natural logarithm to the cumulative dose of allergen theoretically eliciting a 20% drop in baseline FEV_1 (PD_{20}). PD_{20} was estimated from the logistic model which means that the information contained in all the FEV_1 observations was used and not only the FEV_1 responses to the last two doses administered, as in previous studies. The non-linear regression model allows for estimating differences in PD_{20} corresponding to different exposures.

The association between PD_{20} and baseline characteristics of the study participant was investigated using a linear regression model with PD_{20} on a logarithmic scale. Since each subject contributed with two or three measurements of PD_{20}, random subject effect was included in the analysis.

Groups were compared using post-hoc t-tests based on the non-linear mixed effects regression analysis. All results were obtained using the nlme package in the R Programming Environment for Data Analysis and Graphics, Version 3.2.3 (21). Statistical significance was set at $p = 0.05$.

Results

Ozone exposure

Mean ozone exposure-level in the chamber throughout the study was 120.9 (SD 1.8) ppb. Outdoor background ozone exposures were examined for exceedances of the EU limit value of 120 $\mu g\ m^3$ (61.1 ppb) within 14 days before study days. Measurements from an urban background station in Aarhus were applied (part of the Danish Air Quality Monitoring Program (22;22;23)) (appendix 1). Only one study day fell outside these criteria. Three participants were investigated on 24th of May 2011, 13 days after an exceedance of 124 $\mu g\ m^{-3}$. Since this was neither a prolonged nor a very high episode, we assume no influence from outdoor ozone concentrations on our study data.

Airway responsiveness to methacholine

44% of the participants experienced a 20% drop in FEV_1 during the unspecific bronchial methacholine challenge test. Of these, 40% dropped below 20% only after the highest dose. The methacholine PD_{20} estimate for all participants was 4.4 (95%- CI 2.8; 6.8) mg methacholine bromide.

SIC for grass and birch allergen

There was no significant difference in baseline FEV_1 between SICs with allergen alone and with co-exposure to ozone (birch, $p = 0.81$; grass, $p=0.96$). 82% and 76% of individuals exposed to birch or grass allergen, respectively, dropped more than 20% in FEV_1 during the SIC; the numbers were 71% and 74% for birch and grass with co-exposure to ozone.

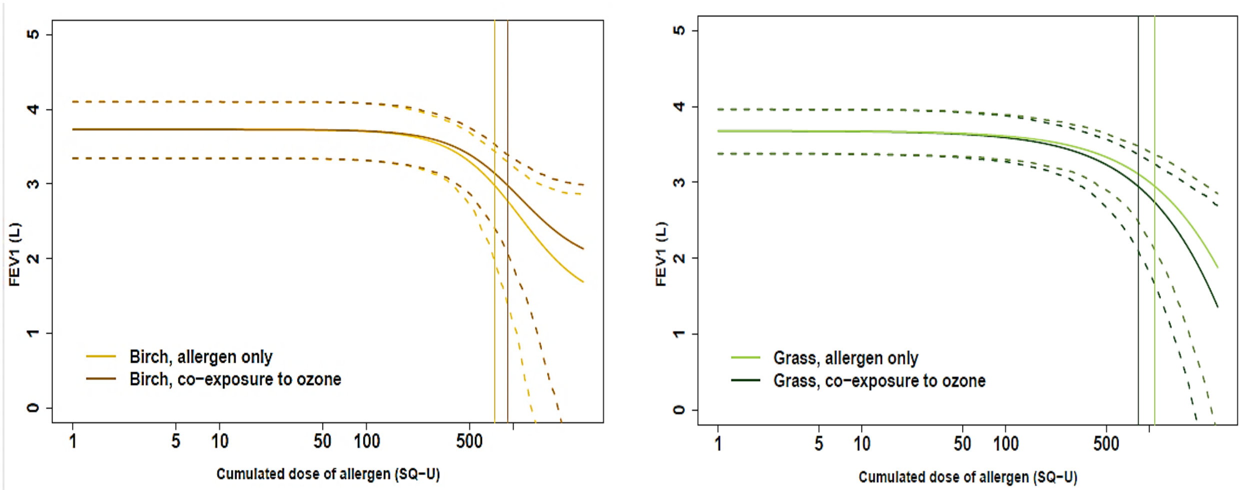

Figure 3 Modelled dose-response relationships for birch (left) and grass (right) with 95% confidence intervals. The vertical lines indicate the modelled PD20 estimates.

The four-parameter logistic model was adapted on all individual SICs providing a good fit to measured data (an example in appendix 2), and no systematic errors in the residuals were seen.

PD_{20} estimates were modelled using all SIC´s assuming 1) no difference between exposures (allergen alone and co-exposure), and 2) allowing for a difference between exposures. No significant difference between the two models was found (birch, p= 0.17; grass, p= 0.08). PD_{20} estimates (95%-CI) for birch were 739 (426-1283) SQ-U for allergen alone and 906 (517– 1590) SQ-U for co-exposure to ozone. For grass PD_{20} estimates were 1092 (517– 2305) SQ-U for allergen alone and 836 (397– 1759) SQ-U for co-exposure to ozone. The validity of assumptions was checked by inspecting plots of residuals against fitted values and QQ-plots of residuals. The modelled FEV_1 by log cumulated dose of allergen curves are shown in Figure 3. For grass, 15 of the 19 participants had lower PD_{20} when co-exposed to ozone. For birch this was true for 9 of the 17 participants. However, differences between PD_{20} for allergen alone and co-exposure to ozone showed no statistical significant findings (birch, p =0.35; grass, p= 0.14).

Association between baseline characteristics and PD_{20}

Table 2 and Figure 4 displays the association between four baseline characteristics and PD_{20} among study participants and PD_{20}.

Only the size of the SPT for grass sensitised participants was clearly associated to PD_{20}. On average a 1 mm larger reaction in SPT resulted in a 27% lower PD_{20}.

	% change in PD_{20}	P
Methacholine	% change in PD_{20} allergen per 10 % change in PD_{20} methacholine	
Birch	2.72 (-2.05 ; 7.71) %	0.288
Grass	1.46 (-4.29 ; 7.55) %	0.634
Skin prick test	% change in PD_{20} per mm change in SPT	
Birch	- 13.12 (-29.57 ; 7.18) %	0.211
Grass	- 27.46 (-40.24 ; 11.94) %	0.0049 *
Number of pos. SPT	% change in PD_{20} per allergy	
Birch	-23.88 (-47.22 ; 9.78) %	0.167
Grass	28.21 (-16.08 ; 95.90) %	0.268
Baseline FEV_1	% change in PD_{20} per (l/sec) change in Baseline FEV1	
Birch	-27.04 (-62.22 ; 41.83) %	0.363
Grass	1.96 (-57.16 ; 142.99) %	0.965

Table 2 Associations between baseline characteristics and allergen PD_{20} ; Methacholine PD_{20}, size of SPT, number of positive SPT and baseline FEV1.

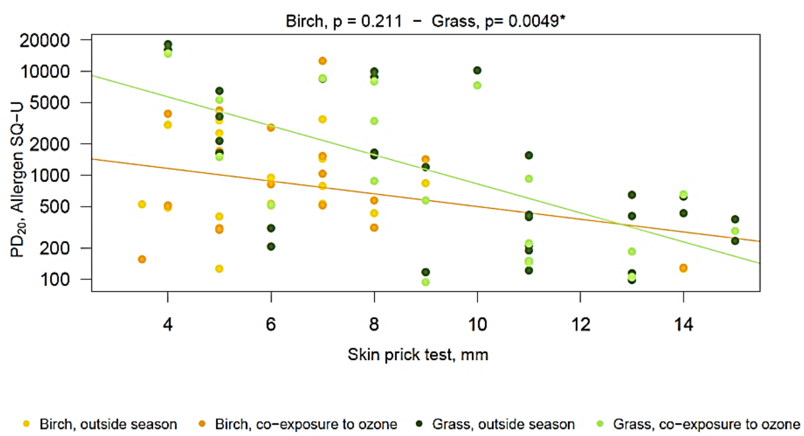

Figure 4 Association between size of skin prick test and PD_{20} for all SIC.

Discussion

In this study we revealed no additional effect of co-exposure to ozone compared to allergen alone for either grass or birch pollen. This was thrue for both the modelled dose response profiles and the modelled PD_{20}. The modelled dose-response curves showed a good fit to individual data, resulting in a reliable estimation of PD_{20} for all SICs.

Ozone and pollen concentrations have been shown to have simultaneous annual peak concentrations. This can be explained by the effect of solar radiation on both the pollen emissions and the ozone formation. Ozone formation increases with sunlight and springtime is high season for intrusions of stratospheric ozone (24). For Denmark a common source of O_3 is the transport of air masses from southern Europe (25). Such episodes take place during situations with high pressure systems over central Europe. The potential effects of simultaneous co-exposure to pollen and O_3 may be higher in central or southern Europe where ozone concentrations exceed levels in Denmark.

Theoretically, any combination of the timing of exposure to pollen and ozone could increase the effect. Exposure to ozone prior to allergen exposure has been shown to induce an increased reaction (5) and also late phase reactions to allergen exposure 4-6 hours after exposure can induce an increased response to ozone exposure (5;26). The effect of prior exposure to ozone could be explained by induced inflammation and mucosal irritation leading to an increased reactivity to the later exposure to allergens (3;5).Exposure to high levels of allergen could also induce inflammation, making the airways more susceptible to a later ozone exposure. High levels of ozone may therefore be of relevance to pollen sensitized individuals both when occurring simultaneous, before or after pollen allergen exposure.

Studies on the effects of pollen exposure can either be conducted in a natural setting, e.g. park studies, or in a more controlled environment in an exposure chamber. When examining the effect of ozone, the exposure chamber does not only provide the possibility of setting a specific ozone level, but also assures elimination of all other exposures which is a strength. On the other hand only short term exposure is feasible to assess in a chamber. One explanation for our negative finding could be the short time exposure to lower levels of ozone. Still our results indicate that short term exposure to ozone levels higher than normally encountered in Denmark do not increase bronchial reactivity pollen sensitized individuals.

Previous studies have shown inconsistent results. Molfino et al (3) found a significant lowering of PC_{15} after exposing 7 asthmatic subject to 120 ppb for 1 h. Jörres et al (5) also found an early bronchoconstrictor response in mild asthmatics, but at higher ozone concentrations of 250 ppb and at exercise. Also Kehrl et al (9) showed an effect on bronchoconstriction in mild asthmatics exposed to 160 ppb during 7 h of exercise. Other studies such as those by Ball (8), Hanania et al (10) and Chen et al (7), did not find significantly higher bronchoconstrictor response to allergen after exposure to ozone. Some suggest that this is due to differences in protocol and levels of ozone. Yet, it has been suggested that there is no lower limit for the effects of ozone on the respiratory system (27). If we assume this to be true, perhaps the effect is not seen after short exposures, but needs to be examined over a longer period, for example in a large scale register based study comparing pollen sensitized persons living under different ozone exposures. Also, the effect of prior exposures to prolonged and high allergen exposure, inducing BHR, could be relevant, for example persons with allergies towards indoor allergens (9).

The only baseline characteristic among participants associated to PD_{20} was the size of the SPT for grass. The negative finding for bronchial reactivity was unexpected. Barnig et al (28) found a clear correlation between PD_{20} for grass allergen and methacholine reactivity for 27 allergic subjects. Among our participants only 44% had a 20% or greater drop in FEV_1 during the methacholine bronchial challenge test. The PD_{20} estimate of 4.4 (SD 2.9; 6.8) mg methacholine bromide is almost the maximum administered dose. We can therefore not exclude that our negative finding is caused by a relatively mild unspecific

bronchial reactivity among our participants. Furthermore a more reliable methacholine PD_{20} could have been modelled if we have used higher concentrations of methacholine.
In general our participants constitute a highly homogenous group which can also serve as an explanation for the apparently low impact of the baseline characteristics. A homogenious group was prioritized as the main objective of the study was to evaluate the effect of ozone on allergen responsiveness in sensitised but otherwise largely healthy participants. None of the participants used asthma medication, and all had a normal baseline lung function. Though, almost half of the participants were hyper-responsive, a feature associated to, but not synonymous of asthma (29). For safety reasons, most studies are performed on mild asthmatics, however the more severely affected may have very different and greater responses (7;9) which can not be evaluated in the current study.

The applied model for the log-dose response curves were based on predicted values from the whole individual curve adjusting for all information added on all curves. In previous studies PD_{20} was estimated solely from the last two measurements. The modelled PD_{20} clearly showed individual differences in the response, but it appears from the model that there is a typical pattern of response, similar for both birch and grass pollen with and without co-exposure to ozone. The width of the CI increased with increasing exposure; however with a high percentage of participants decreasing more than 20% in FEV_1 during challenges, modelled PD_{20} was based on a reliable model. The curves are robust until reaching PD_{20}-levels, where the estimates become more uncertain. To the author's knowledge, these log-dose response curves for grass and birch allergen, showing similar response patterns independent of ozone, have not previously been shown.

We applied a protocol that allowed for doses to be administered according to the reactions, primarily for safety reasons, and in order to avoid exhaustion of the participants. Earlier studies (3;7) also allowed for individual differences to affect the dose administered, by calculating the theoretical allergen concentration needed to elicit a 15% drop in FEV_1, and applied this to evaluate the start dose of allergen for the challenge. They assumed a correlation between the size of the SPT and the PC_{15}, a correlation confirmed in this study.

The maximum dose of allergen provided should be high enough to elicit a response in all sensitized subjects (30). However, due to an assumption in our protocol of no response to allergen at a less than 5% drop at 1400 SQ-U, 8 participants were not given the maximum dose. We may thereby have lost information about a potential rapid drop in FEV_1 at this dose, which could have contributed to the resulting modelled curve. The applied protocol was originally developed for clinical application, and did therefore not take this into account. We recommend in future studies all participants should be given the maximum dose, if their FEV1 is more that 80% of the initial value.

The study was originally designed as a double blinded randomised study, but due to a change in the setup, the initial 13 extra SIC´s with solely grass allergen were performed in the small chamber, while participants were seated in the large, and the exposure was not blinded to the investigators at these times. This could have resulted in an unintentional bias towards measurements, however, since no effect of the co-exposure to ozone is seen, no such bias is assumed.

Conclusions

In this study we found no difference in bronchoconstriction measured as modelled dose response profiles and modelled PD_{20} between solely allergen exposure and co-exposure to allergen and low level ozone. Of the baseline characteristics of the participants only the size of the grass skin prick test showed a significant association with PD_{20}. We demonstrate that a non-linear four-parameter logistic model provides a good fit to the measured data, allowing for a calculation of PD_{20} that is based on all points on the dose-response curve. This method also allows for the estimation of a theoretic PD_{20} for those participants that do not drop 20% during the SIC. Modelled dose-response curves for specific inhalation challenge show a similar pattern for grass and birch allergen. Participants in this study were only short time exposured to low levels of ozone, but still our results indicate that exposure to ozone a limited amount of time at levels normally encountered in Denmark do not increase bronchial reactivity in pollen sensitized individuals.

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Contributors

PVØ, corresponding author, has contributed to the experimental study, the analysis, full-text screening, drafting the article and final approval of the version to be published. JB, TS and VS have contributed to the experimental study, revising the article critically for important intellectual content and final approval of the version to be published. PR has contributed to the experimental study, revising the article critically for important technical content and final approval of the version to be published. OH has contributed to revising the article critically for important intellectual content and final approval of the version to be published. BB has contributed to the statistical analysis, revising the article critically for important intellectual content and final approval of the version to be published. PVØ and VS are responsible for the overall content as guarantor(s).

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Ref Type: Generic

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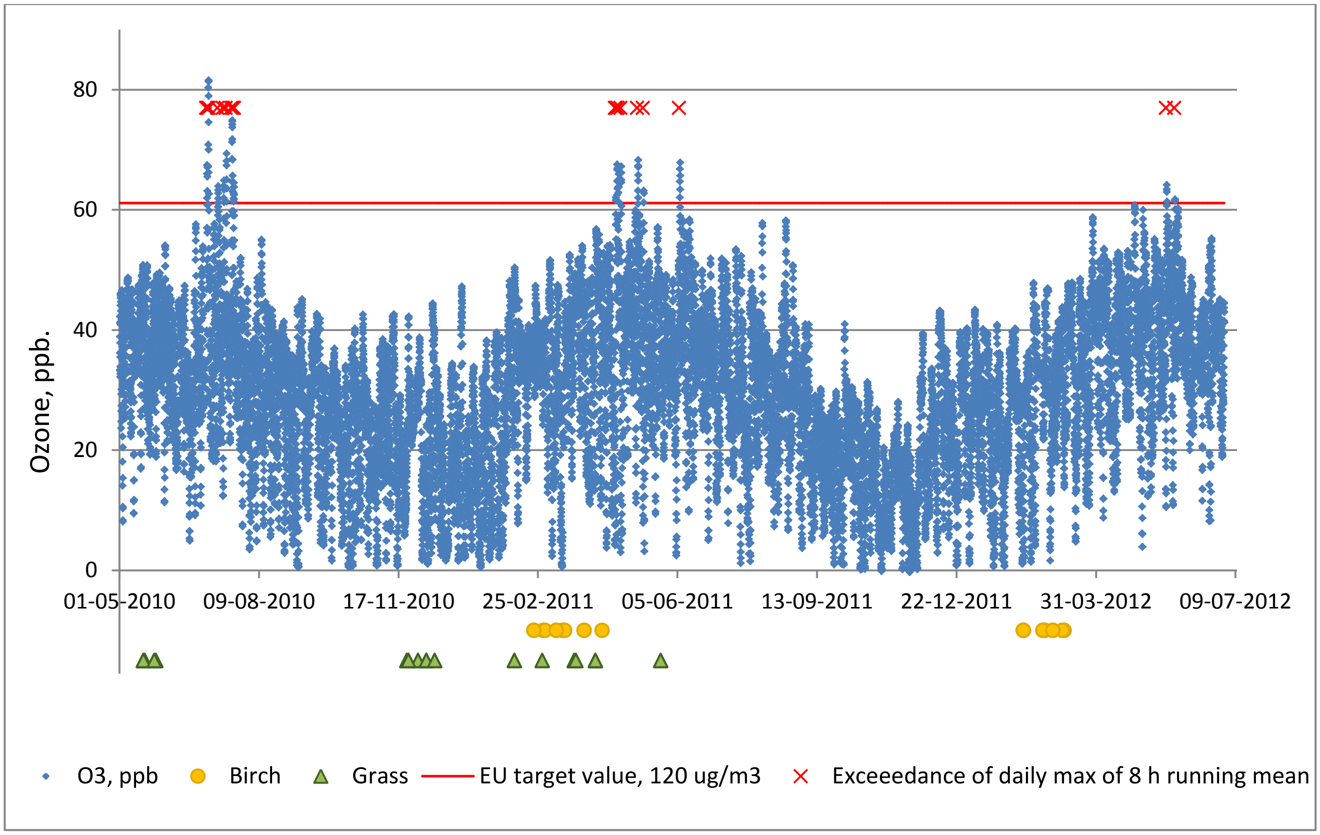
Appendix

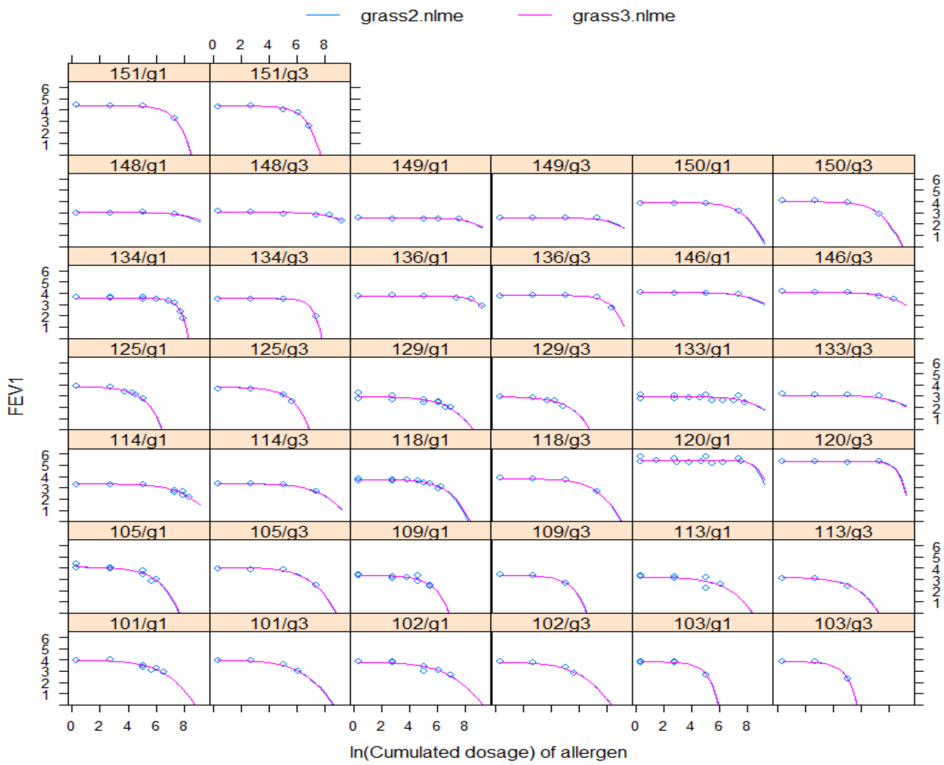
Appendix 1

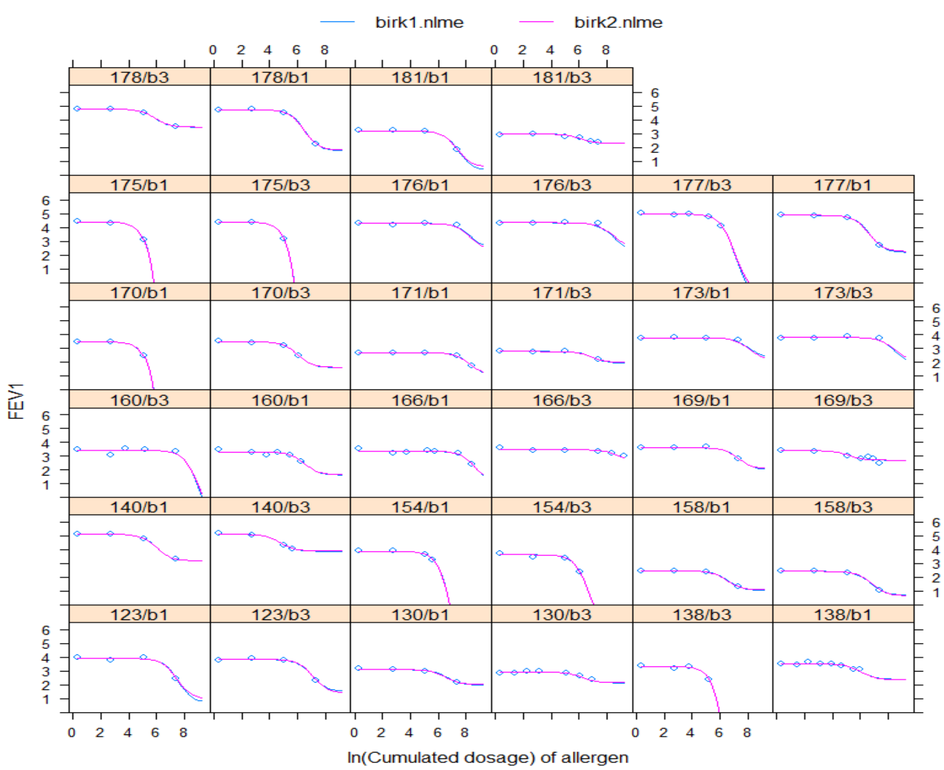
Ambient ozone concentrations measured at Valdemarsgade in Aarhus. Measurements are part of the Danish Air Quality Monitoring Program. Half hourly data is shown as well as indications of exceedances and the timing of the individual study days.



Appendix 2

Modelled dose-response curves for all SIC and measurements as circles. The two different curves in each chart show the model result for the combined dataset (blue) and the model run for data seperated into co-exposure to ozone and allergen alone (pink). The modelled curves fit data well, and the two curves are allmost identical.





Co-author statements

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Declaration of co-authorship

Full name of the PhD student: Pia Viuf Ørby

This declaration concerns the following article/manuscript:

Title:	Identifying urban sources as cause of elevated grass pollen concentrations using GIS and remote sensing
Authors:	C. A. Skjøth, P. V. Ørby, T. Becker, C. Geels, V. Schlüunssen, T. Sigsgaard, J. H. Bønløkke, J. Sommer, P. Søgaard, and O. Hertel.

The article/manuscript is: Published ☐ Accepted ☒ Submitted ☐ In preparation ☐

If published, state full reference: Skjøth CA, Ørby PV, Becker T, Geels C, Schlünssen V, Sigsgaard T, Bønløkke JH, Sommer J, Søgaard P and Hertel O. Identifying urban sources as cause of elevated grass pollen concentrations using GIS and remote sensing. *Biogeosciences* 10: 541-554, 2013.

If accepted or submitted, state journal: Biogeosciences

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

No ☒ Yes ☐ If yes, give details:

The PhD student has contributed to the elements of this article/manuscript as follows:

A. No or little contribution
B. Has contributed (10-30 %)
C. Has contributed considerably (40-60 %)
D. Has done most of the work (70-90 %)
E. Has essentially done all the work

Element	Extent (A-E)
1. Formulation/identification of the scientific problem	A
2. Planning of the experiments and methodology design and development	A
3. Involvement in the experimental work/clinical studies	B
4. Interpretation of the results	B
5. Writing of the first draft of the manuscript	B
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The article/manuscript is: Published ■ Accepted ☒ Submitted ☐ In preparation ☐

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Seasonal variation in diurnal atmospheric grass pollen concentration profiles, R. G. Peel, P. V. Ørby, C. A. Skjøth, R. Kennedy, V. Schlünssen, M. Smith, J. Sommer, and O. Hertel., Biogeosciences, 11, 821-832, 2014, doi:10.5194/bg-11-821-2014

If accepted or submitted, state journal: Biogeosciences.

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

No ☐ Yes ☒ If yes, give details: Part of the material described in this article, but not all, was also used in Chapter 5 in the dissertation of Robert G. Peel; Towards the integrated assessment of human exposure to grass pollen in urban environments, 2013, University of Worcester in collaboration with the Department of Environmental Science, Aarhus University.

The PhD student has contributed to the elements of this article/manuscript as follows:

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E. Has essentially done all the work

Element	Extent (A-E)
1. Formulation/identification of the scientific problem	C
2. Planning of the experiments and methodology design and development	C
3. Involvement in the experimental work/clinical studies	B
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5. Writing of the first draft of the manuscript	B
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This declaration concerns the following article/manuscript:

Title:	Cluster analysis of variations in the diurnal pattern of grass pollen concentrations in Northern Europe (Copenhagen) and Southern Europe (Córdoba)
Authors:	P. Alcázar, P. V. Ørby, J. Oteros, C. A. Skjøth, O. Hertel, C. Galán

The article/manuscript is: Published ☐ Accepted ☐ Submitted ☒ In preparation ☐

If published, state full reference:

If accepted or submitted, state journal: Aerobiologia

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

No ☒ Yes ☐ If yes, give details:

The PhD student has contributed to the elements of this article/manuscript as follows:

A. No or little contribution
B. Has contributed (10-30 %)
C. Has contributed considerably (40-60 %)
D. Has done most of the work (70-90 %)
E. Has essentially done all the work

Element	Extent (A-E)
1. Formulation/identification of the scientific problem	D
2. Planning of the experiments and methodology design and development	C
3. Involvement in the experimental work/clinical studies/data collection	C
4. Interpretation of the results	C
5. Writing of the first draft of the manuscript	C
6. Finalization of the manuscript and submission	C

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Declaration of co-authorship

Full name of the PhD student: Pia Viuf Ørby

This declaration concerns the following article/manuscript:

Title:	The effect of seasonal priming on birch and grass allergen specific inhalation challenges among persons with allergic rhinitis
Authors:	Ørby, P. V., Bonløkke, J. H., Bibby, B. M., Ravn, P., O. Hertel, Sigsgaard, T., Schlünssen, V.

The article/manuscript is: Published ☐ Accepted ☐ Submitted ☒ In preparation ☐

If published, state full reference:

If accepted or submitted, state journal: Clinical and Experimental Allergy

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

No ☒ Yes ☐ If yes, give details:

The PhD student has contributed to the elements of this article/manuscript as follows:

A. No or little contribution
B. Has contributed (10-30 %)
C. Has contributed considerably (40-60 %)
D. Has done most of the work (70-90 %)
E. Has essentially done all the work

Element	Extent (A-E)
1. Formulation/identification of the scientific problem	C
2. Planning of the experiments and methodology design and development	C
3. Involvement in the experimental work/clinical studies/data collection	C
4. Interpretation of the results	D
5. Writing of the first draft of the manuscript	E
6. Finalization of the manuscript and submission	E

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This declaration concerns the following article/manuscript:

Title:	An assesment of the potential for co-exposure to allergenic pollen and air pollution in copenhagen, Denmark
Authors:	Ørby, P. V., Peel, R. G., Skjøth, C. A., Schlünssen, V., Bønløkke, J. H., Ellermann, T., Brændholt, A., Sigsgaard, T., Hertel, O.

The article/manuscript is: Published ☐ Accepted ☒ Submitted ☐ In preparation ☐

If published, state full reference: An assessment of the potential for co-exposure to allergenic pollen and air pollution in Copenhagen, Denmark. P.V. Ørby,R.G. Peel,C. Skjøth,V. Schlünssen,J.H. Bønløkke,T. Ellermann,A. Brændholt,T. Sigsgaard,O. Hertel, Urban Climate, Volume 14, Part 3, December 2015, Pages 457–474,New Sensing Technologies and Methods for Air Pollution Monitoring.

If accepted or submitted, state journal: Urban Climate

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

No ☒ Yes ☐ If yes, give details:

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B. Has contributed (10-30 %)
C. Has contributed considerably (40-60 %)
D. Has done most of the work (70-90 %)
E. Has essentially done all the work

Element	Extent (A-E)
1. Formulation/identification of the scientific problem	D
2. Planning of the experiments and methodology design and development	D
3. Involvement in the experimental work/clinical studies	C
4. Interpretation of the results	D
5. Writing of the first draft of the manuscript	E
6. Finalization of the manuscript and submission	E

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This declaration concerns the following article/manuscript:

Title:	Bronchoconstriction among pollen sensitized persons during co-exposure to pollen allergen and ozone
Authors:	Ørby, P. V., Bønløkke, J. H., Bibby, B. M., Ravn, P., O. Hertel, Sigsgaard, T., Schlünssen, V.

The article/manuscript is: Published ☐ Accepted ☐ Submitted ☒ In preparation ☐

If published, state full reference:

If accepted or submitted, state journal: Allergy

Has the article/manuscript previously been used in other PhD or doctoral dissertations?

No ☒ Yes ☐ If yes, give details:

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Element	Extent (A-E)
1. Formulation/identification of the scientific problem	C
2. Planning of the experiments and methodology design and development	C
3. Involvement in the experimental work/clinical studies/data collection	C
4. Interpretation of the results	D
5. Writing of the first draft of the manuscript	E
6. Finalization of the manuscript and submission	E

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