The interaction of Pollution, Meteorology and Bioaerosols: Implications on Human Health.

By

MAHMOOD MARLIYYAH ABDULLAHI

School of Geography, Earth and Environmental Sciences, University of Birmingham,
United Kingdom.

This dissertation is submitted for the degree of

Doctor of Philosophy

August 2017
ABSTRACT

The global incidence of allergic reaction has been rising for years, especially within westernised urban areas with the underlying reasons still unclear. There is strong evidence that the interaction between pollen grains, environmental pollution and meteorological change is increasing the allergenicity of the pollen grain and consequently, increasing the misery of hay fever sufferers. Recent laboratory experiments have shown that the interaction of pollen with atmospheric oxidants such as ozone (O₃) and nitrogen dioxide (NO₂) can alter protein molecules that are present within pollen grains via post-translational modification (PTM) of the protein.

Within the laboratory, key allergenic birch pollen was exposed to atmospherically relevant exposures of gas phase NO₂ and O₃ under a range of environmentally relevant conditions (temperature and relative humidity RH). The effects of the exposures on the biochemistry of the pollen grains were probed using a proteomic approach. The morphological changes of unexposed and exposed pollen samples to RH, rainwater and NO₂, where observed under fluorescence microscopy and scanning electron microscope (SEM). The discoveries suggest that interaction between gas pollutants and pollen do exist and cause protein specific modifications; in particular, nitration that occurs on tyrosine residues. These observations suggest a possible reason for increased allergies in reaction to such chemically altered protein.

Secondly, a detailed analysis of London Ambulance data is presented and compared to London temperature data recorded at a central London site (St James Park). The baseline relationships established in this work will allow for the prediction of likely changes in ambulance demand (and illness types) that will be caused by seasonal temperature changes and increased frequency and intensity of extreme weather events, due to climate change, in the future.

Lastly, the study applied statistical analyses (time series and odd ratio) to examine short-term associations between birch pollen count with allergic related illnesses recorded in the London Ambulance data, temperature and NO₂.
DECLARATION

I hereby declare, that except where specific reference is made to the work of others, the contents of this thesis are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This thesis is my own work and contains nothing, which is the outcome of work done in collaboration with others, except as specified in the text and acknowledgements. This thesis contains fewer than 50,000 words excluding appendices, references, tables and has fewer than 150 figures. The model used in Chapter 2 of this research was partly developed by Dr. Francis Pope and the discussions include input from both Dr. Francis Pope and Prof. John Thornes.

The work presented in Chapter 1 has been published and is available online in the journal Climate:

**Impact of air temperature on London ambulance call-out incidents and response times**
Marliyyah Mahmood, John Thornes, Francis Pope, Paul Fisher, Sotiris Vardoulakis


The work presented in Chapters 3 and 4 forms the basis of a publication currently in preparation for the journal Atmospheric Measurement Techniques

**Detection and quantification of birch pollen nitration using multiple techniques**
Mahmood, M., Pope F., Grant M., Pasha S.

Additional work done in collaboration with University of Worcester not discussed in this thesis is also currently being prepared for publication (Comparison of untreated stock birch pollen (*Betula pendula*) against pollutant treated determined by ELISA).

Some of the work in Chapters 2 and 4 have been presented in several conferences:

1. **Superior Ambulance Rate Forecasting Using Meteorological Data**

2. **Superior Ambulance Call Out Rate Forecasting Using Meteorological Data**
   Marliyyah A. Mahmood, Francis Pope, William Bloss and John Thornes *Royal Meteorological Society Student Conference, Birmingham, UK (1st -3rd July, 2015), (Oral presentation).*
3. **Posttranslational modification of Birch and Ragweed allergen proteins by common gas phase pollutants, NO₂ and O₃**  
Mahmood, M. A., Pope, F., Bloss, W.  
*American Geophysical Union, Fall Meeting 14th – 18th December, 2015, abstract #A21A-0111. (Poster presentation).*

4. **Superior Ambulance Call Out Rate Forecasting Using Meteorological Data**  
Mahmood, M. A.; Thornes, J. E.; Bloss, W.; Pope, F.  
*American Geophysical Union, Fall Meeting 14th – 18th December, 2015, abstract #NH51A-1851. (Poster presentation).*

5. **Posttranslational modification of bioaerosol protein by common gas pollutants: NO₂ and O₃**  
Abdullahi Mahmood, Marliyyah; Bloss, William; Pope, Francis  
*EGU General Assembly 2016, held 17th -22nd April 2016 in Vienna Austria, p.725. (Poster presentation).*

6. **Posttranslational modification of Birch protein by common gas pollutants: NO₂ and O₃**  
Marliyyah A. Mahmood *, William J. Bloss and Francis D. Pope  
*Chemistry in the Urban Atmosphere: Faraday Discussion, 6th - 8th April, 2016, London, United Kingdom. (Poster presentation).*

7. **Resilience of urban ambulance services under future climate, meteorology and air pollution scenarios**  
Pope, Francis; Chapman, Lee; Fisher, Paul; Mahmood, Marliyyah; Sangkharat, Kamolrat; Thomas, Neil; Thornes, John  
*19th EGU General Assembly, EGU2017, proceedings from the conference held 23th - 28th April, 2017 in Vienna, Austria., p.1775. (Poster presentation).*

8. The study has been presented in the 3MT (3 minutes thesis competition):  
https://www.youtube.com/watch?v=plON3yjJxV0

9. And lastly, a summary (600 words) of the thesis has been published in the Birmingham Brief articles on Birmingham brief publication website:  

Marliyyah A. Mahmood  
August 2017
DEDICATION

In loving memory of our mother, whom I never got to know (May her gentle soul continue to rest in peace) and the most wonderful and exceptional dad who has always stood by me. To my remarkable husband, adorable children and amazing siblings for their infinite love, prayers and support.

"For surely if it was not for the grace of God on you and His mercy, you would have been among the losers" (Al-Baqarah 2:64).
ACKNOWLEDGEMENTS

All praise and gratitude is due to Almighty Allah for His infinite blessings, for seeing me through everything in my life and outdoing each struggle.

My sincere gratitude goes to my remarkable supervisors: Dr Francis Pope for his professional guidance, understanding, availability, patience, encouragement, and believing in me, working with you was indeed a privilege and Prof William Bloss for his time and valuable advice in order to accomplish this research work. Francis’ supervision helped me in all the time of my research and writing of this thesis. Through him, I have learnt how to do a lot of great stuff! It was a real pleasure to be under the supervision of these great gentlemen and forever remain indebted.

I am so grateful to many university staff for their unfailing support and assistance, especially Gretchen, Jamie and Richard, the Petroleum Technology Development Fund for funding the PhD research and Nigerian Nuclear Regulatory Authority for allowing me to pursue this study.

I would like to extend my gratitude to all my PhD colleagues, especially colleagues in Room 412 and Nick who were always willing to help. It was an honour to work in your presence and learn from you all. Without all of your help and kindness it would have been much more challenging. To all my friends, cousins (especially Ma’asoom), uncles, aunties, nieces, nephews and all family members, I appreciate the honourable support, visit and prayers throughout my journey.

My sincere thanks also go to Dr. Melissa Grant, Sabah and Jinglei who helped me in amending several protocols and results interpretation; and Laine and Norman who assisted in the early days of my lab work. They all assisted in providing friendly and conducive atmosphere for the work amidst them in the two laboratories (Biosciences and Genomics) and with the use of their supplies. Thanks to Alex for his time and help in fluorescence microscopy analysis.
Nobody has been more important to me in the pursuit of this project than the members of my family who provide unending inspiration. Without their precious support and prayers, it would not have been possible.

My hearty thanks go to my beloved husband, Lawal Jibrin Ahmed for his love, prayers, patience and sacrifices, the biggest of which was for him to be away from his family. Words cannot express how grateful I am. I thank him so much for making us happy and supporting us in all our needs. The company of our three remarkable children: Abdullah Ayman, Ruqayyah Bahirah, and Rahmah Bariah made me more focused in pursuing this work. They have remained a source of happiness throughout my life and this incredible journey. Very understanding, helpful and loving children, I am so proud of them. Thanks for being wonderful! Special and genuine thanks go to my dear stepmothers: Aunty Sugra (Hafsat) and Aunty Hadiza for their precious support, advice, encouragement, praises, prayers and patience throughout my life. Thanks for looking after our children and me! I would also like to extend my unique thanks to all my in-laws (especially my Father in-law, May his soul continues to rest in peace) for their continuing support, encouragement and prayers. I am indebted to the most magnificent and supportive sibling: Yaya Muslim, Adda Tabia, Ya Husna, Yaya Labib, Yaya Ali, Zaheed, Thamina and Saadiq for their endless love, help, prayers and unconditional support throughout this journey and my life in general. I owe everything and cannot express my love and gratitude towards you, I thank you from the bottom of my heart. My utmost appreciation goes to the best and adorable daddy, Engr. Abdullahi Mahmood for his unconditional love, time, patience, prayers and his continuous support not only during this PhD but also throughout my life. For worrying and listening to me and helping out with our children always. With you by my side, I am scared of nothing. Words can’t express my gratitude. I am glad I have a father like you. I can’t thank you all enough.

I am so grateful to all for their precious support, phone calls and visits.

Thanks for all your love, encouragement and prayers!
# Table of Contents

## CHAPTER INTRODUCTION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 Background</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Pollution</td>
<td>3</td>
</tr>
<tr>
<td>1.1.1 Air pollution</td>
<td>3</td>
</tr>
<tr>
<td>1.1.2 Pollutants</td>
<td>7</td>
</tr>
<tr>
<td>1.2 Bioaerosols</td>
<td>13</td>
</tr>
<tr>
<td>1.2.1 Pollen</td>
<td>14</td>
</tr>
<tr>
<td>1.2.2 Birch pollen</td>
<td>15</td>
</tr>
<tr>
<td>1.2.3 Pollen allergy</td>
<td>18</td>
</tr>
<tr>
<td>1.2.4 Allergenic protein release of pollen grain</td>
<td>19</td>
</tr>
<tr>
<td>1.2.5 Pollen protein nitration</td>
<td>20</td>
</tr>
<tr>
<td>1.3 Climate change</td>
<td>21</td>
</tr>
<tr>
<td>1.3.1 Climate change and ambulance operations</td>
<td>22</td>
</tr>
<tr>
<td>1.4 Air pollution, climate change, pollen and human health</td>
<td>23</td>
</tr>
<tr>
<td>1.5 The Research Questions</td>
<td>27</td>
</tr>
<tr>
<td>1.6 Aims and objectives</td>
<td>27</td>
</tr>
<tr>
<td>1.7 Hypotheses</td>
<td>29</td>
</tr>
<tr>
<td>1.8 The Scope and Limitation of the Research</td>
<td>29</td>
</tr>
<tr>
<td>1.9 Thesis structure</td>
<td>30</td>
</tr>
</tbody>
</table>

## CHAPTER 2 IMPACT OF AIR TEMPERATURE ON LONDON AMBULANCE CALL-OUT INCIDENTS AND RESPONSE TIMES

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 Abstract</td>
<td>32</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>33</td>
</tr>
<tr>
<td>2.1.1 London weather</td>
<td>34</td>
</tr>
<tr>
<td>2.1.2 London Ambulance Service</td>
<td>36</td>
</tr>
<tr>
<td>2.1.3 LAS Ambulance Call out Categories</td>
<td>38</td>
</tr>
<tr>
<td>2.2 Related studies</td>
<td>40</td>
</tr>
<tr>
<td>2.3 Methodology</td>
<td>44</td>
</tr>
<tr>
<td>2.3.1 Data sets</td>
<td>44</td>
</tr>
<tr>
<td>2.3.2 Data analysis</td>
<td>45</td>
</tr>
<tr>
<td>2.3.2.1 Time series analysis</td>
<td>46</td>
</tr>
<tr>
<td>2.3.2.3 Air temperature versus ambulance response time</td>
<td>47</td>
</tr>
<tr>
<td>2.4 Results and Discussion</td>
<td>48</td>
</tr>
<tr>
<td>2.4.1 De-trending effect</td>
<td>49</td>
</tr>
<tr>
<td>2.4.2 Long-term increase in ambulance usage</td>
<td>50</td>
</tr>
<tr>
<td>2.4.3 The influence of air temperature on ambulance response times</td>
<td>52</td>
</tr>
<tr>
<td>2.4.4 Basic relationship between temperature, RH and respiratory chest infection</td>
<td>52</td>
</tr>
<tr>
<td>2.4.5 Ambulance Illness codes versus Air Temperature</td>
<td>54</td>
</tr>
<tr>
<td>2.4.6 Weekly correlation between temperature and illness codes</td>
<td>56</td>
</tr>
<tr>
<td>2.4.7 Daily temperature time lag effects on illness codes</td>
<td>56</td>
</tr>
<tr>
<td>2.4.8 Illness Codes versus winter and summer seasons</td>
<td>61</td>
</tr>
<tr>
<td>2.4.9 Forecasting of ambulance call out rates</td>
<td>64</td>
</tr>
<tr>
<td>2.5 Conclusion</td>
<td>67</td>
</tr>
</tbody>
</table>

## CHAPTER 3 LABORATORY ANALYSES

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 Introduction</td>
<td>72</td>
</tr>
<tr>
<td>3.1 Methodology</td>
<td>73</td>
</tr>
<tr>
<td>3.1.1 Materials</td>
<td>74</td>
</tr>
<tr>
<td>3.1.1.1 Pollen samples</td>
<td>74</td>
</tr>
</tbody>
</table>
CHAPTER 4 DETECTION AND QUANTIFICATION OF BIRCH POLLEN NITRATION

4.0 Synopsis ................................................................. 108
4.1 Introduction .............................................................. 109
  4.1.1 Biochemistry of PTM .................................................. 112
  4.1.2 Challenges in charactering post-translational modification of pollen .... 112
  4.1.3 Impact of O₃ exposure on pollen grain ................................ 114
  4.1.4 Impact of NOₓ exposure to the pollen grain ...................... 115
4.2 Results and Discussion ................................................. 116
  4.2.1 Summary of technical and experimental replicates .................. 117
  4.2.2 Protein concentration ............................................... 118
  4.2.3 SDS-PAGE gel separation ......................................... 118
  4.2.4 Trypsin digestion .................................................. 121
  4.2.5 Protein/Peptide identification ..................................... 122
  4.2.6 Pollen rupture and Gas exposure .................................. 133
  4.2.7 Dot blot ............................................................ 136
  4.2.8 Western blot ....................................................... 143
  4.2.9 MS analysis ....................................................... 144
  4.2.10 In-gel digestion .................................................. 146
  4.2.11 TMT labelling experiment outcome ................................ 150
4.3 Conclusion ............................................................. 158

CHAPTER 5 POLLEN GRAIN IMAGING ........................................... 161
5.0 Synopsis ................................................................. 162
5.1 Objectives ............................................................... 162
5.2 Introduction ............................................................. 163
  5.2.1 Auto fluorescence .................................................. 164
  5.2.2 Fluorescence microscopy (FM) ..................................... 166
## Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>–NO₂</td>
<td>Nitro group</td>
</tr>
<tr>
<td>3NT</td>
<td>3-nitrotyrosine</td>
</tr>
<tr>
<td>A &amp; E</td>
<td>Accident and emergency</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile acid</td>
</tr>
<tr>
<td>AGC</td>
<td>Automatic gain control</td>
</tr>
<tr>
<td>AMPDS</td>
<td>Advanced Medical Priority Dispatch System</td>
</tr>
<tr>
<td>BALM</td>
<td>Birmingham Advance Light Microscopy</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Ca.</td>
<td>Circa</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>CO</td>
</tr>
<tr>
<td>Carboxyhemoglobin</td>
<td>COHb</td>
</tr>
<tr>
<td>Cat A</td>
<td>Category A</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan Fluorescent Protein</td>
</tr>
<tr>
<td>ChA</td>
<td>Chlorophyll A</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CR</td>
<td>Crushed</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>FR</td>
<td>Flow rates</td>
</tr>
<tr>
<td>FM</td>
<td>Fluorescence microscopy</td>
</tr>
<tr>
<td>GP</td>
<td>General practitioner</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>Sulphuric acid</td>
</tr>
<tr>
<td>HCD</td>
<td>High energy collisional dissociation</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>LAS</td>
<td>London Ambulance Service</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption</td>
</tr>
<tr>
<td>MFC</td>
<td>Mass flow controller</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>ND</td>
<td>Nitration degree</td>
</tr>
<tr>
<td>NHS</td>
<td>National Health Service</td>
</tr>
<tr>
<td>NitroY</td>
<td>Tyrosine nitration</td>
</tr>
<tr>
<td>NOx</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO₂</td>
<td>Nitrogen dioxide</td>
</tr>
<tr>
<td>NOx</td>
<td>Nitrogen oxides</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>O₃</td>
<td>Ozone</td>
</tr>
<tr>
<td>OECD</td>
<td>Organisation for Economic Co-operation and Development</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PALMs</td>
<td>Pollen-derived lipid mediators</td>
</tr>
<tr>
<td>PBA</td>
<td>Primary biological aerosols</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBAP</td>
<td>Primary biological aerosol particles</td>
</tr>
<tr>
<td>Phe</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>PM</td>
<td>Particulate matter</td>
</tr>
<tr>
<td>PSM</td>
<td>Peptide spectrum match</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modifications</td>
</tr>
<tr>
<td>PTN</td>
<td>Protein tyrosine nitration</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen species</td>
</tr>
<tr>
<td>ROIs</td>
<td>Reactive oxygen intermediates</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTIs</td>
<td>Respiratory tract infection</td>
</tr>
<tr>
<td>RW</td>
<td>Rainwater</td>
</tr>
<tr>
<td>SAR</td>
<td>Seasonal allergic rhinitis</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE SDS</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SJP</td>
<td>St James Park</td>
</tr>
<tr>
<td>SO</td>
<td>Sulfur oxide</td>
</tr>
<tr>
<td>SO₂</td>
<td>Sulfur dioxide</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with Tween 20</td>
</tr>
<tr>
<td>TEAB</td>
<td>Triethylammonium bicarbonate</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic</td>
</tr>
<tr>
<td>TMT</td>
<td>Tandem Mass Tag</td>
</tr>
<tr>
<td>TNM</td>
<td>Tetranitromethane</td>
</tr>
<tr>
<td>Trizma-HCl</td>
<td>Hydrochloride</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>TSP</td>
<td>Total soluble protein</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UNEP</td>
<td>United Nations Environment Programme</td>
</tr>
<tr>
<td>USEPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VOC</td>
<td>Volatile organic compounds</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
## Symbols

- °C: Celsius
- °F: Fahrenheit
- Da: Daltons
- kDa: Kilodaltons
- L/min⁻¹: Liters per minute
- mg: Milligram
- mg/m³: Milligram per Cubic Meter
- ml: Millilitre
- mm: Millimeter
- mM: Millimolar
- nm: Nanometer
- pH: Potential of hydrogen
- pKa: Ionization constant
- ppb: Parts Per Billion
- ppm: Parts Per Million
- R²: R-squared
- sccm: Standard cubic centimeters per minute
- w/w: weight/weight
- µg: Microgram
- µg/m³: Micrograms per Cubic Meter
- µg/ml: Microgram per milliliter
- µl: Microliters
List of figures

Figure 1.1: Interrelationships between bioaerosol, pollution and meteorology................................. 1
Figure 1.2: The route of pollution from its source to human health and environment......................... 3
Figure 1.3: Sources of primary and secondary pollutants................................................................. 7
Figure 1.4: Schematic drawing of causes and effects of air pollution............................................... 11
Figure 1.5: Characteristic size ranges of atmospheric particles and bioaerosols with exemplary illustrations................................................................................................................................. 14
Figure 1.6: Description of the pathways through which climate parameters and air pollutants can influence the release, potency, and effects of allergens................................................................. 26
Figure 1.7: Thesis structure................................................................................................................ 30
Figure 1.8: London Ambulance Service on response to emergency calls.......................................... 31
Figure 1.9: Location of London in United Kingdom.......................................................................... 33
Figure 1.10: Histograms of London temperatures............................................................................ 35
Figure 1.11: London Ambulance 999 calls 2005/06 – 2014/15................................................................................. 37
Figure 1.12: Cumulative plot of the Cat A illnesses showing top 10 and 20 categories making up ~50% and ~70% of the entire call outs, respectively................................................................. 39
Figure 1.13: Seasonal percentage differences in ambulance call-out rates for the top ten categories................................................................................................................................. 49
Figure 1.14: Scatter plots for a negative temperature dependence (respiratory chest infection), no temperature dependence (abdominal pains) and positive temperature dependence (alcohol related) using de-trended data sets............................................................................................................ 57
Figure 1.15: R squared values plots of the top 10 illnesses against time lag within 0 -15 days........... 63
Figure 1.16: Time series series data of Respiratory chest infection before (A) and after de-trending (B) respectively ............................................................................................................................. 71
Figure 1.17: Scatter plots of daily de-trended data for Respiratory /chest infection and total of the entire top twenty illnesses against mean temperature............................................................................................. 78
Figure 1.18: Scatter plots for a negative temperature dependence (respiratory chest infection), no temperature dependence (abdominal pains) and positive temperature dependence (alcohol related) using de-trended data sets............................................................................................................ 84
Figure 1.19: R squared values plots of the top 10 illnesses against time lag within 0 -15 days........... 91
Figure 1.20: Seasonal percentage differences in ambulance call-out rates for the top ten categories................................................................................................................................. 94
Figure 1.21: Forecast prediction of 2012/2013 respiratory/chest infection call out rate, RH and Temperature ................................................................................................................................................................................................. 98
Figure 1.22: Flow diagram of the laboratory techniques used........................................................... 102
Figure 1.23: Experimental setup for pollen grain exposure............................................................... 106
List of Table

Table 1.1: Global updated WHO Air Quality Guidelines ................................................................. 12
Table 2.1: Correlation between temperature and the top 10 of the medical categories .................. 58
Table 3.1: Buffers and reagents used for experimental analyses .................................................. 76
Table 3.2: Conditions used for some experimental exposures ...................................................... 82
Table 4.1: Identified tryptic peptides of birch pollen protein from the control sample (unexposed) 124
Table 4.2: Identified tryptic peptides of birch pollen protein from the RH exposed sample ....... 126
Table 4.3: Identified tryptic peptides of birch pollen protein from the NO2 exposed sample .... 128
Table 4.4: Identified tryptic peptides of birch pollen protein from the O3 / NO2 exposed sample 131
Table 4.5: Some of identified Bet v 1 protein and a comparison between peptide sequence coverage of the 9 samples subjected to different conditions/treatments ........................................... 135
Table 4.6: Degree of nitration of the pollen samples, under different nitration conditions, references to equivalent weight of nitrated BSA protein ............................................................... 142
Table 4.7: List and information of identified proteins from in gel-digested sample ................... 148
Table 4.8: 20 Nitrated tyrosine residues of NBSA sample (Abcam) that were identified after digestion and MS analysis ................................................................. 151
Table 4.9: Identified peptide after increasing the starting material before enrichment ............. 155
Table 4.10: Identified peptide after increasing the starting material and enrichment ................. 156
Table 4.11: Identified tagged peptides (after increasing the starting material by 100%) before and after enrichment ................................................................. 157
Table 6.1: Reports the odd ratio between pollen counts and allergic related illness codes 207
CHAPTER 1

INTRODUCTION

This chapter summarizes the relationships between climate change, air pollution and bioaerosols with human health. In doing so, it provides a concise introduction to air pollution, bioaerosols and climate change. Finally, the chapter provides the overall structure of the thesis, including the aims and objectives, hypotheses, research questions, scope and limitations of the study.

1.0 Background

Figure 1.1: Interrelationships between bioaerosol, pollution and meteorology. Note that all impinge on human health even though not shown visibly, there are countless interactive effects between all variables.

Bioaerosols, climate change and air pollution are important environmental issues because they can all affect human health and natural ecosystems. It is important to note, that they can be harmful even when not visible. Atmospheric aerosols play a vital role in climate change and human health (Calvello et al., 2010; Kroll and Seinfeld, 2008; Lyamani et al., 2010; Rengarajan et al., 2011). The effects of these aerosols on human health and the environment at large has become one of the significant topics in current environmental research (Pöschl,
These environmental issues are interlinked, leading to a variety of medical conditions including allergic diseases (Kim et al., 2014). There is evidence suggesting that the interaction between plant pollen grains, environmental pollution, and climate change is increasing the allergenicity of the pollen thus increasing the misery of hay fever sufferers. Both allergens and allergic issues are influenced by air pollution in various ways, making the former more stronger and increasing the immune reaction of the latter (Bartra et al., 2007). Yet, these processes are not sufficient to explain the increased rate of allergic diseases in humans. It has been reported by Reinmuth-Selzle et al. (2017) that air pollution and climate change are possible drivers for the increasing burden of allergic diseases (Reinmuth-Selzle et al., 2017). The molecular mechanisms of allergic diseases that may be influenced by air pollution and climate factors are complex and elusive (Reinmuth-Selzle et al., 2017).

One of the main characteristics of urban areas — where, worldwide, human population density is at high level — is atmospheric pollution (Sénéchal et al., 2015). In 2014, 54% of the world population resided in urban areas and this trend is only going to increase (http://www.who.int/gho/urban_health/situation_trends/urban_population_growth_text/en/).

The atmosphere is the channel of transit for a wide variety of particles of biogenic origin in addition to pollutant gases and particles emitted repeatedly due to human activities (Sénéchal et al., 2015). Bioaerosols are family of aerosol particles that consist of a variety of airborne biogenic particles, for example, viruses, bacteria, fungal spores, plant fibers, or pollen (Sénéchal et al., 2015).

Allergy to pollen has increased throughout the world especially within westernized urban areas. “In the last years, a rising trend of the pollen allergies in urban areas has been attributed to atmospheric pollution” (Sousa et al., 2012). However, the reasons for this increase remain unclear. This indicates a need to understand the various relationships that
exist between bioaerosol, meteorology, pollution and human health. Although, several factors have been suggested regarding the 20th century increase (Beggs, 2004; Beggs and Bambrick, 2006; D’amato and Cecchi, 2008; D’amato et al., 2007) in the burden of allergic respiratory diseases, the reasons are not completely understood (Reid and Gamble, 2009).

1.1 Pollution

The term pollution refers to contamination. “Contamination is simply the presence of a substance where it should not be or at concentrations above background that results in or can result in adverse biological effects to resident communities” (Chapman, 2007). There are numerous types of pollution that can come from different sources and have diverse effects on human health and the environment at large. However, this thesis will concentrate on air pollution and its impact on human health because it is one of the most crucial contemporary global environmental issues (Briggs, 2003; WHO, 2014).

1.1.1 Air pollution

![Diagram showing the route of pollution from source to the end effect on human health and environment.]

Figure 1.2: The route of pollution from source to the end effect on human health and environment.
Air pollution is a problem of major national and international interest where public awareness and concern continues to rise (Lave and Seskin, 2013). Polluted air has a severe impact on human health resulting in different kinds of medical conditions. The health effects can result from acute or chronic exposure, leading to either mild short-term impacts such as irritation to the eyes, nose and throat, or more serious long-term effects such as exacerbations of respiratory and cardiovascular diseases resulting in hospital admission or death (Halonen et al., 2009; Peng et al., 2008; Poloniecki et al., 1997). The effects of the pollution on health can partly be determined by certain combinations of air pollutants, which may be changed by other environmental, and behavioural patterns (Kanakidou et al., 2011; Katsouyanni, 1995). Air pollutants contribute to increased death and hospital admittances (Brunekreef and Holgate, 2002). Figure 1.2 describes the route of pollution from source to the end effect on human health and the environment.

Historically important air pollution episodes, where clear deleterious effects on human health were observed include the Meuse Valley of Belgium in 1930, Donora in Pennsylvania of USA in 1948, and London smog episode in 1952 (Pope III, 2004). Between 1948 and 1962, eight air pollution incidences happened in London, but the well-described ‘Great Smog’ episode in December 1952 was the most significant where smoke concentration rose to greater than 50 times above the regulatory limit, and visibility was so poor for individuals not to see their own feet at the National Gallery (Chauhan and Johnston, 2003). These episodes encouraged the implementation of legislative and regulatory measures so as to control outdoor air pollution in several countries within Europe and United States of America (Pope III, 2004). Through the 1960s to 1980s, numerous population-based studies confirmed that air pollution has a negative impact on human health (Lave and Seskin, 1973; Pope 3rd, 2000). Worldwide, it has been observed over the last few decades, that environmental changes appear to be associated with an increased rate of allergic diseases, particularly in countries
with a western lifestyle (Ayres, 2010).

Particulate matter (PM) has been identified as the primary mediator of toxic effects of air pollution following a series of epidemiological studies conducted within a short period of six years (1989 to 1995) (Dockery et al., 1993; Dockery et al., 1992; Pope et al., 1995). These results provided epidemiological and toxicological insight into particulate matter air pollution (Gupta et al., 2013). Air pollution can negatively affect the cardiovascular and respiratory systems of the body (Brook et al., 2004; Pope III, 2004). Gupta et al. (2013) reported that prolonged and recurrent contact with air pollution increases the collective threat of chronic pulmonary, cardiovascular disease and loss of lives (Gupta et al., 2013). In fact and somewhat counter-intuitively, more mortalities transpire due to cardiovascular causes rather than pulmonary diseases following air pollution exposure (Pope et al., 2004).

“Air pollution is now fully acknowledged to be a significant public health problem, responsible for a growing range of health effects that are well documented from the results of an extensive research effort conducted in many regions of the world” (Kelly and Fussell, 2015). The effects of air pollution on human health can include breathing problems, coughing, asthma and causing or worsening existing respiratory and cardiac illnesses (WHO, 2014). These effects can lead to increased medicine use, hospital admissions, and early death (WHO, 2014). According to WHO, “2.4 million people die yearly from causes directly attributable to air pollution, with 1.5 millions of these deaths attributable to indoors air pollution” (WHO, 2002). The United Nations Environment Programme has estimated that 1.1 billion people breathe harmful air (UNICEF and Organization, 2002), globally. It was mentioned in another research that despite earlier enhancements in air quality, large populations in urban areas still breathe air that is not up to national (European) and international (WHO) standards of Air Quality Guidelines (Kelly and Fussell, 2015), even
with the legislative measures in place.

Epidemiological studies suggest that cardiopulmonary disease related to inhalation of fine particle air pollution results to more than 500,000 deaths in the USA each year (Khan, 2011). A convincing association between pneumonia related casualties and air pollution from automobiles have been demonstrated in a study by the University of Birmingham (Khan, 2011). Yearly, more deaths are connected to air pollution than to automobile disasters worldwide (Collins, 2012). The WHO has estimated that approximately 800,000 deaths and 4.6 million lost life yearly throughout the world due to urban air contamination exposures (WHO, 2002).

It is now well understood that the key contemporary environmental risk factor related to early mortality is air pollution, which surpasses other risk factors such as poor health and scarcity of drinking water (OECD, 2014). Approximately 3.7 million deaths have been attributed to outdoor urban and rural sources in the year 2012 as reported by WHO (WHO, 2014).
1.1.2 Pollutants

The atmospheric environment becomes polluted when particulate matter and or gases are into the atmosphere, which causes harm to humans, other living organisms, and the environment. Any substance in the air that has unfavorable effects on human wellbeing and or the environment is referred to as air or environmental pollutant. Various air pollutants are known, differing in their properties such as natural structure, reaction properties, release, transportation ability and their subsequent influences on human health (Kampa and Castanas, 2008) and the natural environment. They can be classified into four major types: Gaseous pollutants (e.g. nitrogen oxide (NOx), and ozone (O3)), Persistent organic pollutants (e.g. dioxins), Heavy metals (e.g. lead, mercury) and Particulate Matter (PM) (Kampa and Castanas, 2008). Gaseous pollutants have the greatest impact on atmospheric composition and are predominantly associated with fossil-fuel burning (Katsouyanni, 2003). Figure 1.3 illustrates the sources of primary and secondary pollutants. These pollutants are present in solid, liquid or gaseous states, which can either, be natural (biogenic) or man-made.
(anthropogenic). Notably, all of these pollutants can pose health risks, with the possibility of associated allergies even at low levels of exposure, particularly amidst those individuals with pre-existing health complications. Pollutants are classified as primary, directly produced from source or secondary those that are not produced directly. The most common sources of air pollution include:

**Particulate matter** (PM): is a type of air pollutants, comprising of complex and varying mixtures of particles (including diesel exhaust) suspended in the air that are produced by a variety of natural and human activities (Pöschl, 2005). PM vary in size and composition. “The pathogenicity of PM is determined by their size, composition, origin, solubility and their ability to produce reactive oxygen” (Xing et al., 2016). Factories, fire, power plants, waste burners, automobile, manufacturing, and environmental windblown dirt are all regarded as sources of this pollutant (Kampa and Castanas, 2008). Increased airborne concentrations of PM have a strong connection with early mortalities, hospital admittances, and asthma attacks, where the aged and individuals with pre-existing breathing conditions are at more danger (SEI, 2012).

**Ozone** (O₃): It is formed as a “secondary pollutant in the troposphere from complex photochemical reactions following emissions of precursor gases such as NOₓ and non-methane volatile organic compounds (NMVOCs), deriving from paint application, road transport, dry-cleaning, and other solvent uses” (Ferrante et al., 2015). The high oxidizing potential causes O₃ to damage mucus and respiratory tissues of animals and plant and similarly harms vegetation, specifically, trees and plants throughout their cultivation, and ecosystems, resulting in decreased farming returns and plant development (Ferrante et al., 2015). Ground-level O₃ has a negative influence on human wellbeing, however, it helps protect the earth from the sun’s damaging rays at upper atmospheric level (Ferrante et al.,
Combined evidence from different studies have shown that O₃ exposure is linked with increased respiratory related illnesses (Chen and Kan, 2008). Significant individual differences do exist in response to O₃ where children, aged individuals, asthmatics and people with existing persistent airway ailments are more susceptible to O₃ related ailments (Chen and Kan, 2008). It has been reported that during the warmer season, the impact of O₃ on respiratory hospital admittances appear stronger (Chen and Kan, 2008).

**Nitrogen dioxide** (NO₂): is a reddish-brown toxic gas with a harsh, stinging smell (Patocka and Kuca, 2014). It largely originates from the exhaust gas of vehicles, other elevated temperature burning, and is also formed naturally through thunderstorm due to processes related to the electric discharge associated with lightning (Kenneth and Monica, 2014). NO₂ is toxic by inhalation (Kelly and Fussell, 2011), especially for children, the ageing individuals, those with existing respiratory related illnesses and people with chronic obstructive pulmonary disease (COPD) (Kenneth and Monica, 2014). NO₂ causes multiple negative respiratory effects associated with increased emergency hospital visits and admittances for respiratory illness (Galan et al., 2003). Additionally, a well-defined impact of NO₂ on occurrence of viral infections among asthmatics patients have been reported among studies assessing intensification of asthma in children (Linaker et al., 2000). Atmospheric NO₂ is also known to be either harmful or beneficial to plants depending on the concentration and plant species (Capron and Mansfield, 1977; Sandhu and Gupta, 1989; Wellburn, 1990). Because combustion processes typically produce both PM and NOₓ, it is often difficult to epidemiologically separate the effects of NOₓ and PM.

**Sulfur dioxide** (SO₂): is usually formed by volcanoes and in several manufacturing procedures is often a local pollutant, especially in moist atmospheres (McGranahan, 2012). At inhalation exposure, SO₂ can cause severe irritation of the nose and throat (Patocka and
It forms $\text{H}_2\text{SO}_3$ when in contact with moist membranes (White and Martin, 2010). At high concentrations it can cause life-threatening accumulation of fluid in the lungs (Patocka and Kuca, 2014). Highest levels of $\text{SO}_2$ in the atmosphere can result in short-term breathing struggle for active outdoors individuals with asthma (El-Sharkawy, 2013) while longer-term exposures to the excessive levels of this gas leads to respiratory illness and worsen heart ailments (Nguyen and Kim, 2006). “Gaseous $\text{SO}_2$ can remain in dry atmospheres for many days and combine with other pollutants to form sulphate particles, which can persist and be transported considerable distances as a fine particulate and it can be an important component of haze” (SEI, 2012). Even though some researchers reported connections between $\text{SO}_2$ exposure and daily death, emergency hospital admissions for asthma, COPD and cardiac illness (Wong et al., 1999), others debated that $\text{SO}_2$ may function as a “surrogate” for city air pollution from fossil fuel burning (Buringh et al., 2000). Note that in the UK and many other countries the concentrations of $\text{SO}_2$ are now much lower than their historical highs due to the use of cleaner non-sulphur containing fuels.

**Carbon monoxide** (CO) - is the most abundant atmospheric pollutant released by our technological society and also a natural by-product of different mammalian, plant and bacterial cell systems (Uffen, 1981). It is colourless, odourless, non-irritating but very toxic gas and a product of partial combustion from vehicles and engines where its major source is road transport (Kampa and Castanas, 2008). CO binds in the lungs with hemoglobin in the blood to form carboxyhemoglobin (COHb) that impairs the conveyance of oxygen within the body system (Schwela, 2000). This reduces the amount of oxygen reaching the body’s organs and tissues, thereby aggravating heart illness that results in chest pain and other symptoms. Other health effects include hypoxia, neurological deficits and neurobehavioral changes, and that persists even at very low CO levels, signifying no threshold for the commencement of
these consequences (Schwela, 2000). Formation of COHb at lower levels may have more serious health consequences than ambient CO (Hampson et al., 2012).

Figures 2.1 illustrates the schematic drawing of causes and effects of air pollution as a result of natural and man-made activities. These ambient pollutants do have a greater impact on human health that is associated with a number of disease consequences (Kelly and Fussell, 2011).

![Figure 1.4: Schematic drawing of causes and effects of air pollution: (1) greenhouse effect, (2) particulate contamination, (3) increased UV radiation, (4) acid rain, (5) increased ground level ozone concentration, (6) increased levels of nitrogen oxides. CH₄-Methane, O₃- Ozone, SOₓ- Sulphur oxides, CO₂- Carbon dioxide, NOₓ- Nitrogen oxides and CFC- Chlorofluorocarbons (Adopted from Kenneth and Monica 2014).](image)

Even though, the main air pollutants encountered in daily life are PM, O₃, CO, NO₂ and SO₂, in this study only NO₂ and O₃ were studied in relation to their effects on pollen grain. This is partly because SO₂ levels have been decreasing in most parts of the world in contrary to traffic-related air pollutants, while NO₂ and (away from cities) O₃ levels tend to increase due
to the increased number of motor vehicles. Additionally, NO\textsubscript{2} and O\textsubscript{3} were studied because of the well-known health effects of these traffic-related pollutants in relation to pollen grain.

<table>
<thead>
<tr>
<th>Pollutants</th>
<th>Averaging time</th>
<th>AQG values (µg/m\textsuperscript{3})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Particulate matter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PM2.5</td>
<td>1 year</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>24 h (99th percentile)</td>
<td>25</td>
</tr>
<tr>
<td>PM10</td>
<td>1 year</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>24 h (99th percentile)</td>
<td>50</td>
</tr>
<tr>
<td>Ozone O\textsubscript{3}</td>
<td>8 h daily maximum</td>
<td>100</td>
</tr>
<tr>
<td>Nitrogen dioxide NO\textsubscript{2}</td>
<td>1 year</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>1 h</td>
<td>100</td>
</tr>
<tr>
<td>Sulfur dioxide SO\textsubscript{2}</td>
<td>24 h</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td>500</td>
</tr>
</tbody>
</table>

Table 1.1: Global updated WHO Air Quality Guidelines (AQG) (Adopted from WHO Regional Office for Europe; 2006). Short-term values are defined such that the 99\textsuperscript{th} percentile of the 24-hour averages measured throughout a calendar year does not exceed the stated value. Averaging time is the amount of time frame taken into consideration the concentration of pollutant that will have short-term (acute) and long-term (chronic) effect on human health post exposure. AQG values are the recommended maximum concentrations of the pollutant in the air over the averaging time.

The WHO Air Quality Guidelines outlines an international reference on the negative effects of exposure to air pollutants on human wellbeing. The scientific knowledge on health hazards related to air pollutants, providing risk estimates for exposure to air pollutants and recommending air quality guidelines for member states to develop their own national air quality standards are all summarized on the guidelines (Table 1.1 above). Table 1.1 shows the latest global updated version of the WHO AQG published in 2006, which focused on four classical air pollutants, namely PM, NO\textsubscript{2}, O\textsubscript{3} and SO\textsubscript{2}. 
1.2 Bioaerosols

Primary biological aerosols (PBA), referred to as bioaerosols, are a subset of atmospheric particles, which are directly discharged from the biosphere into the atmosphere (Fröhlich-Nowoisky et al., 2016). As the name implies bioaerosols are biological particles suspended in the air.

“Bioaerosols are airborne viable and non-viable biological particles (e.g fungi, bacteria, pollen and viruses), their fragments and by-products” (Exley et al., 2014).

Bioaerosols are abundant in all environments, their concentration depends on several factors, including environmental position, climate and time of day (Bertolini et al., 2013; Brodie et al., 2007; Fierer et al., 2008; Lee et al., 2010). The aerodynamic behavior of bioaerosols depends upon their length dimensions, density and form (Jones and Harrison, 2004). The size of a biological particle will affect its survivability in the atmosphere where the “interaction of a particle with its environment depends upon its surface area, while its ability to sustain damage depends upon its volume, resulting in smaller particles being more susceptible to environmental damage” (Jones and Harrison, 2004).

As illustrated in Figure 1.5, PBA particle diameters range from nanometers up to about a tenth of a millimetre (Fröhlich-Nowoisky et al., 2016) with the upper limit of the aerosol particle size range determined by rapid loss via sedimentation (Hinds, 2012; Pöschl, 2005). PBA play a key role in the dispersal of reproductive units from plants and microbes (pollen, spores, etc.), for which the atmosphere permits their transportation over geographic barriers and long distances (Brown and Hovmøller, 2002; Després et al., 2012; Womack et al., 2010). Bioaerosols undergo further chemical and physical transformation, stress, and biological aging upon interaction with UV radiation, photo-oxidants, and various air pollutants (e.g
acids, NO$_2$, aromatic compounds, and soot) during the process of atmospheric transport (Estillore et al., 2016; Franze et al., 2005; Santarpia et al., 2012; Shiraiwa et al., 2012b).

![Figure 1.5: Characteristic size ranges of atmospheric particles and bioaerosols with exemplary illustrations: (A) protein, (B) virus, (C) bacteria, (D) fungal spore, and (E) pollen grain adapted from (Pöschl and Shiraiwa, 2015).](image)

### 1.2.1 Pollen

“Pollen grains are biological structures produced by superior plants to perform the vital task of sexual reproduction” (Sousa et al., 2012). Pollen is a fine to coarse powder comprising the micro gametophytes (sperm producing gametophytes) of seed plants (Pastorius, 2014) and is part of the coarse fraction of air particulate matter (particle diameters >10 µm) (Després et al., 2012; Després et al., 2007; Elbert et al., 2007). Anemophilous plants develop compensatory mechanisms enabling successful fertilization, such as the discharge of huge quantities of airborne pollen, making dispersal of the grain easier (Sousa et al., 2012). Anemophilous plants are plants that rely on the wind to transport their pollen (D’amato et al., 2007). Pollen fragments are typically located within the fine PM fraction (PM2.5 which is less than 2.5 micron in length) that can be easily deposited into the human respiratory tract and alveolar areas of the lung (Fröhlich-Nowoisky et al., 2012; Fröhlich-Nowoisky et al., 2009; Müller-Germann et al., 2015). “These features, associated with the allergens present in both inner part of the pollen wall (intine and cytoplasm) and pollen outer wall
(submicroscopical sites of the exine and orbicules) make these aerosol biological particles to trigger respiratory allergic reactions” (Sousa et al., 2012). Structural and architectural features of allergenic proteins appear to play a role in their allergenicity, however, research has shown that pollen allergens from almost all plant species belong to only a few (29 out of 7,868) protein families (Radauer and Breiteneder, 2006; Radauer et al., 2008). Some pollens are more allergenic than others and some are produced in larger quantities (Jackson-Menaldi et al., 2002). The combination of abundance and allergenicity dictates the level of associated human allergy.

1.2.2 Birch pollen

Birch (Betula) belongs to the Betulaceae family (Asam et al., 2015) that is assigned to Fagales order (APG II 2003). Betulaceae (Fagales) contain roughly 120-150 species of trees or shrubs, appearing typically in the northern temperate zone (Grimm and Renner, 2013). The birch is a tree or shrub that are medium sized trees with small, triangular, serrated leaves and white bark. Birch trees have yellow catkins that produce the pollen and the fruit composed of layers of small seeds in a catkin shape, green at first, then brown (https://www.worcester.ac.uk/discover/nparu-pollen-types-birch.html). Birch pollen has a unique morphology that allows the species to be identified easily. The genus Betula species are typically similar, where dissimilarities between the species might be found in the size, abundance, and organization of the different structural features. Due to the similarities in structure and other components of trees belonging to the Fagales order, their major allergens have a degree of cross-reactivity (Puc 2003; Rodriguez-Rajo et al. 2004). More than 96% of patients allergic to tree pollen react toward Bet v 1 (Jarolim et al., 1989), which is the major allergen in birch trees (Ipsen and Løwenstein, 1983).
Birch is mainly distributed in the UK (countrywide), Europe, Asia and Northern USA, limited to cooler climates or found at altitude (https://www.worcester.ac.uk/discover/nparu-pollen-types-birch.html). In England, there are large amounts of Betula (Skjøth et al., 2013), and high concentrations have been identified in London (Skjøth et al, 2009) and Worcester (Adams-Groom et al, 2002). Worldwide, different birch trees species do exist. However, the native to UK are: Betula pendula (silver birch), B. pubescens (downy birch), and B. nana (dwarf birch) where B. pendula and B. pubescens are the most common and widespread (Preston et al. 2002; Stace 1997). In the UK, the flowering of birch normally happens between April and May (Skjøth et al., 2009). Dry and damp conditions are favourable to birch trees with B. pubescens being more common in humid surroundings (Skjøth et al., 2009).

Temperature is one of the main factors determining the start date of a pollen season; specifically for trees, flowering in spring it is the temperature during the period prior the onset of flowering is most important (Spieksma et al., 1995). The aerosols that contain allergens as well as those governing their release due to fragmentation of pollen and spores might occur as a result of weather change (Taylor et al., 2004). Records on the start of birch pollen seasons monitored across three sites (Cardiff, Derby and London) in the UK for forty-two years showed a trend for the pollen season to begin earlier (Emberlin et al., 1997). The study on these sites have shown that a clear biotic response in the timing of the birch pollen seasons is occurring in relation to the warmer spring temperatures of recent years (Emberlin et al., 1997). The most influential weather on the start dates of the birch pollen season at the three sites was from early February to middle of March (Adams-Groom et al., 2002). Overall, it has been established that climate change has a significant impact on airborne pollen since it induces faster plant growth, increases the amount of pollen production from each plant and
pollen season, increases the amount of allergenic proteins contained in pollen and increases the start time of plant growth (D’Amato et al., 2013).

When birch trees are flowering and become exposed to moisture followed by drying winds, they can generate particulate aerosols containing pollen allergens (Taylor et al., 2004). Due to the small nature of the particles, they can be deposited in the peripheral airways, which have the potential to stimulate an inflammatory response (Taylor et al., 2004) and upon interaction with air pollutant, can cause pollen protein specific modification that in turn might lead to increased pollen allergenicity. “Usually the onset, duration, and intensity of clinical symptoms in patients with respiratory allergy to pollen are correlated with counts of atmospheric pollen grains, although this relation is not always simple” (D’Amato et al., 1996). Skjøth et al. (2009) reported that 90% of patients allergic to birch pollen show mild symptoms when the pollen count is above 80 grains/m$^3$ at the start of the birch pollen season (Skjøth et al., 2009). However, 80% of patients do show indications of allergic reaction at a level below 30 grains/m$^3$ during the late season (Emberlin 1997; Koivikko et al. 1986; Viander and Koivikko 1978). It is reported in another study, exposure to pollen concentration of 20 pollen grains/m$^3$ of air showed noticeable symptoms in patients allergic to grass. At concentration of 65 and 120 (several hours exposure) pollen grains/m$^3$, the symptoms were intensified and causes dyspnoea in some patients respectively (Rapiejko et al., 2007). Comparable symptoms transpired after contact with birch pollen. Thus, it can then be established that clinical signs of allergic disease are dependent on the concentration and the kind of aeroallergen the individual was exposed to (Rapiejko et al., 2007) and the time of exposure.
1.2.3 Pollen allergy

Pollen grains are a major concern in allergy (ANSES, 2014). An allergy occurs when a specific sensitivity to a foreign material recognised as allergen causes the body's immune system to respond using the defence mechanisms (Brain, 2004). Asthma in sensitized individual can be triggered by pollen. In the atmosphere, the allergenic content of pollen varies according to weather, geography and vegetation (D’Amato et al., 2007). The main allergenic period, exact timing and high pollen seasons will differ yearly based on climate change and biological factors (http://www.worcester.ac.uk/discover/pollen-calendar.html). The most common allergic conditions is hay fever, with more than 10 million estimated sufferers in the UK alone (Pashley et al., 2015).

“Allergies are generally thought to be a detrimental outcome of a mistargeted immune response that evolved to provide immunity to macroparasites” (Palm et al., 2012). The immune system treats the pollen as an intruder and responds by mobilizing to attack by producing large amounts of antibodies. Seasonal allergic rhinitis (SAR) has been known to develop as a result of plant pollens in the atmosphere and its indications overlap with pollen season (Davies et al., 1998). However, there are several anomalies that suggest additional factors must be considered because the relationship is not direct and simple (Davies et al., 1998). The occurrence of respiratory allergic reactions stimulated by pollens has increased in recent years (D'Amato et al., 1998). Currently, the prevalence of pollen sensitivity is expected to be up 40% (D’Amato et al., 2007). Regarding pollen allergenicity, some studies suggested that pollen-derived lipid mediators (PALMs), can also interact with the immune system thereby resulting in the modification of the allergenic response (Bashir et al., 2013; Traidl-Hoffmann et al., 2003).
1.2.4 Allergenic protein release of pollen grain

Pollen is a naturally occurring atmospheric environmental allergen where in several cases is accountable for severe human health diseases (Bosch-Cano et al., 2011; Cresti and Linskens, 2000; D'Amato et al., 2010; Traidl-Hoffmann et al., 2009). The release of allergenic proteins from pollen and spores usually occurs after cell destruction or under moist conditions (Knutsen et al., 2012). Particularly, pollen rupture can occur as a result of osmotic shock during rainfall and can lead to occurrences of thunderstorm asthma (Behrendt and Becker, 2001; Cecchi et al., 2010; D'Amato et al., 2016; Taylor and Jonsson, 2004). These weather conditions may stimulate hydration and disintegration of pollen grains that discharge allergenic bioaerosols into the air (D'Amato et al., 2010). Additionally, elevated concentrations of pollen, fungal spores, and other PBA particles have also been detected following the onset of heavy rainfall and moist weather conditions (Elbert et al., 2007; Huffman et al., 2013; Müller-Germann et al., 2015). Increased concentrations of free allergen particles in fine air PM have also been seen after rainfall (Schäppi et al., 1997). The interaction of PBA particles with air pollutants, like NO₂ and O₃, can also damage the particles’ envelope, and it has been hypothesized that this facilitates the release of allergenic materials, such as cytoplasmic granules from pollen (Reinmuth-Selzle et al., 2017). Behrendt et al. (1997) showed that there is morphological evidence for preactivation of pollen by organic extracts of airborne particulate that may then induce local allergen release, resulting in either allergenic extrusion followed by generation of allergenic aerosols or adsorption of pollen-derived proteins to airborne particles (Behrendt et al., 1997). Furthermore, Okuyama et al. (2007) studied the acid adsorption properties of the pollen and concluded that nitric acid is not only adsorbed on the surface but also dissolved into the inner part of the pollen, thus changing the chemical balance (Okuyama et al., 2007) of the pollen grain.
1.2.5 Pollen protein nitration

At least 50 different diseases have been reported in association with protein nitration (Abello et al., 2009; Greenacre and Ischiropoulos, 2001) and it has been shown to modify the immunogenic potential aeroallergens like Bet v 1 allergen protein of birch pollen (Gruijthuijsen et al., 2006; Karle et al., 2012). High concentrations of traffic-related air pollutant particularly NOX and O₃ enhanced asthma and allergic diseases suggested by numerous studies (D’Amato et al., 2007; Shiraiwa et al., 2012b). The advancement of allergies by traffic-related air pollution might be due to post-translational modification (PTM), nitration and associated modifications in the immunogenicity of allergenic proteins (Pöschl, 2005). PTM (post-translational modification) occurs when there is an addition of a functional group on a protein, affecting one or more amino acids (building blocks of protein). The modification is catalyzed by enzymes after the completion of protein translation by ribosomes (Kumar et al., 2008).

Although the allergen content remained unchanged, birch pollen from urban regions had a greater allergenic potential than pollen from rural regions (Bryce et al., 2009). Additionally, proteins can be modified upon exposure to pollutants such as NO₂ and O₃ while still in the atmosphere. “Laboratory and field studies showed that proteins were efficiently nitrated upon exposure to gas mixtures of NO₂ and O₃ or polluted urban air (summer smog)” (Franze et al., 2005). Nitrated and oxidized proteins as well as protein degradation all occur upon the heterogeneous reaction of the protein with the gaseous reactants O₃ and NO₂ (Shiraiwa et al., 2012b). A higher degree of nitration (ND) was reported to happen when the protein was pretreated with O₃. Shiraiwa et al. (2011) reported that the deduction from these studies was, the nitration reaction of proteins with O₃ and NO₂ develop via long-lived reactive oxygen
intermediates ROIs (Shiraiwa et al., 2011). ROIs are products of PAH (Polycyclic aromatic hydrocarbon) and protein nitration (Shiraiwa et al., 2011).

In another study, Bet v 1 was nitrated using standard laboratory reagent for protein nitration Tetranitromethane (reagent that reacts with tyrosine and cysteine residues of protein leading to modification) (TNM) and two naturally occurring nitrating reagents, that is, Peroxynitrite (derived from NO) (ONOO\(^-\)) imitating inflammation and oxidative/nitrosative stress and \(O_3/NO_2\) representing the effect of air pollution (Reinmuth-Selzle et al., 2014). It was revealed in their findings that the effectiveness and specificity of the protein nitration is incumbent on the nitrating agent and the reaction conditions used during an experiment. It was also demonstrated that the nitration percentages were greater for sample of protein solutions (20% per day) than for solid or semisolid protein samples (2% per day) (Reinmuth-Selzle et al., 2014). Thus, it can be assumed that under moist conditions, the allergenic potential of allergen protein might be specifically amplified (Garland et al., 2008).

1.3 Climate change

The term climate is generally understood to mean the usual weather of a place but can be different for seasons. Climate change can then be described as a variation in the usual weather of a particular place that changes in how much it rains in a year or change in temperature for a month or season.

Climate change denotes a huge risk to worldwide health that could affect numerous disease factors in the 21\(^{st}\) century because of its impact on certain food supplies, air and water quality, season, finances, and several extra serious wellbeing causes (Gennaro et al., 2015). “There is also a link between climate change and air pollution; an individual’s response to air pollution depends on the source and components of the pollution as well as on climatic
agents” (Gennaro et al., 2015). It has been suggested that fundamental changes on the atmosphere and the climate created by human activity, affects the biosphere and the human environment at large (D'amato and Cecchi, 2008). This has affected and will continue to affect the human health (Beggs, 2004). Over the last 30 years, the Intergovernmental Panel report of February 2007 on climate change agrees that worldwide temperature has risen significantly due to amplified greenhouse gas emissions, mainly from anthropogenic sources (Jones et al., 2007).

The occurrence of severe weather events, which includes heat waves, heavy rainfall, and thunderstorms, have been reported to increase in recent years (Ayres et al., 2009; Beggs, 2004; D'amato and Cecchi, 2008). Heat-related prevalence of hospitalization and death resulting from cardiovascular and respiratory diseases have also been testified (Baccini et al., 2008; Michelozzi et al., 2009; Stafoggia et al., 2006).

1.3.1 Climate change and ambulance operations

There have been very few studies of the effect of severe weather conditions and climate change on ambulance operations in the UK (Thornes et al., 2014) but there have been several recent studies in other countries namely: Australia, Brisbane: Sydney: Canada, Toronto, China, Hong Kong: Italy and Switzerland. Most of these international studies are concerned with the negative impacts of heat waves on ambulance; however, the study carried out by Thornes et al. (2014) on the impact of extreme weather on ambulance performance has examined the negative impacts of extreme cold weather as well. It was shown in the study that there is a considerable scope to improve understanding across a number of issues and there are potentially significant links between ambulance demand/performance and extreme weather and climate change. Even though, more research is required to establish a complete
understanding and identification of temperature thresholds, this study (impact of extreme weather on ambulance performance by Thornes et al. (2014)) has shown that hot and cold temperatures have significant negative impact on ambulance performance. Both hot and cold weather significantly increase the total number of incidents and in particular Category A (Cat A) incidents. “In the UK, there is a target response rate of 75% of life threatening incidents (Category A) that must be responded to within 8 min” (Thornes et al., 2014). More research is needed to evaluate which illness codes increase in hot and cold weather (Thornes et al., 2014).

A meaningful correlation between severe weather, increased ambulance call-out and response times was evidently established when daily air temperature data was compared with ambulance call-out data for Birmingham within 2007-2011 (Thornes et al., 2014). The influence of cold weather on health is predictable and mostly preventable reported by Public Health England. Studying the effects of extreme weather will widen the knowledge of the relationships between temperature and human health, and also help in generating public health policy that will aid in preventing the unfavorable impacts of weather change on the population (Lin et al., 2009). It will also help ambulance response time.

1.4 Air pollution, climate change, pollen and human health

In Europe, allergic diseases are increasing (Frank and Ernst, 2016) and climate change and anthropogenic air pollution are the probable reasons examined for this trend (Krämer et al., 2000). Laboratory outcomes indicated that diesel exhaust particles increase sensitivity to allergens while epidemiological analyses propose an interaction between allergic illnesses and traffic contamination (Davies et al., 1998). Evidence from studies have shown that two most important air pollutants; O₃ and NO₂, can have negative effect on human wellbeing such
as the initiation of lung inflammation by O₃ (Uysal and Schapira, 2003) and can enhance other allergic related illnesses such as asthma. To a lesser extent, SO₂ also has possible influence on allergic reactions (Saxon and Diaz-Sanchez, 2005). Obviously, there are extra factors influencing the prevalence of allergic reactions apart from the already known cause, pollen. Thus, it can be assumed that an interaction between air pollutants and allergens do exist that aggravates the development of atopy and the indicators of allergic ailment (Davies et al., 1998).

In the past few decades, a link between air pollutants and pollen on the severity of respiratory allergy signs have been highlighted (Bosch-Cano et al., 2011; D’amato et al., 2010; Sousa et al., 2011; Traidl-Hoffmann et al., 2009). It has also been proposed that contact with extreme levels of pollutants such as NOₓ and SOₓ can increase allergic sensitization, however, the role of the air pollutants is not well-defined (Bosch-Cano et al., 2011; Sousa et al., 2011).

In recent years, climate variation has altered exposure to air pollutants that profoundly influence public health via exposure to ambient PM 2.5 (Mimura et al., 2014). These recent changes are related to an increase in asthma and allergic respiratory diseases (Mimura et al., 2014). Build-up of air pollutants, such as O₃, at ground level has an impact on occurrences of rhinitis and asthma exacerbation (Cecchi et al., 2010; Viegi and Baldacci, 2002). Direct interference of air pollution on individuals with respiratory allergies induces serious effects, however, the indirect consequences on pollen proteins are still under investigation (Sousa et al., 2012). The reactions to air pollutants by each individual is based on the kind of pollutant exposed to, the degree/time of the exposure, the person's wellbeing status and genetics (Vallero, 2007). It is important to note that the impact of air pollutants on individual can occur as a direct or indirect effect and its presence also exert vital actions on aeroallergens (Bartra et al., 2007).
Nonetheless, individuals with already existing allergic related illness such as asthma are susceptible to developing obstructive airway exacerbations upon contact with gaseous and particulate components of air pollution as suggested by considerable evidence (D'amato and Cecchi, 2008). These pollutants also affect plants and their pollen specifically; its morphology, cell wall, protein release and pollen protein (Frank and Ernst, 2016). The pollen coat, comprising of a complicated combination of pigments, waxes, lipids, aromatics and proteins (Edlund et al., 2004) might be damaged upon contact with air pollution as well as other factors (Frank and Ernst, 2016) such as humidity. It has also been shown that pollen growth in the plant (Schoene et al., 2004) and airborne pollination (Wang et al., 2010) may also be interfered by pollutants. The release of NO$_2$ in urban setting is largely by transportation and housing heating (Chassard et al., 2015). The threshold for human health protection on an annual basis with in Europe, has been fixed at 40 mg/m$^3$ ~213 ppb/0.213 ppm (European Union directive 1999/30/CE) (Chassard et al., 2015). However, in the event of urban pollution, hourly concentration may extent up to 350 mg/m$^3$ (Airparif, 2009). It is not clear whether such levels of NO$_2$ will have an impact on pollen grains (Chassard et al., 2015).

Changes in the environment (global warming, air pollution, etc.) will result in an earlier and longer pollen season, enhanced pollen production and an increase in pollen allergenicity with a negative effect on atopic patients (D'amato and Cecchi, 2008). Climate change might alter allergic disease through other potential mechanisms, which includes: prolonged pollen periods thereby increasing the time of human exposure to aeroallergens; likelihood of longer allergy signs in individuals with existing allergic disease and lastly, elevated levels of pollen counts in the air may increase the gravity of sensitized symptoms (USEPA, 2008; Ziska et al., 2011).
Figure 1.6: Description of the pathways through which climate parameters and air pollutants can influence the release, potency, and effects of allergens: temperature (T), relative humidity (RH), ultraviolet (UV) radiation, particulate matter (PM), ozone and nitrogen dioxides (O$_3$, NO$_2$) (modified from Reinmuth-Selzle et al., 2017). Pollen grains on their own carry allergens that cause allergic reactions. Upon interaction with pollutants and climate change, pollen becomes more allergenic and pollen season extends thereby increasing misery of sufferers. While climate change directly affect human health via extreme weathers, pollutants also affect human health directly causing various medical conditions. All these might lead to increase in ambulance call out rate.

This interaction between pollen and mentioned variables in the atmosphere may lead to pollen pre-activation, morphological changes on the pollen surface, alteration of allergen and protein release, generation of more potent allergenic aerosols and increase pollen season thereby increasing allergic season and misery of sufferers.
1.5 The Research Questions

1. What are the likely impacts of interaction between air pollution, meteorology and bioaerosols on human health?

2. Are there observable differences between pollens that have been exposed to air pollution to those which have not?

3. Do correlations exist between meteorological variables (temperature and relative humidity), pollen and different ambulance callout categories? (callout categories are defined in Chapter 2)

1.6 Aims and objectives

The length of the pollen season of some of the most allergenic pollen species (e.g. ragweed and birch) is increasing as a result of climate change and hence it is expected that adverse health effects will become an even more severe problem in the nearest future. However, much less is known about the mechanism linking air pollution and climate change to increased pollen allergenicity. Birch pollen was chosen because of its high allergenic properties which makes it one of the major cause of pollinosis (hay fever caused by allergic reaction to pollen) and ranked one of the most important allergic pollen type.

The overall goal of this project was established not only to better understand birch pollen allergenicity but also ascertain the ability to relate and predict the link between temperatures, and pollen counts with several medical conditions including allergic diseases.
The research used laboratory techniques to understand and measure changes that occur in pollen composition upon exposure to particular atmospheric components. The laboratory results and London data sets will then be incorporated into a statistical model, to see if there will be clear signal of increased allergic illnesses when both the pollutant concentration and pollen counts are high. The basic relation between some illness codes of the London ambulance with the meteorological variable of St James Park, London was also explored using a statistical model. Consequently begin to link the impact of these mechanisms to human health.

To achieve the aim, this research:

- Designed a laboratory setup and protocol to measure post-translational modifications on bioaerosols.

- Investigated the post-translational modification of key allergenic pollen species (Birch) through exposure of the pollen grains to atmospherically relevant exposures of gas phase of NO₂ and O₃ within a dedicated and highly-instrumented laboratory for the investigation of particle (pollen) and gas phase species interactions.

- Studied the effect of air temperature and pollen counts on ambulance callout rates for different medical categories using a statistical model.

- Explored real time imaging of pollen exposure (to RH and NO₂) to visualize any morphological changes on the pollen grain using light microscopy.

- Probed the impact of NO₂, RH and rainwater on surface of the pollen grain using SEM (scanning electron microscope) microscopy.
1.7 Hypotheses

1. Ozone (O₃) and nitrogen dioxide (NO₂) pollution cause post-translational modification on pollen grain proteins.

2. There are relationships between meteorological variables with some medical conditions.

3. There is a relationship between pollen counts and allergic illnesses.

4. Exposure to pollutants, humidity and hydration affects pollen grain morphologically and enhances the release of its particles.

1.8 The Scope and Limitation of the Research

The thesis will limit its assessment on the impacts of air pollutants, O₃ and mostly NO₂ upon pollen grain. It will investigate one type of pollen, namely birch, which is known to be a major allergenic species in the UK and Europe. It will explore the relationship between air temperature of St James park, London with key illness category codes used by the London Ambulance Service, in particular the top 20 callout categories. It will also study the morphological changes on the pollen grain after subjecting it to different treatments and conditions. Lastly, relationship concerning pollen counts with allergic related conditions amongst illness codes of London ambulance callouts data set will be assessed using time series and odds ratio analyses.

In this thesis, all empirical Chapters have a specific literature survey section and related experimental and analytical methodologies. The exceptions to this rule are Chapters 3 and 4 that are reliant on one and another. Where Chapter 3 presents the detailed laboratory protocols required for the acquisition of the results shown in Chapter 4.
1.9 Thesis structure

Figure 1.7: Thesis structure
CHAPTER 2

Impact of air temperature on London ambulance call-out incidents and response times

This chapter establishes the relationships between observed weather and the number of ambulance calls incidents and response times. A detailed analysis of London Ambulance callout data (2003-2013) is presented and compared to London weather data. These results are compared, where possible, to published research for other cities around the world.

Figure 2.1: London Ambulance Service on response to emergency calls as snow hit parts of the capital on 30th November 2010. Adopted from (http://www.londonambulance.nhs.uk)

This chapter has been presented at conferences and is available from online conference abstract database and records. This work has also been published in the Journal: Climate (Mahmood, M.A.; Thornes, J.E.; Pope, F.D.; Fisher, P.A.; Vardoulakis, S. Impact of Air Temperature on London Ambulance Call-Out Incidents and Response Times. Climate 2017, 5, 61.).
2.0 Abstract

Ambulances are an integral part of a country’s infrastructure ensuring its citizens and visitors are kept healthy. These services are in operation continuously (24/7) around the world and yet, until recently, ambulance data have only been used for operational purposes rather than for assessing public health. The impact of weather and climate change on ambulance services around the world has received increasing attention in recent years but most studies have been single medical condition specific.

In England in 2013/14 more than 8.4 million emergency calls were received of which 71% required an emergency response. Ambulance call-out data offers a new and valuable (near) real-time source of public health morbidity information that can also be used to assess the impact of environmental conditions, such as temperature, upon human health. A detailed analysis of London Ambulance data is presented and compared to London temperature data recorded at a central London site (St James Park). Ambulance services are susceptible to disruptions from both hot and cold weather; disruptions primarily occur due to the increased number of emergency calls under such conditions. In London, the speed of ambulance response begins to suffer when the mean daily air temperature drops below ca. 2 °C or rises above ca. 20 °C. The degradation in response times is more rapid, with respect to change in temperature, at lower temperatures compared to higher temperatures, which result in three distinct temperature regimes <2, 2-20, >20°C. The baseline relationships established in this work will allow for the prediction of likely changes in ambulance demand (and illness types) that will be caused by seasonal temperature changes and increased frequency and intensity of extreme/severe weather events, due to climate change, in the future.
2.1 Introduction

London is the UK’s largest city, and covers 1572 km² (Smith, 2015). See Figure 2.1 for a map of London’s location in the UK. In terms of population density, London is by far the most densely populated city in the UK, with 4,779 people per km² (Allen et al., 2012). The Census that took place on the 27th March 2011 indicated that London’s population has reached 8.2 million, making it the most populous city (www.ons.gov.uk). The biggest rise in London’s population is forecast to be in the 65 plus age group and the overall population is forecast to exceed 9 million by 2021 and to be almost 10 million by 2031 (Dunnell, 2007). To ensure an effective ambulance service there must be capacity to answer all callouts in a timely manner. Therefore, accurate prediction of the daily demand for ambulances is critical to meet targets every day.

This study sets out to investigate the basic relationship between mean temperature (also some work on RH) and London ambulance callouts of Category A (Cat A) incidents.

Figure 2.2: Location of London in United Kingdom. Adopted from (https://www.google.co.uk/maps/place/United+Kingdom)
2.1.1 London weather

London, the capital city of the United Kingdom has broadly similar climates to the rest of the UK, having cool summers, mild winters, no wet or dry season, and often moderate to strong winds (Parker, 2016). London has a moderate oceanic climate (Köppen-Geiger climate classification: warm temperate, fully humid, warm summer (Kottek et al., 2006). Nonetheless, absolute minimum temperatures have reported to range from $-10.0 \degree C (14.0 \degree F)$ at St James Park, in central London down to $-16.1 \degree C (3.0 \degree F)$ at Northolt during January 1962 - the lowest official temperature in the London area. Kew's record showed temperature up to $38.1 \degree C (100.6 \degree F)$ which is recorded as the highest temperature in the London area, however, the lowest temperature to occur in recent years (21st century) is $-14.2 \degree C (6.4 \degree F)$ at Northolt during 2010 (Simon, 2010).

The Figure (2.2 A, B and C) below displays daily mean temperature histograms for the years 2003 and 2010 (which were the hottest and coldest year for over 100 years), and for the years 2000 to 2013. The histograms give clear indications of the range of temperatures experienced in London within the years.
Figure 2.3: Histograms of London temperatures. A- Year 2003 daily mean temperature record that indicates a very hot year having a significant summer heatwave with highest temperature over 30°C. B- Year 2010 daily mean temperature record that indicates a very cold weather having a significant winter coldwave. This has been reported in other papers. C- Represents the daily mean temperature from year 2000 to 2013, which shows an averagely warm weather from the past 13 years.
2.1.2 London Ambulance Service

The London Ambulance Service (LAS) is the busiest ambulance service in the UK. It is an integral part of London’s infrastructure ensuring that the residents and visitors to the capital of the UK are kept healthy. As such, the smooth running of the LAS is vital.

Within England there are currently 11 National Health Service (NHS) organisations that provide ambulance services and more than 8.4 million emergency calls were received in the year ending March 2014, of which 71% required an emergency (face to face) response (HSCIC, 2014). This is an average of 23,216 calls per day (16.1 calls per minute). The total figure of emergency patient journeys was 5.02 million and 1.99 million patients were cured at the scene (HSCIC, 2014). “The total cost of the NHS ambulance service is close to £2 billion per year, of which about £1.5 billion is spent on emergency services and the rest on ambulatory (pre-arranged) services” (Thornes et al., 2014).

The London Ambulance Service (LAS) employs nearly 5,000 staff, including 3,150 frontline staff across 70 ambulance stations serving the Greater London population of more than 8 million people. In 2014/15 over 1.9 million emergency ambulance calls were received in London (Figure 2.4) of which 1.1 million were responded to (on average 3,000 incidents per day) and nearly half a million were considered life threatening (Category A). This activity levels are steadily increasing with 9% more calls in 2014/15 than in 2013/14 (more than 400 extra calls per day) (Wu et al., 2012).
The LAS uses the Advanced Medical Priority Dispatch System (AMPDS) to initially triage the patient’s chief complaint. In this step, there are approximately 30 complaint types that are then further categorised as either Cat A (designated as life threatening) – with a target response of 8 minutes or less, and all other calls (not serious or life threatening) – with a target time agreed locally which is normally up to 19 minutes. The NHS specifies that 75% of Cat A incidents must be responded to within the 8 minutes target time that is from the time of the 999 call to the ambulance arriving at the scene of incident.

After an ambulance crew has seen the patient, a further refined illness code is specified with just over 100 categories used. For example, a patient originally identified as having breathing problems may, after assessment, be further considered as asthma, COPD, hyperventilating, respiratory or dyspnoea. This data is recorded on patient report forms, which are available for analysis a few weeks after the event. It is noted that whilst LAS ambulance staff are trained medical professionals their diagnostic categories may be changed or updated once the patient arrives at hospital. Some category codes such as “other medical conditions”, “generally
unwell” and “pain (other)” are general and non-specific but are understandable, in that in the short time available the patient could be urgently rushed to hospital without the exact illness/injury being obvious. There is also some potential for diagnosis error within the other illness categories. For example, asthma diagnosis is relatively easy in part because the patient is very likely to know the condition from which they are suffering, whereas “respiratory chest infection” is more prone to misdiagnosis because of the commonality of its symptoms with other call-out categories such as COPD. Overall the illness data is as reliable as other data sources confirmed by the consistency and repeatability of results.

2.1.3 LAS Ambulance Call out Categories

There are a hundred and three (103) Cat A illnesses with a total number of 3677454 call outs in only the year 2013. Figure 2.5 illustrates the cumulative plot of all Cat A illnesses. The figure reveals that the top 10 call-outs as defined by total incidence rate account for approximately 50% of all Cat A callouts, while the top 20 call-outs accounts for approximately 75%. The top 20 illnesses include: Other medical condition (illnesses with no designated name), Pain-other (pain with no associated illness), Respiratory chest infection (infection of respiratory tract organs), Dyspnnoea (shortness of breath or difficulty in breathing), Pain-Chest (likely chest pain), Generally unwell (fatigue), Alcohol related, Abdominal pains (pain in chest and pelvic region), Dizzy near faint/loss of coordination, Vomiting (throwing up), Collapse reason unknown (sudden falling with unknown reason), Cardiac chest pain ACS (acute coronary syndrome) (chest discomfort related to heart), Hyperventilation panic attack (over breathing that causes panic/anxiety attack), Epileptic fit (seizure), No injury or illness, Head injury minor, Seizure non ep, Pyrexia of unknown origin (fever of unknown origin greater than 38.3°C on several times), Pain back (pain in the back)
and Asthma (long-term lung disease that inflames and narrows the airways). These illnesses are shown on the bar chart (Figure 2.6) according to their respective ranking. For this reason, this study has limited its investigations to the top 20 medical conditions.

Figure 2.5: Cumulative plot of the Cat A illnesses showing top 10 and 20 categories making up ~50% and ~70% of the entire call outs, respectively.
2.2 Related studies

In recent years there has been an increasing awareness of the impact of weather and climate change on public health. In particular, the role of heat waves on public health has been widely investigated (Vardoulakis and Heaviside, 2012). Extreme weather condition impacts directly on ambulance services through additional calls because of the increased prevalence of temperature dependent call-out categories. Response times are affected by increasing call...
volume but weather can also directly impede response times by creating obstacles to reaching patients, for example, flooding, snow, ice, fallen trees and fog.

Previously, studies have investigated the impact of hot and cold temperatures in relation to same-day and lagged-days exposures (e.g., heat waves) (Guo et al., 2011; Yu et al., 2011). The study carried out by Thornes (2014) on the impact of extreme weather on ambulance performance examined the negative impacts of extreme cold weather. It was shown in the study that there is a considerable scope to improve understanding across a number of issues and there are potentially significant links between ambulance demand/performance and extreme weather and climate change. However, limited efforts to study the impact of temperature on ambulance attendances have been made (Cerutti et al., 2006; Nitschke et al., 2011). An increased ambulance call-out and response times with significant link between severe weather were evidently revealed after comparing 5 years data of daily air temperature with ambulance call-out for Birmingham (Thornes et al., 2014). Furthermore, the figure of ambulance call-outs increased through the heat wave of August 2003, while during the coldest December (beyond 100 years), “the response rate fell below 50% for 3 days in a row (18-20 December 2010) with a mean response time of 15 min” (Thornes et al., 2014). The results showed that a decrease in the air temperature by 1°C leads to a reduction of 1.3% in ambulance call-out performance (Thornes et al., 2014). The ambulance call-out performance is the number of Cat A that is responded to in the 8 mins. Nonetheless, there have been very few studies of the impact of severe weather and climate change on ambulance operations in the UK (Thornes et al., 2014) but there have been several recent studies where the ambulance based studies looked at the negative impact of heat waves on ambulance performance in other countries namely: Australia: Adelaide (Nitschke et al., 2011), Brisbane (Turner et al., 2012), and Sydney (Schaffer et al., 2012); Canada: Toronto (Bassil et al., 2010; Dolney and Sheridan, 2006); Italy: Emilia-Romagna (Alessandrini et al., 2011) and Florence (Petralli et
al., 2012); Switzerland: Ticino (Cerutti et al., 2006) and United Kingdom: London (Thornes et al., 2014; Wolf et al., 2014).

For example, during warm weather in London, for every 1°C above a mean temperature of 20°C, it has been shown that the total number of ambulance incidents increases by 1% on average. There are fewer studies that looked at cold waves: Australia: Brisbane (Turner et al., 2012) and the United Kingdom: London (Thornes et al., 2014, showed that for December 2010, the coldest December for 100 years, the daily number of Cat A incidents for the London Ambulance Service was nearly 20% higher than November 2010). These results show that severe cold weather has a significant negative impact on ambulance performance. For example, during cold weather in London, for every 1°C below a mean temperature of 2°C, it has been shown that Category A performance declines by 1.5% (Thornes, 2014).

A study in Hong Kong looked at the impact of a range of weather parameters (temperature, humidity, air pressure and cloud) on the daily demand for ambulances (Wong and Lai, 2010) and concluded that:

“The presence of strong weather effects among different target groups indicates the possibility for the development of a short-term forecast system of daily ambulance demand using weather variables. The availability of such a forecast system would render more effective deployment of the ambulance services to meet unexpected increases in service demands” (Wong and Lai, 2010).

Such a forecast system would enable much better handling of ambulance demand during severe/extreme weather events likely to be enhanced by climate change. In Germany (Bavaria) the impact of a range of weather conditions (2006-2007) on COPD and the effect on ambulance incidents has been examined (Ferrari et al., 2012). Also a few studies have
looked at the carbon footprint of ambulance services for example in Australia (Brown et al., 2012) and the United States (Chung and Meltzer, 2009).

Most of these international studies are concerned with the negative impacts of heat waves on ambulance. And a number of the investigations have stated that a strong association between same-day temperature and emergency admissions for cerebrovascular (Makie et al., 2002; Wang et al., 2006) and respiratory disease do exist (Abe et al., 2009). Particularly, the ambulance response calls and intense heat study in Toronto Ontario, Canada (Dolney and Sheridan, 2006). After studying the difference in calls across the town, the finding indicated that increased call rates during hot days were linked to both day-of-week factors and population travels (Dolney and Sheridan, 2006). Among the recent studies carried out, Toronto, Ontario, Canada (Bassil et al., 2010) and Emilia-Romagna, Italy (Alessandrini et al., 2011) tried to measure the temperature-ambulance attendance association for cardiovascular and respiratory disease and temperature exposure, after controlling for interfering factors. An increase in ambulance attendances for both and other non-traumatic diseases was observed that is linked particularly to the summer days temperature (Turner et al., 2012). The impact of air pollution (PM$_{10}$) on ambulance incidents has previously been shown to be significant in the Italian Region of Emilia-Romagna (Sajani et al., 2014). Since particular “effects on ambulance attendances were found to differ from those on hospital admissions or mortality and also between the different attendance categories; it would therefore be useful to compare different exposure-response relationships in future research” (Cerutti et al., 2006). Based on public health policy (Dolney and Sheridan, 2006), detecting the initial indications of temperature effects on human health that cannot be investigated using death and hospital admissions data could be supported by studying ambulance attendances data instead (Turner et al., 2012). A time-series study in Huainan, China examined their impacts on emergency ambulance dispatches under different temperature metrics and reported that both extreme
heat and heatwaves were significantly associated with increases in emergency ambulance dispatches, and their effects appeared to be acute (Cheng et al., 2016). A significantly increased risk for all attendance categories was found to be associated with cold temperature effects indicating that the short exposure lags considered in the Italian study (Alessandrini et al., 2011) might have followed in an underestimation of the cold impact. In another study by Turner et al. (2012), related trends across all the attendance groups were observed, after immediate exposure to heat.

2.3 Methodology

2.3.1 Data sets

London meteorological variables (temperature and relative humidity (RH)) and ambulance callouts for Cat A illnesses data sets for the period of 01-04-2003 to 31-07-2013 were analyzed. LAS provided daily-anonymised ambulance data, which provided information on callout category. Meteorological data was that of St James Park observatory (SJP, 54.97554 °N and -1.62162 °E), which occupies a central position in London, a location that is approximately within the centre of the LAS’s geographical remits. SJP is the longest-record Central London meteorology available (Jones and Lister, 2009). The hourly output temperature data from this station was obtained via the British Atmospheric Data Centre (BADC) (Bhaskaran et al., 2013). Temperature is the measurement of hotness or coldness of an environment or object, while RH is the percentage of the partial pressure of water vapour referenced to the saturation vapour pressure of water at a given temperature (Seinfeld and Pandis, 2016) that varies, depending on the interplay between temperature and gas phase water concentration.
2.3.2 Data analysis

The meteorological hourly data set was converted to daily mean averaged data using the “dplyr” package in R statistical software (Wickham and Francois, 2015). There were no missing values for the entire London ambulance data set analysed, however, after conversion of the hourly temperature data to daily mean data, 10 days data were missing amongst 3134 days. These days were omitted from the analysis. Among the 103 categories A (life threatening) illnesses, only the top 20 most common call out categories were analysed in this study. All data sets used were initially tabulated in excel where the mean data were calculated and subsequently analysed in R statistical software (R version 3.0.2) using the appropriate packages (lubridate, timeSeries, TTR, zoo, xts, akima, abline, car and plotrix). The highest mean temperature and RH in the data were 27.8°C and 100% respectively while the lowest were -2.4°C and 40.9% respectively. Initial tests showed that there was little difference in outcomes between the use of mean average daily temperature, versus minimum or maximum daily temperature. However, Guo et al., (Guo et al., 2011) mentioned that mean temperature was found to be a better predictor and thus was used as the temperature indicator in this study.

In this study, various statistical analyses were performed, including simple descriptive statistics and time series analysis. For the time series analysis, the ambulance callout data was de-trended to remove any long-term trend using either a linear fit or polynomial fit of the total time series. The de-trending analysis splits the data into three components, namely: long-term trend, seasonality and residual components. The seasonality component was used for understanding the relationship between illness codes and temperature using linear regression analysis. The joint effects of temperature and RH on some of the illness codes were also observed using multi linear regression. Lastly, the statistical method used to test for statistical significance of the results was the lm function in R statistical software, which
provide p-values. The p-value test was performed on individual de-trended callouts versus temperature.

2.3.2.1 Time series analysis

To investigate the relationship between the meteorological variables (temperature and RH) and the illness codes, both data sets were converted into weekly data sets from daily data. The daily ambulance callout data was transformed into weekly data through summing of the daily data average over seven days. The weekly temperature data used is the mean average temperature. It is worth noting that strong correlation does not imply causation. Correlation denotes a statistical relationship between variables. The strength of the correlation can be measured using the R-squared ($R^2$) metric, which can be a strong, medium, weak or no relationship. In this study, the $R^2$ values are defined as: strong (0.5-0.7), medium (0.3-0.49), weak (0.19-0.29) and insignificant (0-0.18) correlations respectively. The $R^2$ metric measures the fraction of the sample variance that can be explained by the correlating variable.

To probe the effect of temperature, and hence seasonality on the call-out rates, the long-term trends need to be removed from the data. There are various statistical methodologies that can be used to remove long-term trends (Bhaskaran et al., 2013). In this study, the de-trending was achieved through division of the observed data with the cubic least squares fit (by subtracting cubic model from the data produced) of the observed. De-trending removes background trend and any long-time trend from the data alongside the weekly conversion. De-trending data also focuses analysis on the variations in a data set and enables future prediction of values because the background trend and long-time trend have been eliminated. After the long-term trends of the different callout categories have been removed, then the temperature dependence of the different categories was analysed. Time series graphs of the
variables were then generated to visualize the effect of the temperature on the illness codes in terms of ambulance usage and a scatter plot for the de-trending outcome was also plotted.

For the correlation analysis, scatter plots of the relationship between mean temperatures versus de-trended illness code callouts were created. Time lag of the data was also carried out, with the time lag either in days or weeks: 0 to 3 weeks, and 0 to 15 days. The $R^2$ values were recorded. The term time lag effect refers to the delay between the time of an intervention or exposure onset, such as the date on which a person gets exposed, and the subsequent development of a health outcome (Gail, 2005). The lag analysis defines how $x$ affects $y$ over time, that is the effect of a regressor $x$ on $y$ occurs over time rather that all happening at once. Generating $R^2$ values of the relationship between the variables of interest explained the time lag relationship of the variables. Lastly, scatter plots of the lag days against the $R^2$ values were generated.

Further analyses were performed which investigated the joint effects of temperature and RH using multi linear regression. Linear regression and cubic model were performed as well as residual analysis (observed differences between dependent variable and predicted value) to enable prediction. Multi linear regression was for predicting a dependent variable using values of more than one independent variables, while linear regression was used to ascertain the linear relationship between dependent and independent variables. This aids in forecasting dependent variable based on the values of independent variable.

### 2.3.2.3 Air temperature versus ambulance response time

Daily data was extracted from the LAS for 2003-2013 including the number of calls, responded incidents, the number of Cat A calls, the % of responses within 8 minutes and
illness codes. During those years 14,717,476 calls were received; 9,947,927 incidents were responded to with a frontline vehicle (68%) and 3,359,572 Cat A life threatening calls were assessed (23% of all calls). The response data was then compared to mean daily temperature data from St James Park (SJP, longitude and latitude coordinates 54.97554 °N and -1.62162 °E) in London over the same period. The heat waves of 2006 and the warm summer of 2013 plus the very cold December of 2010 are present within the dataset providing a wide cross section of weather events.

2.4 Results and Discussion

An increase on the rate of ambulance usage was observed over the time period studied i.e. ambulance usage is increasing year on year. The rate of increase is larger than the population increase over the same time period. The callout frequencies of many different medical conditions are dependent upon the meteorological conditions. In particular, temperature is a good predictor of callout rate with both negative and positive temperature dependencies observed for different medical categories. Different categories of ambulance callout have different time lags associated with them depending on the category. In broad terms, categories involving illness, which require an incubation period, will have a time lag (time interval between two related spectacles that is, a cause and its effect, here is temperature /RH and their effect on human health) whereas accidents tend to have zero time lags. The outcomes show statistically significant relationship between mean temperature and some of the analyzed illnesses of the London ambulance callout even though some were weakly correlated. Furthermore, there were time lagged-effects observed within 0-15 days and 0-3 weeks as well. Among all the top 20 illnesses, respiratory chest infection had the highest correlation with temperature.
2.4.1 De-trending effect

The outcome of de-trending the data of respiratory chest infection is illustrated in Figure 2.7 A & B below respectively. The figures show how long-term features that obscure the relationship of interest have been eliminated. Additionally, the noise level was removed using weekly data as opposed to daily data. The cubic fit line was fitted along the whole data, which indicated that all the data has been analysed.

![Graph A](image1)

![Graph B](image2)

Figure 2.7: Time series data of Respiratory chest infection before (A) and after de-trending (B) respectively. The data is represented in black and a cubic fit line in red, which indicates de-trending.
2.4.2 Long-term increase in ambulance usage

Example of ambulance time series data are shown in Figure 2.8, which shows time series data from 2005-2013 of the daily rate for the total LAS ambulance Cat A life threatening call-outs; as well as “respiratory chest infection” and “abdominal pains” and daily air temperature. The two call-outs categories represent temperature dependence and no temperature dependence call-out categories respectively. All the time series data show a significant long-term increase in call-out frequency. The increase from 2005-2013 is non-linear and is dependent on category but the approximate increase over all categories is a near doubling. This increasing trend can be roughly separated into two distinct phases characterised by a slow increase between 2005 and 2009, followed by a much more rapid increase between 2009 and 2013. This increase can only partly be explained by the increasing population of London as there have been other changes within London’s population demographics such as the increasing age of the population. In broad terms, the young and the old are more susceptible to conditions requiring the ambulance service, and health care in general, compared to the in between ages. However, it is noted that we did not have access to age dependent data; so, all analyses are conducted on the total London population. Furthermore, it is likely that significant drivers of the change in ambulance call-out rate are due to how the London population utilizes the ambulance service. Some of this change may likely be driven by an increasing pervasiveness of mobile phone usage (Wu et al., 2012), that makes calling an ambulance easier whenever required. This may also suggest that more people are aware of using ambulance rather than going to the Accident and Emergency unit because of the long waiting time and or there is an increase in immigrants and/or difficulty in scheduling an appointment with a GP. Other social changes will also likely influence call-out rates such as changing habits with respect to use of the National Health Service (NHS).
Figure 2.8- Time series data from 2005 to 2013 showing the London Ambulance daily Category A call-out frequency for selected categories compared to the average mean temperature as recorded at St James Park, London. The ‘respiratory’ and ‘abdominal’ refer to the call-out categories ‘respiratory chest infection’ and ‘abdominal pains’, which represent temperature dependence and no temperature dependence call-out categories respectively.
2.4.3 The influence of air temperature on ambulance response times

Figure 2.9 (below) demonstrates how both call-out volume and response rate performance vary with mean daily temperature. The call-out volume has the long-term trend removed as detailed above and values shown are representative for the call-out volume received in 2013. It can be seen that as temperature rises above ca. 20 °C and goes below ca. 2 °C the total ambulance call-out volume increases and the percentage of responses within the 8 mins target reduces. Both call-out volume and percentage of responses rise in a near-linear fashion beyond the hot and cold threshold temperatures. Figure 2.9 (bottom panel) shows that, performance drops off more quickly as the mean temperature drops below 2 °C compared to the reduction in performance when the temperature rises above 20 °C. A 20% increase in daily callouts, compared to the average, leads to a decrease in performance (measured as % response in 8 min) of 14.4% and 8.2% for temperatures below 2 °C and temperatures above 20 °C, respectively. This difference between hot and cold periods can partly be explained because slippery roads due to ice and snow affect performance at low temperatures whereas in warm temperatures the roads and traffic are less likely to be affected. This Figure shows that the weather does not have to be severe for an impact on ambulance services to occur at low or high temperatures.

There is also a seasonal temperature change in the number and type of incidents, which means that the performance of the ambulance service in London is marginally better in spring and autumn than in summer and winter. Unseasonably warm and cold weather both exaggerate these variations and cause a significant reduction in performance especially during ‘heatwaves’ and ‘coldwaves’.
Figure 2.9: London mean daily temperature versus ambulance call-out metrics (2003-2013). Top panel- temperature dependence of response rate as % of category A incidents responded to within 8 minutes averaged for each 1°C temperature bin. Error bars represent 1°. Red dashed line shows NHS target of 75% or responses to be completed within 8 minutes. Middle panel illustrations the temperature dependence of ambulance call-out volume. Long-term trend in data has been removed (see main text for details) and the values provided are representative for the year 2013. Error bars
2.4.4 Basic relationship between temperature, RH and respiratory chest infection

Figure 2.10 (A and B) shows the time series of weekly averages of daily data (2009-2013) for temperature, RH and LAS ambulance call outs of respiratory chest infection and contour plot of the three variables. Comparing the time series data of the three variables reveals the relationship to be seen clearly. As temperature increases, RH declines which is normally the default relationship between the two variables. However, the correlation between temperature and respiratory chest infection is negative ($R^2 0.5$), while respiratory chest infection with RH is positive ($R^2 0.2$). This is in partial agreement with a study conducted by Mäkinen et al. (2009) who concluded that cold temperature and low humidity were linked with increased episode of RTIs, and a reduction in temperature and humidity preceded the beginning of the infections (Mäkinen et al., 2009). The contour plot (Figure 2.10 B) demonstrates interestedly how respiratory chest infection call out rates rise with temperature fall and RH increase. In particular, there is a vital spot at temperature below ~5°C and RH above ~75 % where respiratory chest infections are most common. Among the analyzed categories, respiratory chest infection callout rate had the strongest correlation with the variables.
Figure 2.10 A: Time series of weekly averages of daily data (2010-2013) of respiratory chest infection call out rate (blue), RH (black) and Temperature (red). B: presents a contour plot of RH, Temperature and Respiratory chest infection (log10 transformed) indicating the visual relationship between the 3 variables. The arrows signify clear temperature (red) and of RH (purple) effects. For the contour plot legend, any value beyond 1 represents above average callout and less than 1 is below average callout.
2.4.5 Ambulance Illness codes versus Air Temperature

The data collected were studied using time series analysis where all the time series data showed a significant long-term increase in callout frequency. The long-term increase is typically non-linear and is category dependent. It is also observed that certain categories show a clear seasonality. This seasonality, where present, is hypothetically driven by temperature.

The rationale behind the use of weekly data is twofold: firstly, it increases the statistical robustness of any correlations observed with temperature. Secondly, weekly data removes ‘day of the week’ effects. For example, call-out categories associated with alcohol show a clear weekly cycle: data for Monday to Thursday have near identical frequencies but a significant frequency increase is associated with the weekend that peaks on Saturdays. It is noted that weekly data does not remove the effects of holiday/special days on the data.

2.4.6 Weekly correlation between temperature and illness codes

Sometimes time series data can display what appear to be obvious trends, however, it may be hard to explain this data as an overall trend. Breaking down the series into different components may allow easy modelling of each part. In this example, the scatter plots of weekly de-trended data for respiratory chest infection and total of the entire top twenty illnesses against mean temperature is demonstrated (Figure 2.11 A & B). This indicated that analysing the data of a single variable or in smaller groups in comparison to analysing mass of data sets with multiple variables may yields better results and increases possibility of future forecasting.
Figure 2.11: Scatter plots of weekly de-trended data for respiratory chest infection (A) and total of the entire top 20 illnesses (B) against mean temperature.

Table 2.1 shows the weekly correlation coefficients between $R^2$ values of de-trended data of the top 10 Cat. A illnesses and mean temperature from 0 to 3 weeks lag. There exists statistically significant correlation between temperature and some of the illness codes analyzed. However, some were only weakly correlated. Respiratory chest infection and dyspnoea were found to have the strongest correlations with the mean temperature (1-week
time lag). For each time lag, there is little or insignificant (about 1%) increase in the $R^2$ value for all the illnesses. With the exception of dizzy/near faint/loss of coordination, however, there is also an insignificant (1%) decrease in the values. Many of the categories show the correlations at time lag = 1 week (see Table 2.1) with the exception of alcohol related and dizzy/near faint/loss of coordination.

<table>
<thead>
<tr>
<th>Illnesses</th>
<th>0 week time lag</th>
<th>1 week time lag</th>
<th>2 week time lag</th>
<th>3 week time lag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory chest infection</td>
<td>0.55</td>
<td>0.58</td>
<td>0.55</td>
<td>0.42</td>
</tr>
<tr>
<td>Dyspnoea</td>
<td>0.53</td>
<td>0.58</td>
<td>0.54</td>
<td>0.49</td>
</tr>
<tr>
<td>Generally unwell</td>
<td>0.45</td>
<td>0.52</td>
<td>0.50</td>
<td>0.41</td>
</tr>
<tr>
<td>Vomiting</td>
<td>0.35</td>
<td>0.47</td>
<td>0.42</td>
<td>0.35</td>
</tr>
<tr>
<td>Alcohol related</td>
<td>0.48</td>
<td>0.30</td>
<td>0.24</td>
<td>0.30</td>
</tr>
<tr>
<td>Other medical condition</td>
<td>0.17</td>
<td>0.28</td>
<td>0.28</td>
<td>0.23</td>
</tr>
<tr>
<td>Pain chest</td>
<td>0.12</td>
<td>0.19</td>
<td>0.25</td>
<td>0.18</td>
</tr>
<tr>
<td>Pain other</td>
<td>0.05</td>
<td>0.08</td>
<td>0.12</td>
<td>0.10</td>
</tr>
<tr>
<td>Abdominal Pains</td>
<td>0.00</td>
<td>0.01</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Dizzy/loss of coordination</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Total for all 103 categories</td>
<td>0.11</td>
<td>0.24</td>
<td>0.24</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Table 2.1: Correlation between temperature and the top 10 of the medical categories. Each box on the table represent 0 – 3 weeks lag time relationship between temperature and the specific category, which are highlighted according to the level of correlation strength. **Red** =Strong correlation (0.5-0.7), **Yellow** = Medium correlation (0.3-0.49), **Green** = Weak correlation (0.19-0.29), **Grey** = Insignificant correlation (0-0.18)
Note: All values were rounded up to 2 decimal places.

The simple linear temperature dependence model used on the long term de-trended data set of the top 20 callout categories revealed some illnesses having statistically significant negative temperature dependence such as other medical conditions ***(p<0.001)***, dyspnoea...
**(p<0.001)**, generally unwell ***(p<0.001)**, pain (other) ***(p<0.01)**, respiratory chest infection ***(p<0.001)**. While only alcohol related shows statistically significant positive temperature dependence ***(p<0.001)**, some among the remaining categories show no statistically significant temperature dependence such as pain (chest), abdominal pain, dizzy (near faint or loss of coordination) and so on. P value is the probability that results have occurred by statistical accident. The lower the p value the higher the statistical significance and visa versa. Here, the P values are low which suggest that the results obtained did not occur due to statistical accident. However, it is noted, that having low statistical significance is not a conclusive result that relationship does not exist between the variables. Figure 2.12 A, B and C respectively illustrate the scatter plot for a negative (respiratory chest infection), positive (alcohol related) and no temperature dependence (abdominal pains) respectively. This indicates that not all categories are temperature dependent according to this research; however, some might be dependent on other environmental factors such as pollutant and pollen count (see Chapter 6). The time series analysis methodologies developed in this chapter can be used for assessing the effect of other environmental variables on ambulance callout rates.
Figure 2.12: Scatter plots for a negative temperature dependence (respiratory chest infection), no temperature dependence (abdominal pains) and positive temperature dependence (alcohol related) using de-trended data sets.
The linear temperature dependence model does not provide any indication of the rationale behind the temperature dependencies but the patterns are clear to see and hypotheses can be generated from the data patterns.

2.4.7 Daily temperature time lag effects on illness codes

The daily results mirror the weekly results from above but potentially give a better idea about incubation rates albeit with poorer statistics (because of less data density). The study carried out in Toronto described huge increase in ambulance response calls that was observed in the summer period. While in Italy, it was seen for increasing temperature, and was linked to same-day heat effects precisely for other non-traumatic and respiratory illnesses (Alessandrini et al., 2011). However, in this study, for all the callouts analysed, with the exception of dizzy/near faint/loss of coordination that had same-day effect (which could be as result of the impact of abrupt change in weather) and alcohol related had one day effect, it was observed that all the remaining top illnesses had a similar pattern of time-lagged effects of at least 5 days when analysed as daily data (data not shown). Figure 2.13 shows the $R^2$ values plots of the top 10 illnesses against time lag within 0-15 days. Respiratory chest infection had 5 days lag effect, which may suggest an incubation period of about 5 days for the infection to develop fully. For alcohol, it was just a 1-day lag effect and a drastic drop afterward, perhaps due to hangover. In the case of asthma, studies have shown that it is influenced by temperature, however the statistical relationship in this study is weak. This may be as a result of “an initial large number of transfers and subsequent admissions of seriously ill patients to hospital, thereby removing high-risk individuals from the general population that would further use ambulance services” (Turner et al., 2012) and/or the increased use of antihistamine (drug for allergies).
The common pattern among the top categories, which is the slight drop in the lag effects when analyzed as daily data, excluding dizzy/near faint/loss of coordination is consistent with the harvesting phenomenon discovered in studies of temperature and death (Yu et al., 2011). Although in this part of the study, cold and hot temperature were not segregated but analyzed as a whole, the results have proven a relationship between temperature and some medical conditions. Other studies have proven that heat effects on some diseases are short term and detected instantly (Bassil et al., 2010; Ye et al., 2012), which is usually strongest within the first 1-3 days following exposure, before reducing in magnitude (Ostro et al., 2010). The lagged effect results of temperatures on the illnesses of up to 15 days following exposure support opinions from earlier research (Hajat et al., 2002). This opinion also supports the research findings.
Figure 2.13: R squared values of the correlation of the top 10 illnesses with temperature, plotted against time lag within 0-15 days.
Note for some categories there appears to be a lagging effect, e.g. abdominal pains, but the \( R^2 \) values are very low so no correlation can be assumed. For some categories, there exist longer time-lagged effects for the categories: generally unwell, other medication and vomiting amongst the other illnesses, which may suggest a longer or different incubation time for the medical condition incorporated within the categories.

2.4.8 Illness Codes versus winter and summer seasons

Figure 2.14 (below) provides the percentage changes for the summer (June, July and August) and winter (December, January and February) seasons compared to the total de-trended data set. It can be seen that the warmer temperatures associated with the summer season lead to a reduction in the top 10 callout categories associated with negative temperature dependence and an increase in the “alcohol related” category, which has positive temperature dependence. The winter season has the opposite effect to summer for the same reasons; lower average temperatures lead to an increase of incidence for the negatively temperature correlated categories and a decrease in the “alcohol related” category. In addition to the summer and winter seasons, the individual months of July and December are investigated which allows for the effect of a heat wave (July 2006) and a cold wave (December 2010) to be investigated with the de-trended data sets. The average mean weekly temperatures in July 2006 and December 2010 were 21.4 ± 3.4 °C and 1.4 ± 1.2 °C, respectively. However, there can be a standard error of 1. The average July and December temperatures over the eight-year study period were 18.6 ± 2.8 °C and 6.0 ± 1.4 °C, respectively. The average summer and winter temperatures over the eight-year study period were 17.7 ± 2.7 °C and 5.9 ± 1.4 °C, respectively. As expected, the 2010 cold wave led to significant increases, compared to the average December and winter, in all of the negatively temperature correlated categories. The
“alcohol related” call-out did not reduce as much as might be expected (based on temperature alone) in December 2010 and the average December categories, but this is likely due to the confounding influence of seasonal Christmas period alcohol drinking. The average summer, average July and the July 2006 heat wave call-outs behave mostly as expected with temperature dependencies playing a central role.

However, there are a few unexpected results. For example, the “respiratory chest infection” category call-out frequency increases in the July 2006 heat wave when a prediction based just on a linear temperature dependence would suggest a decrease in call-out rate. This indicates that factors, in addition to temperature, were likely to be significant during the heat wave or alternatively, the temperature dependence is non-linear at higher temperature. The increased levels of pollution (such as O3 often increases in hot temperatures because of increased VOC loading) associated with the heat wave are a likely cofactor, which the simple temperature dependent model does not take into account (Stedman, 2004). The impact of air pollution (PM10) on ambulance incidents has previously been shown to be significant in the Italian Region of Emilia-Romagna (Sajani et al., 2014)
Figure 2.14: Seasonal percentage differences in ambulance call-out rates for the top 10 categories. Winter is defined as the months of December, January and February. Summer is defined as the months of June, July and August.

The fact that the research was limited to the top 20 categories does not mean that the outstanding 83 categories are not temperature dependent. They may display a significantly larger temperature effect or other influential factors such as pollutants and pollen counts. The overall outcome of the findings suggests a statistical significant relationship can be found between most call-out categories and temperature.
2.4.9 Forecasting of ambulance call out rates

Currently daily estimates of the number of LAS ambulances likely to be required for the week ahead are based on statistics for the same days of the year for the last 3 years (Thornes et al., 2014). This takes into account weekends, national and school holidays, but only accounts for changes in the weather on a seasonal basis. However, the weather is rarely the same on a particular day, or in a particular week, from year to year. Therefore, further research to enable bespoke weather forecasts to be built into the ambulance service prediction models is recommended. Better prediction of call-out rates would allow for improved operational resilience, as well as reducing air pollution from idling ambulances on urban streets. Warming temperatures, due to climate change, may reduce the total number of ambulance callouts in winter and conversely increase them in summer, although changes in population size and structure and in other climatic factors (e.g. precipitation), not examined in this study, may have the opposite effect. There is also evidence that heatwaves and coldwaves could increasingly cause increased demand and ambulance response time delays.

Using the relationship between temperature and ambulance call-outs, after controlling for air pollution and other confounding factors such as influenza, could inform future studies and help forecast ambulance callout numbers up to a week ahead (the time period for which robust meteorological forecasts are available). In addition to short term forecasting, the effect of climate could be used for yearly projections. Figure 2.15 below shows an example of statistical prediction of the 2012/2013 respiratory chest infection callouts. The model uses the previous three years of data to train both the long-term trend and temperature dependence of the model. It is noted that the actual measured temperature used in the forecast model would not be available in advance. However, long term climate projections are available. It can be seen that the model captures much of the detail of the actual ambulance callouts. The only
major feature missed by the model is the influenza season peak which occurs at the end of 2012. It indicates that good long-range ambulance callout rate projections can be generated if the appropriate meteorological forecasts, of sufficient skill, are available.

Figure 2.15: Forecast prediction of 2012/2013 respiratory/chest infection call out rates. The model is trained using 3 years of data from 2009-2012 shown by black line. The 2012/2013 forecast is shown by the red line and the actual call out for 2012/2013 is shown in green. The residual between the forecast and actual is shown by the blue line. The grey dotted lines indicate the +10 and -10 daily call out rates, and the dashed line gives the call out rate equal to zero.

The residual part of the graph represents the percentage of the validation data not clarified by the model. Agreeing to the independence test criteria, a good model has residuals uncorrelated with previous records (Sharma and Sutton, 2012). “Evidence of correlation indicates that the model does not describe how part of the output relates to the corresponding input. For example, a peak outside the confidence interval for lag k means that the output y(t) that originates from the input u(t-k) is not properly described by the model” (Sharma and Sutton, 2012). This residual is as a result of flu peaks that is usually not temperature
dependent but as a result of holidays when people tend to move around sharing their gems and so on. For example, it is well known that New Year’s Day is the busiest day of the year for the ambulance service as mentioned previously and weekly data does not remove the effect of New Year’s Day, or other holiday/special days, but it does lessen their impact. For now, it was challenging to predict the residual peaks using this model. Going further, an attempt to uncover any cyclical trends or use more sophisticated methods might allow more accurate forecasting analysis to be carried out. The evidence presented in this section supports the opinion that ambulance attendance callouts records are an effective and well-timed source of data that can be used for health early warning systems. The more accurate the forecast is the better an early warning tool for health surveillance systems will be. An effective forecast will allow the ambulance services to prepare ahead of time leading to more lives being saved.

2.5 Conclusion

Ambulance services are placed under stress due to increased demand whenever the weather is severe – often at a time when patients are also under increased stress – particularly during heatwaves and cold weather. In this study, the effect of mean temperature was investigated but in theory any meteorological or pollutant related parameter could be investigated. This research illustrates the concept with air temperature but information on snow, ice, gales; heavy rain, floods, air pollution and fog could also be beneficial. The study established that there is statistically correlation between temperature and different medical condition within 0-15 days and 0-3weeks lagged effects on some of the illnesses. The weather impacts directly on day-to-day operations whilst the climate contributes to the level of service required (e.g. the total number of staff and ambulances). Given the existing lack of research into
temperature effects on London ambulance callouts, the study will contribute to an understanding in this perspective and may also serve as an early warning information for ambulance driver and hospital staff to prepare way ahead of time. As the climate changes affecting the frequency of hot and cold weather events, the ambulance service needs to become more resilient and would be better prepared by using bespoke weather forecasts and climate predictions.

In summary, it can be concluded that temperature has influence on many categories of illnesses. Albeit, different patterns are observed for different callout categories. Callouts in most categories show significant relationship with temperature. The sign of correlation varies with category (e.g alcohol vs dyspnoea) with most negatively correlated and time lag effects were observed for some of the call-out categories (typically human-contact related illnesses). This is the first study that has shown the effects of ambient temperature and RH on London ambulance callouts for specific illness categories. The relationships elucidated in this chapter need to be understood before the pollen work in Chapter 6 is undertaken because the temperature is a dominant effect.
CHAPTER 3

Laboratory analyses

In this chapter, the experimental methodologies and analytical techniques used in both exposing pollen grain to atmospheric gases and determining the degree of pollen nitration are described. Certain protocols were used according to manufacturer’s instructions while others required bespoke tailoring to meet the study aims. For precision and accuracy, all experiments were performed in triplicate.

Figure 3.1: Flow diagram of the laboratory techniques used in this study.
In line with the objectives of this thesis, Figure 3.1 provides a graphical overview of the laboratory analyses used for the study. It starts with the pollen grain, which contains proteins (including the allergenic proteins), being exposed to atmospheric gases. Post exposure, the pollen is subjected to analysis to explore the effects at a proteomic level using four different techniques (explained in detail in the methodology section), which includes dot blot, Western blot, in-gel digestion and TMT labeling (Tandem Mass Tag) experiments.

3.0 Introduction

Air pollution has shown to interfere with human health, however, its effect on pollen proteins are still under study (Sousa et al., 2012). It has been reported in earlier studies that these pollutant cause PTM (post-translational modification) of the pollen protein, in particular the incorporation of a nitro group (NO$_2$) on the amino acid (tyrosine) of the protein a process termed nitration. Laboratory findings on the kinetics and degree of the interactions of pollution, in both gas and particulate phase, are relatively scarce (Chassard et al., 2015).

In this chapter, the PTM of birch pollen proteins are measured after interaction of the pollen with well constrained environmental conditions. In particular, the pollen grains are subjected to different RH conditions, and different concentrations of NO$_2$ and O$_3$. Furthermore, the physical condition of the pollen is modified either by crushing or immersion in rainwater to emulate real life cycle scenarios of the pollen.

As mentioned earlier, RH is the percentage of the partial pressure of water vapour referenced to the saturation vapour pressure of water at a given temperature (Seinfeld and Pandis, 2016). NO$_2$ comes predominately from automobile exhaust, either directly from the exhaust (largely from diesel vehicles) or from the partitioning of nitric oxide (NO, from both petrol and diesel
vehicles), that comes from the exhaust, and is converted to NO$_2$ in the atmosphere via reaction with O$_3$.

$$\text{NO} + \text{O}_3 \rightarrow \text{NO}_2 + \text{O}_2$$

O$_3$ in the troposphere (lower atmosphere where pollen largely resides) comes predominantly from the interaction of nitrogen oxides (NO$_x$ = NO + NO$_2$) and volatile organic compounds (VOCs) and sunlight (Seinfeld and Pandis, 2016).

The concentrations of NO$_2$ and O$_3$ used were such that the pollen exposures to these pollutants were atmospherically realistic. The crushing of the pollen mimicked the mechanical and chemical stresses encountered by the pollen grains in the outside real world. Immersion in rainwater mimicked the effect of rain on the pollen whilst still in the flower (catkin) of the birch tree.

### 3.1 Methodology

Typically, proteomics workflow consists of protein extraction, quantification, separation, identification, data analysis and interpretation (Carpentier et al., 2008). The success of the entire experiment is determined by the solubilization/precipitation process which is also a vital step and strongly affects the quality of the final results (Martínez-Maqueda et al., 2013). This process leads to separation of proteins in the sample selectively from different substances that may affect the proteomic assay (Berkelman, 1998). Taking into account the immense diversity of proteins and the huge amount of interfering contaminants present, simultaneous solubilization of all proteins remains a great challenge (Martínez-Maqueda et al., 2013) and requires a robust technique for a successful extraction product. In view of all that has been mentioned so far, one may accept that sample preparation has an intense effect
on the final product of protein and peptide separation and their subsequent analysis.

The preparation of sample from most materials for proteomic analysis requires homogenization/cell disruption, which enables access to protein content usually concealed with the cell wall of the organism or plant material. Various chemical and physical techniques can be used to destroy the cell wall such as mechanical homogenization, ultrasound homogenization, pressure homogenization, temperature treatments, and osmotic and chemical lysis (Martínez-Maqueda et al., 2013). Because their tissues are rich in proteases and other intrusive compounds, plants are generally more problematic for protein extraction (Wang et al., 2008) so cell disruption is required before they can be totally solubilized and extracted. In this study, the mechanical homogenization (automated) was used because pollen grains have an even harder cell than other plants cells; in another section, grains were ruptured in rainwater (osmotic lysis).

3.1.1 Materials

3.1.1.1 Pollen samples

Birch (Betula pendula) is an ornamental tree that produces staminate flowers (Cheng et al., 1999). Birch pollen flowers usually in April and May and is one of the main causes of allergic reactions particularly hay fever in the UK and parts of Europe affecting around 15-20% of the population (Khwarahm et al., 2017). The Pollen was purchased form ALK-Abello laboratory and stored dry at ambient temperature (~23-25 degrees).
### 3.1.1.2 Reagents

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Application</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Dodecyl Sulphate (SDS) Solution (10%)</td>
<td>Buffer solution for extraction or re-solubilisation of proteins</td>
<td>Sigma</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Reduces protease release (additive to buffer solution)</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Trizma® hydrochloride solution</td>
<td>Lysis buffer</td>
<td>Sigma</td>
</tr>
<tr>
<td>Protein Inhibitor Cocktail</td>
<td>Protein degradation (by protease)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Acetone</td>
<td>Protein precipitation</td>
<td>Sigma</td>
</tr>
<tr>
<td>Trizma-HCl (1 M, pH7.5)</td>
<td></td>
<td>Sigma</td>
</tr>
<tr>
<td>Nitrated BSA (Bovine serum albumin)</td>
<td>Standard (tagging and blotting experiments)</td>
<td>ABCAM</td>
</tr>
<tr>
<td>BCA Kit</td>
<td>Protein quantification</td>
<td>Thermo scientific</td>
</tr>
<tr>
<td>100 mM TEAB (triethylammonium bicarbonate)</td>
<td>500 µl of 1 M TEAB into 4500 µl of water</td>
<td>N/A</td>
</tr>
<tr>
<td>200 mM TCEP (20 µl of 1 M TCEP into 80 µl of 100 mM TEAB)</td>
<td>Quenching (alkylation reaction)</td>
<td>N/A</td>
</tr>
<tr>
<td>375 mM Iodoacetamide (IAA)</td>
<td>Alkylation</td>
<td>N/A</td>
</tr>
<tr>
<td>230 mM DTT</td>
<td>Reduction</td>
<td>N/A</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Protein digestion</td>
<td></td>
</tr>
<tr>
<td>Extraction buffer (50 mM Tris-HCl, 10% sucrose and inhibitory proteases)</td>
<td>Protein extraction</td>
<td>N/A</td>
</tr>
<tr>
<td>Acetic anhydride</td>
<td>Acetylation</td>
<td>Sigma</td>
</tr>
<tr>
<td>18% Ammonia solution (1.03 ml of ammonia solution with 0.97 ml of water)</td>
<td>Acetylation</td>
<td>N/A</td>
</tr>
<tr>
<td>5% Hydroxylamine (10 µl of 50% hydroxylamine stock solution in 90 µl of water)</td>
<td>Quenching (TMT labelling reaction)</td>
<td>N/A</td>
</tr>
<tr>
<td>TEAB (1 M)</td>
<td>Buffer</td>
<td>Fluka</td>
</tr>
</tbody>
</table>
**Table 3.1: Buffers and reagents used for experimental analyses.**

Table 3.1 list the reagents and recipes used in this study. The analytical reagents were of highest purity available and were purchased from either Sigma-Aldrich or Fisher Scientific.
Ultrapure (deionised) water was supplied in a quality higher than 18.2 MΩ.cm by a Milli-Q Plus 185 system (Millipore S.A., Molsheim, France). Synthetic Air and nitrogen dioxide (50ppm) were purchased from BOC (UK) respectively. Ozone was generated using the uv lamp.

### 3.1.1.3 Equipment

**Precellys homogenizer**- this is a highly efficient rapid cell lysis and homogenizing instrument designed to liberate DNA, RNA and proteins from a wide range of specimens. It improves sample preparation by breaking cell walls open thereby giving more access to proteins.

**LC-MS**- Liquid Chromatography (LC) / Mass Spectrometry (MS) is powerful and sensitive analytical tool for proteomics research. LC separates the sample components by passing the proteins through a column and introduces them to the MS. The MS provides information about the sample molecular weight.

**Centrifuge**- this is a device for separating particles in a solution according to their size, shape, density, viscosity nature of the medium. It spins down samples using centrifugal force for separating heterozygous aqueous solutions and suspensions of various densities in approved test tubes.

**Odyssey infrared imaging system** – this is a scanner that uses infrared fluorophores for imaging applications and supports a wide range of applications that benefit from sensitive and near-infrared fluorescence detection such as the blotting techniques.
3.2 Experimental set-up for gas exposure

3.2.1. Setup for the pollen exposure

Pollen samples were exposed to gases in vitro in a purpose built experimental setup. All exposure experiments were performed with the following procedure. Figure 3.2 shows a schematic diagram of the experimental exposure setup.

Figure 3.2- Experimental setup for pollen grain exposure. NO$_2$ (nitrogen dioxide and NO$_X$ (nitrogen oxide).
Pollen grains were weighed onto Whatman TE 35 Membrane Filter (PTFE, supports) (GE Healthcare UK Limited) with 0.2µm pores and 47mm diameter and a second filter paper was added on top of the pollen sample to prevent the pollen from escaping before putting it securely into the filter holder. It is noted that the pore size of the filters is significantly smaller than the investigated pollen grains and hence the grains could not escape from the filter holder setup.

3.3 Pollen exposure to gases

Experiments were typically performed with 10-50 mg of dry weight pollen. The gases flowing through the system were controlled using mass flow controllers (MFC) (Brooks Instruments; Model SLA5850S). The NOx monitor is a 42C Thermo Environmental instrument that quantifies NO₂ via chemiluminescence detection system. The NO₂ must first be transformed into NO before it can be measured. The NO and NOₓ calculated concentration are stored in the memory and the difference between the concentration are used for determining the NO₂ concentration. For each gas, the set flow rate was displayed in terms of the volume of the gas and expressed either in units of liters per minute (Lmin⁻¹) or standard cubic centimeters per minute (sccm). The MFC used to flow synthetic air, O₃ and or NO₂ in the gas flow tubes were controlled through the IGI lab interface software (LAB Interface 130410, v1.0) on a laptop and the RH required was monitored using the RH probe (Sensirion SHT/1) that monitors the RH during the duration of the experiment. Varying the combined ratios of dry and wet (through a water bubbler) airflow through the gas flow tubes generated the desired RH. The temperature inside the chamber was found to vary between 21-25 ºC during an experiment lasting 1-2 hours. The MFC and RH probe were calibrated
using external meters to ensure accuracy. The RH probe measures both temperature and RH with an accuracy ± 1.8% RH and ± 0.3°C.

After placing the pollen sample onto the filter paper holder, the filter holder was then connected to the gas flow tubes and gas was introduced at constant flow rates (FR). The FR was controlled by the MFC that allowed exposure to the desired gas/gases, under different concentrations and variable flow rates. Several experiments were performed as presented in Table 3.2 below, which all happened between 1-2 hours with different flow rates, RH, gas mixtures and concentrations. The exposure = time × concentration. The maximum concentration of NO₂ used was 10 ppm, and this value was based on the calculation that the exposure is of 24 hours for 7 days. 7 days was chosen as it represents a typical upper limit for the time that atmospheric aerosol particles are airborne. For a 1 hour exposure, at 10 ppm of NO₂, this is equivalent to a weeklong exposure of 0.059 ppm (59 ppb) , which is below present atmospheric national hourly-limit value acceptable for human health protection in Europe (0.11 ppm, 110 ppb for NO₂) (Cuinica et al., 2014).

Once the required exposure time was reached, the filter paper was carefully removed using tweezers and placed onto a petri dish, then sealed with parafilm and stored in the -80 degrees freezer until it was required for protein extraction and proteomics analysis. Typically, the analysis was carried out on the same day as the exposure. Non-exposed pollen samples were used as controls. The controls are subjected to exactly the same protocol minus the reactive gases. Figure 3.3 illustrates the experimental set up for pollen grain exposure to RH, NO₂ and O₃.
Figure 3.3- Experimental setup for pollen grain exposure to RH (relative humidity), NO₂ (nitrogen dioxide) and O₃ (ozone). MFC- mass flow controller.

The NO₂ uptake by the pollen was also investigated under the influence of pre-treatment with O₃ as studied by Chassard et al. (2015). The O₃ used was generated using an ultraviolet generator (UV ozone generator) and the concentration was monitored with an O₃ analyzer (2B Technologies, Model 205). 10 mg of the pollen was exposure to flows of O₃ between 21-26 ppmv (2100 – 2600 ppb which is not atmospherically realistic but for the lifetime exposure representing a week is equivalent to a modest concentration of 12.5-15.4 ppb) at a flowrate of 1 Lmin⁻¹ of synthetic air (dry and wet) for 1 hour. After which the gas flow tube
was flushed with dry synthetic air for 5 minutes then exposed to NO$_2$ for 1 hour under the same experimental conditions. Table 3.2 presents the conditions used for some of the experimental exposures.

![Table 3.2: Conditions used for some experimental exposures. Note: 0 means absence of O$_3$, NO$_2$ and RH. All experiments were performed for duration of 1-2 hours. The table displays few of the many exposures carried out during the research.](image)

### 3.4 Protein extraction method

Pollen grains have hard cell walls to withstand many environmental stresses and to protect the genetic information as well as stored compounds (Fila et al., 2011). The pollen surface is probably hydrophobic because the exine is enclosed with waxes and proteins (Pope, 2010). For the proteomics analysis to be achieved, which requires protein to be extracted and
separated, homogenization was employed at the initial stage of the experiment to enable access to the protein content of the pollen grain after the exposure.

3.4.1 Cell disruption (Homogenization)

Cell disruption was performed using the Precellys 24. It utilizes a bead (ceramic, glass and metal) beating process coupled with a powerful figure of 8 motion allowing lysis to be achieved with very short programs typically less than one minute. 10-50 mg of the exposed pollen grain was transferred into the precellys tube (containing 2.8 mm metal beads) and homogenize in 1 ml of 50 mM TrisHCL-pH 6.8, 10% Sucrose and 10 µl inhibitors for 60s at 6500 rpm speeds. The homogenizer was operated at full capacity in order to enhance release of the protein. Proteins were then extracted from the samples as described in the sub section below. Sonication was also adopted during protein extraction to complete cell lysis and shear DNA to reduce sample viscosity. The sonicator was operated with samples in the tube placed on ice during each run at a chosen speed of 15 microns for 15 seconds three times.

3.4.2 Protein extraction from intact pollen samples

The homogenized pollen samples were transferred to 2 ml Eppendorf tube and centrifuged at 16000 ×g for 20 mins at 4°C. Supernatant was kept and to the pellet the buffer (above) with additional 2% SDS was added, mixed and incubated for 60 minutes at laboratory temperature. Samples were sonicated (because of the viscosity nature of the sample) in short pulses with a sonicating probe to break DNA. To the 2 ml of the sample, 5 fold Acetone (10 ml) was added and store to precipitate at -20 °C overnight. The 12 ml sample was centrifuge at 16000 xg for 30 minutes at 4 °C. The supernatant was kept; pellets dried and given to the
facility for proteomics analysis. Several samples were generated from supernatant and pellet so as to compare which part of the sample contains higher allergen protein contents.

### 3.4.3 Pollen rupture

To further explore the applicability of NO$_2$ effect on pollen grain, the grains were ruptured in three different ways; 1) immersed in rainwater (collected from the University of Birmingham using a sterile bottle and funnel) over night for ~ 22 hours, 2) mechanically disrupted dry using metal beads and 3) exposed to ~90% RH for 24 hours. Figure 3.4 shows the diagram illustrating the 3 different pollen-rupturing conditions.

**Figure 3.4:** Diagram illustrating the 3 different pollen-rupturing conditions. The birch pollen grain is easily recognized by its characteristic triangular shape with three germination pores in each angle.
3.5 Sample preparation and gas exposure to ruptured pollen proteins

The ruptured samples were dried (using Eppendorf Vacuum Concentrator at 30 °C that can be used to concentrate samples to required amount or dehydrate them completely) on a filter paper and exposure to desired amount of gases (2 ppm, 5 ppm and 10 ppm of NO₂) as described in the previous section 3.2. The exposed sample was recovered from the filter paper using the extraction buffer (50 mM Tris-HCl pH6.8, 10% sucrose and inhibitory proteases) and protein concentration was estimated with Bicinchoninic Acid (BCA) (is a two-component, high-precision, detergent-compatible assay reagent available for measuring absolute protein concentration by comparing to a protein standard) protein assay kit as described in section 3.6 below. The rainwater was also checked using the same protein assay protocol to ensure that it does not contain any airborne contaminants that may interfere with the experimental results.

In this experimental setup, only one filter paper was used because the exposure is at the extracted pollen protein samples, which are air-dried on the filter paper so have lesser risk of being blown away by the gases during exposure.

3.6 Protein concentration determination

BCA protein assay kit is an excellent tool for estimating the protein concentration of a sample. The intensity of the coloured reaction product is a direct function of the protein amount that can be determined by comparing its absorbance value to a standard absorbance curve.

The protein concentration of the samples was determined by BCA Protein assay kit and read by Tecan infinite 5200 pro plate readers absorbance spectroscopy at a wavelength of 570nm.
BCA standards ranged from 2 mg/ml to 25 µg/ml. This was carried out by dispensing 10 µl of each standard into a 96 well plate along with the protein samples extracted from the pollen. BCA protein assay reagents were mixed in the ratio of 1:50. An addition of 200 µl of BCA protein assay reagents A and B mixture was added to each well and the plate was incubated for 30 minutes at 37 °C. The absorbance at 570 nm was read and using the excel results sheet, a linear curve with equation was generated from the known concentrations of BCA standards in order to calculate the protein concentration of the samples.

3.7 SDS-PAGE gel procedure

Much proteomic work relies upon the pre-separation of target proteins by one- or two-dimensional gel electrophoresis (Aebersold and Mann, 2003). Two dimensional difference gel electrophoresis has proven to be a powerful technique for examining protein expression, which allows the simultaneous resolution of thousands of proteins (O'Farrell, 1975). The separation of proteins take place in two steps; in the first dimension separation is dependent on their charge using isoelectric focusing (IEF), and in the second dimension on their molecular weight using SDS-PAGE (Marouga et al., 2005). An important advantage of gel electrophoresis compared to gel-free approach is the identification of proteins from polyacrylamide gels, which eliminates low molecular weight contaminations, as well as detergents and buffer components, that are often have negative impact on mass spectrometric sequencing (Shevchenko et al., 2007).

Selecting the correct gel for the protein of interest can be a crucial step in optimizing detection and quantification of the protein and modification of interest. If the target protein is of low molecular weight a high percentage gel gives a better resolution. Here, 12% gradient of precast mini gel from BIO-RAD was used (allows a wide coverage of protein weights).
The gel was held in a cassette and a comb stacked on top of the 10 wells. The samples extracts were run on SDS-PAGE gel in order to observe the amount of protein content in the different sample extracts; supernatant and pellet and in another experiment (Western blot and in-gel digestion) was done to detect the presence of protein modification. Samples (pollen protein extracts) were dried down to 10 µl each (smaller samples were made up to 10 µl with water) and 10 µl of Laemmli buffer solution was added (1:1 ratio) making 20 µl in total for each sample. The glycerol content of the buffer solution aids the samples to effortlessly sink into the wells of the gel. The samples are then kept on a heating block for 10 minutes at 95 °C which denatures the protein while retaining the sulphide bonds. Denaturing the protein ensures that the negative charge of amino acids is not neutralized, assisting the protein to transfer in an electric field applied during the process. Subsequently, it was allowed to cool before loading on to the gel. The gels were placed in the tank (the tank houses a dual cell holder, an electrode assembly, lid with power cables and mini buffer dam) and the comb removed, then about 900 ml SDS running buffer was poured into the tank. Samples were loaded in equal proportions (20 µl) and the pre-stained protein ladder, 10 µl. Finally, the gels were allowed to run at 200 Volts for at least 45 minutes.

3.8 Dot blot technique

The dot blot is a technique for detecting, analysing, and identifying proteins by spotting circular patterned protein samples directly onto the membrane. Dot blot differs from western blot in that no gel is used so protein samples are not separated electrophoretically but are spotted through circular templates directly onto the membrane of choice and probing it using antibody. It can be used to semi-quantify concentration of a protein in the presence of both standard protein and specific antibody against it. This technique was applied to assess the
presence of nitration in the research samples. The ability of this technique to detect nitration is due to the fact that it uses the antibody specific for the target of interest.

In this experiment, the proteins of interest are 3-nitrotyrosine proteins that are supposed to be produced as the result of protein exposure to NO$_2^-$, which were probed using polyclonal Anti-3-nitrotyrosine antibody produced in Rabbit (Sigma Aldrich) as the primary and the secondary was Goat Anti-Rabbit antibody (Odyssey, LI-COR). The primary antibody binds only nitrated proteins on the membrane while the secondary antibody binds to the primary antibody and emits the fluorescence signal during scanning. The molecule responsible for the fluorescence signal in the secondary antibody is IRDye 800CW dye. IRDye 800CW is ideal for antibody labelling and has absorption and emission wavelengths in the NIR spectrum, between 680 and 800 nm as well as higher signal-to-noise ratios (https://www.licor.com/bio/products/reagents/irdye/). Figure 3.5 below demonstrates how the binding of the antibody process works.

![Figure 3.5](image_url)  
**Figure 3.5:** Schematic diagram explaining the process of antibody binding in the detection of nitrated protein
Below are the steps used for the dot blot analysis.

Protein concentrations of the samples were calculated (5 µg/ml). Using a narrow-mouth pipette tip, 2.5 µl of nitrated BSA and amount need from the research samples were spotted onto the Whatman nitrocellulose transfer membrane (pore size 0.45 um) minimizing the area that the solution penetrates (usually 3-4 mm diam.) by applying it slowly and allowing it to dry. A grid by pencil to indicate the region of blotting was drawn on the membrane. Once the spotting was completed, the membrane was allowed to dry completely and 20 ml of blocking buffer (5% milk powder in TBST) was added onto the membrane in a weighing boat then incubated at room temperature for 1 hour on a rocker. This blocks non-specific sites of the membrane. The blocking buffer was discarded and using 1:1000 ratio of the primary antibody (following manufacturer’s instruction), 20 µl in 20 ml of 50% blocking buffer (10 ml of TBST and blocking each), the membrane was incubated with the antibody overnight on a rocker with gentle shaking at room temperature (RT). Afterwards, it was washed three times with TBST (3 x 10 min) and incubated with secondary antibody, 1:10000 (2 µl in 20 ml of TBST) for 1 hour on the rocker with gentle shaking at RT. The secondary antibody was disposed and membrane washed three times, two washes with TBS-T and last wash with PBS to remove residual Tween 20 (3 x 10mins). The membrane was then scanned on Odyssey infrared imaging system (MousePod 9120-MP, LI-COR, Inc USA). The scanner uses infrared fluorophores for imaging applications and supports a wide range of applications that benefit from sensitive and near-infrared fluorescence detection such as the blotting techniques. It is distinctively furnished with two infrared channels (700 and 800 channel laser source) for direct fluorescence detection on membranes (Osterman and Schutz-Geschwender, 2012). The channel used for this study was the 800 laser source that is compatible with the secondary antibody used.
The total fluorescence intensity from the dots was measured in the Odyssey imaging system that has a software for quantifying concentration (picomoles) of the dots. The values generated by subtracting the area of dot from the background were used for plotting graphs. An estimated degree of nitration was also calculated with reference to equivalent weight of nitrated BSA protein.

3.9 Western blot

Western blot, also referred to as immunoblotting is an analytical technique similar to dot blot, however, it is used for identifying specific protein on gel based on band separation and relies on three elements: separation of the protein mixtures by their size according to molecular weight; transfer of the separated proteins onto the membrane and identification of the targeted protein using antibody (Mahmood and Yang, 2012). Following the SDS-PAGE electrophoresis, the gel was placed on to the western blot pads already soaked in transfer buffer (so as to saturate the pads) for approximately 10 minutes. The membrane was placed onto the gel and another pad added on top making sure no air bubbles are trapped using a roller. This was then inserted into the cassette, placed in the transfer tank and transferred for approximately 7 minutes. The electric current used for the transfer induces the migration of the proteins from the gel onto the membrane producing a band for each protein. Once the transfer was completed, 20 ml of blocking buffer (5% milk powder in TBST) was added onto the membrane in a weighing boat and incubated at room temperature for 1 hour on a rocker. This blocks non-specific binding sites of the membrane. The blocking buffer was discarded and using 1:1000 ratio of the primary antibody, 20 ul in 20 ml of 50% blocking buffer (10ml of TBST and blocking each), the membrane was incubated with the antibody overnight on a rocker at RT. Afterwards, it was washed three times with TBST (3 x 10 min) and incubated with secondary antibody (2 ul in 20ml of TBST) for an hour on the rocker at RT. The
secondary antibody was disposed and membrane washed three times, two washes with TBS-T and last wash with PBS (3 x 10mins). The washing eliminates unbound antibody leaving only the bound antibody to the protein of interest. The membrane with bound antibodies was then scanned and detected on Odyssey system. As the antibodies only bind to the protein of interest, single band (if any) should be visible for each gel lane.

Western blot results are typically considered to be semi-quantitative because it offers a relative evaluation of protein levels, instead of a complete measure of quantity (Mahmood and Yang, 2012). There are two reasons for the lack of complete measure of quantity; first, there may be some disparities in loading and transfer rates among the samples in their individual lanes, usually diverse on separate blots and secondly, the signal produced “by detection is not linear across the concentration range of samples” (Mahmood and Yang, 2012).

3.10 In-gel trypsin digestion of Coomassie-stained proteins

“In-gel digestion of proteins isolated by gel electrophoresis is a cornerstone of mass spectrometry (MS)-driven proteomics” (Shevchenko et al., 2007). It is done to cut the protein of interest present within the polyacrylamide matrix. The sequencing of the molecular weight-separated protein bands increase the vigorous scope of the protein mixtures analysis since each peptides created by in-gel tryptic cleavage of each band are sequenced and analyzed separately (Shevchenko et al., 2007). Analyzing complex mixture by spreading out the proteome over 10–20 gel slices dramatically increases the depth of analysis, and hence the number of identified proteins and detected PTMs will also increase (Shevchenko et al., 2007). Figure 3.6 shows the main steps involved during the experiment.
After running the gel (described in section 3.6 above), it was stained in Coomassie (a strong 700 nm fluorophore whose fluorescence is induced upon protein binding) blue protein stain overnight. The molecular weight of the visible polypeptide bands were estimated by comparison with protein markers. The bands of interest (~17 kDa, which is the molecular weight of Bet v1) were excised from the gel, placed in 1.5 ml Eppendorf tubes and cut into several pieces (about 2-3 mm). Cutting the band into smaller pieces increases surface area to assist diffusion. The gel pieces were washed in 500 µl of 100 mM ammonium bicarbonate and 50% acetonitrile/50% 100 mM ammonium bicarbonate respectively for 1 hour on the shaker and wash discarded (washing removes excess stain and SDS that can interfere with mass spectrometry analysis). To break disulphide bonds and obtain a completely unfolded protein for digestion into peptide, 150 µl of 100 mM ammonium bicarbonate and 10 µl of 45 mM dithiothreitol (DTT) were added then incubated at 60°C for 30 minutes in the heating block. For alkylation (prevents reformation of disulphide bonds), sample was cooled to room temperature (~10 mins), 10 µl of 100 mM iodoacetamide (IAA) added and sample incubated in the dark for 30 minutes. Solvent was discarded and gel pieces washed in 500 µl of 50%
acetonitrile/50% 100 mM ammonium bicarbonate (it removes any excess DTT and IAA) for 1 hour on the shaker. Wash was discarded and an addition of 50 µl of acetonitrile to shrink gel pieces. After 5-10 minutes, solvent was removed and gel pieces dried completely in a vacuum centrifuge. Acetonitrile was added to draw water out of the gel. Addition of trypsin (20 µl for average size band) was done gradually until the gel pieces were completely rehydrated to previous size. It is vital to fully cover the gel pieces for good digestion. Finally, sufficient amount of 25 mM ammonium bicarbonate was added to cover just over the gel pieces (~20 µl) and allowed to digest overnight at 37°C in the heating block. The tube was briefly centrifuged to pellet gel pieces and all liquid was transferred to fresh Eppendorf tube being careful not to transfer any gel. 20 µl 5% formic acid was added on to the gel pieces and incubated for 20 minutes on heating block at 37°C. 40 µl acetonitrile was added to the gel pieces in Eppendorf and incubate for another 20 minutes on heating block at 37°C. It was briefly centrifuged and the liquid transferred to the same Eppendorf tube as used for the first extracted liquid. The combined extracts were completely dried down, ziptipped and handed over to the facility for the MS analysis. Figure 3.7 presents the schematic procedure summary of in-gel tryptic digestion.
3.11 Trypsin digestion

Trypsin is a proteolytic enzyme, essential for protein digestion with molecular weight of 24 kDa composed of 220 residues. “Trypsin is an aggressive and stable protease, which very specifically cleaves proteins on the carboxy-terminal side of arginine and lysine residues” (Olsen et al., 2004). This produces suitable peptides for sequencing and with a basic residue at the carboxyl terminus of the peptide that “result in information-rich, and easily interpretable, peptide-fragmentation spectra” (Steen and Mann, 2004).

The tryptic digestion is normally performed overnight using small amounts of trypsin enzyme depending on the required quantity for an experiment. Typically, the ratio of the trypsin-to-protein is in the range of 1:20–1:40 (w/w) where the rationale behind the low ratio is to avoid trypsin autolysis, which reduces trypsin activity and increases the sample complexity (Egeland et al., 2016). For each treatment/condition ~100 µg protein was transferred to new Eppendorf tubes and made up to 100 µl with 100 mM TEAB if required. 5 µl of 200 mM
TCEP was added and incubated at 55 °C for 1 hour. It was removed from heat and leave to cool back to room temperature. 5 µl of 375 mM iodoacetamide was added and incubated for 30 mins in the dark. The reaction was quenched by adding 5 µl of 230 mM DTT and incubated at room temperature for 5 mins. Lastly, 2.5 µl of trypsin (gold standard trypsin) per 100 µg of sample was added and samples digested overnight at 37 °C. This gives a 1:50 ratio of enzyme: protein. The correct ratio of enzyme: protein helps to maintain good digestion time with compromising method sensitivity or digestion repeatability (Egeland et al., 2016).

3.12 TMT labeling experiment

The tagging experiment targets the quantitative identification of nitrotyrosine-containing proteins/peptides using TMT tags (Tandem Mass Tag™). The TMT tags reagents are designed to enable identification and quantitation of proteins in different samples using tandem mass spectrometry (MS). Samples are differentially labeled chemically, combined and simultaneously analyzed by LC-MS/MS, with relative quantitation performed by comparison of intensities of the ‘reporter’ fragments in the MS/MS spectra (Timms and Cutillas, 2010). There were five major steps involved for this experiment as shown in the Figure 3.8 below. At each stage of the experimental step, aliquots of the samples were taken and checked on the MS to ensure investigation was going on as expected. The amount of sample needed for the tagging experiment was digested overnight using trypsin as described in section 3.11. Nitrated Bovine Serum Albumin (NBSA) (Abcam) was used as standard, which was treated with peroxynitrite that modifies tyrosine by acting as an oxidant to produce 3-nitrotyrosine along the length of the BSA protein; however, the number of the nitrated sites of the standard NBSA used was not specified in the manufacture’s manual. Figure 3.8 represents the flow chart of the major steps involved for TMT labelling experiment.
3.12.1 Acetylation of tryptic peptides (to block primary amine groups)

The acetylation of the N-terminus of the tryptic peptides increases the peptide stability. 20 µl of acetic anhydride was added and incubated at 37 °C for 30 min. Then 200 µl of 18% ammonia solution added, incubated at 37 °C for another 30 min and samples were evaporated to complete dryness by vacuum centrifugation. Figure 3.9 below demonstrates the formation of nitrotyrosine (addition of 45Da) and its reduction (addition of 15Da) to aminotyrosine using nitrating (NO$_2$) and reducing (Na$_2$S$_2$O$_4$) agents respectively.
3.12. 2 Reduction and TMT-tagging

The acetylated peptides were re-dissolved in 50 µl of 100 mM TEAB and 50 µl of 50 mM sodium dithionite added with continuous stirring of the reaction at room temperature for 30 min to reduce 3-nitrotyrosine (3NT) to 3-aminotyrosine (3AT). Samples were then evaporated to complete dryness by vacuum centrifugation. Immediately before use, the TMT Label Reagents were equilibrated to room temperature and 41 µL of anhydrous acetonitrile added to each tube. Reagents were left to dissolve for 5 mins with occasional vortexing and briefly centrifuged the tube to gather the solution. Carefully, 41 µL of the TMT Label Reagent was transferred to each sample and incubated for 1 hour at RT. The labeling, if used correctly is efficient for all peptides regardless of protein sequence or proteolytic enzyme specificity; however, labeling does not occur when primary amino groups are modified, such as when N-terminal glutamine or glutamic acid forms a ring (pyro-glutamic acid) or if the group is acetylated (Rauniyar and Yates III, 2014). To quench the TMT labelling reaction, 8
μL of 5% hydroxylamine was added to the sample and incubated for 15 mins. All tagged samples were combined together (equal proportions) and evaporate to complete dryness by vacuum centrifugation. Figure 3.10 explains the detailed conversions of 3NT to 3AT which is expected to take place in the process of the tagging experiment.

Figure 3.10: Flow diagram showing the steps involved in the tagging experiment after formation of 3NT up to the stage of LC/MS analysis. K and Y represents amino acid lysine and tyrosine respectively.
3.12.3 Enrichment of TMT-labelled peptides

Thermo Scientific anti-TMT resin and TMT elution buffer are the main reagents used for the enrichment step. Both solutions are used for specific capture and elution of TMT Reagent-labelled peptides respectively. The anti-TMT resin and TMT elution buffer are effective for reducing sample complexity, and improving dynamic range (Gygi et al., 1999). Anti-TMT resin uses the highly specific anti-TMT antibody to capture peptides labelled with TMT reagents. The antibody is specific for the mass reporter region of the TMT reagents that allows for enrichment of TMT-labeled while the Elution Buffer is a volatile neutral buffer, which competitively elutes captured TMT-labeled peptides (Gygi et al., 1999).

200 µl of anti-TMT resin slurry was transferred into a fresh Eppendorf tube and spun in centrifuge at 2500-x g for 2 min until resin has pelleted. Supernatant was discarded being careful not to dislodge the pellet. 500 µl of TBS was added, vortex to resuspend pellet, and the centrifugation process repeated. The washing process was done three times with TBS. lyophilized peptides was then resuspended with 100 µl of TBS, poured onto the anti-TMT resin pellet and incubated for 2 hours at room temperature with mixing. After incubation, it was centrifuged at 2500-x g for 2 min to pellet resin. The supernatant was removed and washing process (as described above) repeated 5 and 3 times with 500 µl of TBS and water respectively. 200 µl of TMT elution buffer was then added to eluted sample mix resin and centrifuged at 2500 x g for 2 min until resin has pelleted. The supernatant was collected into fresh Eppendorf tube. Then 200 µl of TMT elution buffer mix resin was added and the centrifugation process repeated. Supernatant was collected into the same Eppendorf as previous elution step. The collected supernatant was spun for 2 min at 10,000 x g to remove any residual resin, then the supernatant was transferred into
a new Eppendorf tube and dried to completeness in vacuum centrifuge. Lastly, sample was ziptipped as explained below and handed over for MS analysis. Figure 3.11 below is an example of typical workflow for the Thermo Scientific iodoTMT Reagents used for tagging experiment using the 6plex TMT tags. During this study, the 10plex TMT and TMTzero Reagents were used. TMTzero for trial because is less costly and TMT10 (enables up to 10 different peptide samples to be labelled at a go) due to large amount of the research samples. Both Reagents share identical structure but contain different numbers and combinations of 13C and 15N isotopes in the mass reporter. Note all 10-plex reagents set contains 10 different isobaric compounds with the same mass and chemical structure but each has a unique reporter mass used to measure relative protein expression levels (of each analysed sample) upon fragmentation and tandem mass spectrometry.

![Diagram](image)

**Figure 3.11:** An example of typical workflow for the Thermo Scientific iodoTMT Reagents used for tagging experiment. Modified from Murray et al., 2012. (Murray et al., 2012)

### 3.13 Zip Tipping/Desalting

The liquid samples containing the mixture of peptides are desalted using millipore C18 ZipTips. Tips are cleaned by pre-wetting in 100% acetonitrile (ACN) (x2 twice) and equilibrated with 0.1% trifluoroacetic acid TFA (rinsed x2). Samples are then aspirated
and dispensed 10x (pipette up and down). At this point the sample binds to the matrix. The tip is then washed with 0.1% TFA (x2) trifluoroacetic acid to remove excess salts before elution of peptides with 100 µL of 50% acetonitrile, 0.1% trifluoroacetic acid. The eluted peptides in fresh Eppendorf tube are dried down using the vacuum centrifuge to remove the acetonitrile, and finally re-suspended in 0.1% formic acid solution in water. At this stage, the samples are handed over for the MS/MS analysis.

3.14 Proteomics MS

“Proteomics is the study of complex biological systems by analysing protein expression, function, modifications, and interaction” (Domon and Aebersold, 2006). The field of proteomics is built on technologies to analyse whole proteins in an experiment (Ong and Mann, 2005). Mass spectrometry (mass spec or MS) is a powerful and sensitive analytical tool for proteomics research. It plays a vital role in known and unknown protein detection, identification and quantification by revealing their structural and chemical properties based on their mass to charge (m/z) ratio. The mass spectrometer impacts energy into the peptides causing it to fragment at the peptide bonds between amino acids and the masses of these fragment ions are then recorded. The fragmented peptides can then be used to produce a characteristic sequence to determine the position and identification of the PTM.

MS is efficient for identifying and describing proteins that are present in complex mixtures. The results are mostly qualitative; nonetheless, recently new approaches offer the chance to quantify proteomic information (Ong and Mann, 2005). This involves associating the identified signals from the same peptide generated from dissimilar conditions that produces an approximate abundance of relative protein between two proteomes (Ong and Mann, 2005). Alternatively, and more accurately, peptides are labelled with stable isotopes such as the TMT tags, whereby an estimated mass difference
is introduced between peptides from different experimental conditions/treatment (Ong and Mann, 2005). In this research, this quantitative approach was employed that is supposed to give precise practical evidence and temporal modifications in the proteome of the pollen, which should be captured by MS (Ong and Mann, 2005).

Because the proteome is a complex mixture, there is no particular method for preparing protein samples for the MS analysis, however, the preparation of proteins for an MS analysis normally includes initial isolation or separation of the protein(s) so as to lessen the complexity of the proteome (Gundry et al., 2009). The main benefit of this step is to retain the essential information of the intact proteins prior to enzymatic or chemical digestion processes (Gundry et al., 2009). It also increases the possibility of identifying other protein isoforms, polymorphisms, and PTMs. There are 13 different identified Bet v 1 isoforms (Fernandes et al., 2013). In this study, some isoforms of Bet v 1 protein were identified. “Bet v 1 comes in a variety of isoforms that share virtually identical conformations, but their relative concentrations are plant-specific” (von Loetzen et al., 2015).

Figure 3.12: Typical proteomics experiment workflow illustrating the five stages involved. Adapted from (Aebersold and Mann, 2003).
As explained in a study by Ong and Mann (2005), the typical proteomics experiment consists of five stages (Figure 3.12 above), however, in this study the majority of the experiments skipped the gel electrophoresis step. The advantage of gel based methods is that the dynamic scope of investigation will be increased because sequential separation of proteins is based on molecular weight (Gundry et al., 2009) that will allow for specific selection of targeted proteins. This process was employed only during in-gel trypsin digest and western blot experiments because both techniques require the separation of the complex mixture of protein according to their molecular weight. Ong and Mann (2005) explained the five steps as follows:

- Stage 1, the proteins to be investigated are secluded from cell lysate or tissues using appropriate method,
- Stage 2, proteins are degraded using trypsin enzymes to generate peptides because it is more sensitive for MS analysis identification,
- Stage 3, the peptides are divided by high-pressure liquid chromatography where they are nebulized in minute, highly charged drops, which after vaporization, multiply protonated peptides enter the MS,
- Stage 4, a mass spectrum of the peptides eluting at this time point is taken and,
- Stage 5, the computer generates an arranged list of these peptides for fragmentation and a sequence of tandem mass spectrometric (MS/MS) tests proceeds (Ong and Mann, 2005).

### 3.15 LC-MS/MS Experiment

There are two main approaches of MS; top-down and bottom up where the former provides information on intact proteins and the latter is used for analysis of digested
proteins. The LC fractionates the peptides while MS analyses the peptides (Tsikas and Duncan, 2014). Here, the bottom-up MS was used.

UltiMate® 3000 HPLC series (Dionex, Sunnyvale, CA USA) is used for peptide concentration and separation. Samples are trapped on uPrecolumn Cartridge, Acclaim PepMap 100 C18, 5 um, 100A 300um i.d. x 5mm (Dionex, Sunnyvale, CA USA) and separated in Nano Series™ Standard Columns 75 µm i.d. x 15 cm, packed with C18 PepMap100, 3 µm, 100Å (Dionex, Sunnyvale, CA USA). The gradient used is from 3.2% to 44% solvent B (0.1% formic acid in acetonitrile) for 30 min. Peptides were eluted directly (~ 350 nL min⁻¹) via a Triversa Nanomate nanospray source (Advion Biosciences, NY) into a LTQ Orbitrap Elite mass spectrometer (ThermoFisher Scientific, Germany). The data-dependent scanning acquisition is controlled by Xcalibur 2.7 software. The mass spectrometer alternated between a full FT-MS scan (m/z 380 – 1800) and subsequent collision-induced dissociation (CID) MS/MS scans of the 7 most abundant ions (within the peptide of a protein). This eliminates noise from the spectrum and reduces the number of ions to be considered (Eng et al., 1994). Survey scans were acquired in the Orbitrap with a resolution of 120 000 at m/z 400 and automatic gain control (AGC) 1x10⁶. Precursor ions were isolated and subjected to CID in the linear ion trap with AGC 1x10⁵. Collision activation for the experiment is performed in the linear trap using helium gas at normalized collision energy to precursor m/z of 35% and activation Q 0.25. The width of the precursor isolation window is 2 m/z and only multiply charged precursor ions are selected for MS/MS. The MS and MS/MS scans are searched against Uniprot database (pollen) using Proteome Discoverer 1.4 (ThermoFisher Scientific). Aside from the fixed modification; Carbamidomethylation, variable modifications used included acetylation (N terminus), acetylation (K), oxidation (M) and Nitration (Y). The precursor mass tolerance
is 10 ppm and the MS/MS mass tolerance was 0.8Da. Two-missed cleavages are allowed and accepted as a real hit protein with at least two high confidence peptides.

3.16 Qual browser application

The Proteome Discoverer application includes the Qual browser application used for examining spectra and chromatograms in detail. This is possible after a full MS scan is performed and all the peptide ions are recorded. The browser allows viewing the entire ion chromatogram, individual precursor and MS data. Each peak can be selected and analysed in detail. The chromatogram and spectra of 3 samples subjected to different treatments (RH, O₃ and NO₂) under same environmental condition were generated using the browser.
Figure 3.13: Chromatogram and spectrum of birch pollen protein subjected to A-RH, B- NO₂ and C- O₃ respectively.
The chromatogram (top Figure 3.13 A, B & C) is a graphical display based on the retention time (time taken for a peptide to get eluted from the column) of the peaks generated as the components get separated after passing through the detector. Each peak represents the component present in the sample and its retention time is labeled at the top of the peak. This allows for easier analysis on a specific peak of interest. Peptides eluted from the chromatographic column are ionized, and their m/z values are measured by the MS. The peptides are eluted from the LC column and their ion intensity is recorded at different time points, forming the peptide peak. The mass spectrum (bottom Figure 3.13 A, B & C) is displayed as a vertical bar graph, where each identified bar act for an ion having a certain mass-to-charge ratio (m/z) and the length of the bar describes the relative abundance of the ion (Joshi, 2012).
CHAPTER 4

Detection and Quantification of birch pollen nitration

This chapter presents results of laboratory measurements designed to determine the effect of atmospheric oxidants on the protein of the birch pollen grain. Measurements, conducted at the proteomic level, include dot blotting techniques, in-gel digestion, and LC-MS/MS analysis.

4.0 Synopsis

Air pollution can exacerbate several medical conditions, for example, hay fever and asthma (Mahmood et al., 2015). The global incidence of hay fever has been rising for decades; however, the underlying reasons behind this rise remain unclear (Mahmood et al., 2015).

This study investigates the post translational modification (PTM) on the protein content of the highly allergenic birch pollen that is common in Europe. Within the laboratory through an innovative experimental chamber, the pollen grain was subjected to atmospherically relevant exposures of gas phase NO₂ and O₃ under a range of environmentally applicable conditions. The levels of the gases used were below the atmospheric limit value acceptable for human health protection in Europe. The effects of the trace gas exposures on the biochemistry of the pollen grains were then probed using proteomic approaches (liquid chromatography coupled to an ultra-high resolution mass spectrometer, dot and western blotting and in-gel digestion). The degree of nitration was semi-quantified using nitrated Bovine serum albumin (BSA) as a reference standard. BSA
is a model protein that successfully reacts with NO₂ at atmospheric concentrations (Shiraiwa et al., 2012a). Slightly different polypeptide profiles were revealed by SDS-PAGE gel analysis between exposed and non-exposed pollen. Overall, the findings indicated significant interaction between gas phase pollutants and pollen. These interactions result in protein specific modifications; in particular, the addition of a nitro group (–NO₂) to the phenolic ring of a tyrosine residue is observed. Nitration is seen to occur upon tyrosine residues, and nitrosylation occurs on cysteine residues. Upon the interaction with tyrosine, it forms 3-nitrotyrosine (3NT), a post-translational modification (oxidative) associated with many diseases.

4.1 Introduction

It is a well-known fact that pollens from certain plant species like birch and ragweed can trigger several kinds of human allergic reactions in sensitized individuals. It is assumed that the interaction of pollen with common gas phase pollutants, such as NO₂ and O₃, increases the allergenicity of the pollen and hence increases hay fever incidence (Franze et al., 2005; Reinmuth-Selzle et al., 2014). Protein nitration occurs as a result of oxidative stress stimulated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as NO₂ (Nie et al., 2006). ROS are derived from oxygen molecules and RNS are nitrogen-containing oxidants (Bedard and Krause, 2007).

Previous studies have shown that the reaction of pollen with atmospheric oxidants can alter protein molecules that are present within pollen grains and cause variations in the chemical composition of pollen protein via PTM of the protein (Franze et al., 2005; Reinmuth-Selzle et al., 2014). This change in protein chemistry is likely linked to the
change in allergenicity, and consequently provides the link between urban pollution and increased allergenicity.

Since atmospheric pollutants often have greater concentrations within urban areas, in particular nitrogen oxides \((\text{NO}_x = \text{NO} + \text{NO}_2)\) because their predominant source is from vehicle exhausts, the hypothesis suggests that greater allergenicity should occur in or downwind of urban areas.

A study by Schröder et al. (2015) indicated that there is greater hay fever prevalence within urban in comparison to rural areas (Schröder et al., 2015). Induced chemical changes may affect human immune reaction to the pollen allergen protein, which provides a possible pathway for increased allergenic response to the chemically altered pollen protein that has interacted with atmospheric pollution. Other hypotheses for greater allergenicity in urban areas are indirect. For example, the higher CO\(_2\) levels and higher temperatures (urban heat island effect), which promote greater plant growth.

The main identified effect of NO\(_2\) on the pollen proteins among research done to date is PTM of tyrosine termed, protein tyrosine nitration (PTN), that is irreversible. “This posttranslational modification of allergens provides a rationale for the increase in allergic diseases in air-polluted regions, summarizing the different studies, little is yet known about the molecular mechanisms of the effects of ozone and NO\(_2\) on pollen, and more research is needed on that point, however, the existing research already clearly indicates dose-dependent and species-specific impacts of these air pollutants, which in most cases result in heightened allergenicity” (Frank and Ernst, 2016).
Nitration of tyrosine as a modification has been known for over hundred years (Tsikas and Duncan, 2014), which is facilitated by an amplified concentration of reactive oxygen and nitrogen. Even though nitration has been mainly reported to happen on tyrosine residue (Tyr); “phenylalanine (Phe) and tryptophan (Trp) are also nitrated by RNS; however, their aromatic ring is much less reactive regarding nitrating agents than the activated phenolic ring of tyrosine” (Ikeda et al., 2007). Even though the normal abundance of tyrosine residues make up to 3.2% in most proteins (Clementi et al., 1999), the targeted tyrosine residues for PTN in a given protein sample is unpredictable (Ischiropoulos, 1998; Ischiropoulos, 2003; Souza et al., 1999). Figure 4.1 demonstrates the modification that involves irreversible and reversible changes on a protein with exposure to reactive oxygen (ROS) and nitrogen species (RNS).

![Figure 4.1: Modification of proteins by reactive oxygen and nitrogen species. Upon interaction with reactive oxygen and nitrogen species (ROS/RNS), proteins can undergo a wide range of reversible and irreversible chemical modifications. Adopted from (Bachi et al., 2012).](image)

In this chapter, the changes in the pollen protein with respect to exposure to NO₂, O₃ and RH were assessed and a comparative study was made with other studies where possible.
4.1.1 Biochemistry of PTM

PTMs happen when proteins react with reactive agents and protein structures are chemically altered. A number of different types of these modifications have been identified and new modifications are still being discovered (Khoury et al., 2011; Jensen, 2004). Protein nitration is among the most common modifications and is linked to the onset of diseases. The nitration of a protein often involves the addition of a nitro group (–NO₂) to the phenolic ring of a tyrosine residue (Zhan and Desiderio, 2009). Tyrosine nitration leads to addition of a –NO₂ at the 3rd position of the aromatic ring of amino acid, tyrosine forming 3-nitrotyrosine (NTyr) (Walcher et al., 2003). The NO₂ creates a substantial shift in the pKₐ (which expresses the strength of the compound as an acid) value of the Tyr residue from ~10 to ~7, consequently increasing the acidic nature of the hydroxyl group (Abriata et al., 2009). It also decreases the pKₐ of the neighboring hydroxyl moiety, and may modify protein makeup, role, and interactions (Quint et al., 2006; Sokolovsky et al., 1967). Nitrated tyrosine is usually in low-abundance that is associated with a variety of diseases (Abello et al., 2009; Bakillah, 2009; Pacher et al., 2007; Sultana et al., 2008; Zhan and Desiderio, 2006).

4.1.2 Challenges in charactering post-translational modification of pollen

There are several techniques to quantity protein modification, both using mass spectrometry and non-mass spectrometry methods. PTMs are chemical alterations of proteins that may explain extremely diverse protein activities in living organisms (Theillet et al., 2012). Due to the fact that PTMs are accompanied with mass changes, for example a 45 Da increment for nitration, the characterization of PTMs on a certain protein or on a subset of proteins is possible using mass spectrometry with proteolytic digestions of
sample where necessary. However, it has been reported as a challenging task to reliably identify and quantity protein nitration due to its low stoichiometry in a biological system (Ischiropoulos, 2003; Radi, 2004), which may also contribute to misidentifications of nitrated peptides by “shotgun” proteomics (Prokai, 2009; Stevens et al., 2008). Shotgun proteomics is when an indirect measurement of proteins through peptides derived from proteolytic digestion of intact proteins is achieved (Zhang et al., 2013). Because PTMs generate a mixture of complex and heterogenic gene products, the determination is one of the main challenges in proteomics research (Jensen, 2004). Li et al. (2011) have recently discussed the need for full valuation of proteomic data and the validation of workflows when searching the nitroproteome as a result of the challenges and suggested the usage of well-defined reference standards (Li et al., 2011). “There are thousands of distinct proteins in biological systems and if nitration is specific, establishing exactly which residue(s) within any specific protein(s) is/are nitrated becomes extraordinarily challenging” (Tsikas and Duncan, 2014).

Even though numerous methods (direct and indirect) based on chromatography, mass spectrometry, and/or immunoassay have been developed for identification and quantitation of protein nitration (Agaton et al., 2004; Bigelow and Qian, 2008; Duncan, 2003; Garbis et al., 2005; Koeck et al., 2004; Ryberg and Caidahl, 2007; Tsikas and Caidahl, 2005), recent publications have highlighted limitations associated with these techniques (Bigelow and Qian, 2008; Duncan, 2003; Ryberg and Caidahl, 2007; Tsikas, 2012; Tsikas and Caidahl, 2005). “Liquid chromatography/mass spectrometry (LC-MS) does not offer sufficient selectivity for 3-nitrotyrosine measurement in biological samples” (Tsikas and Duncan, 2014). In previous decades, a variety of mass spectrometric quantification methods have been developed, which include isobaric stable
isotope tagging that allows multiplexing in relative or absolute proteomic quantification experiments via tandem mass spectrometry (Ahrné et al., 2016).

For all of the above-mentioned reasons, in vitro site-specificity studies on protein tyrosine nitration are challenging (Prokai, 2009; Stevens et al., 2008). Clearly, there is the need for additional sample manipulation and high-quality investigations to identify 3NT in a sample with less challenge. Figure 4.2 displays simplified schematic of the procedures used for proteomics analysis on MS/MS.

![Figure 4.2: Simplified schematic of the procedures used for proteomics analysis on MS/MS (adopted from Tsikas and Duncan, 2013).](image)

4.1.3 Impact of O₃ exposure on pollen grain

Pollen grains contain a significant quantity of protein materials (Shiraiwa et al., 2012b). Air pollution can impact this protein materials particularly; the allergen content, the probability of allergen protein release or possible alterations to the allergen proteins (Frank and Ernst, 2016). Beck et al. (2013) revealed that Bet v 1 allergen content is positively linked with increasing O₃ levels (Beck et al., 2013). This is surprising since O₃ itself cannot generate new allergenic proteins. However, the metabolism of both plants
and animals can be affected by \(O_3\) where its toxicity occurs as a result of ROS, such as the superoxide anion radical (\(\bullet O_2^-\)), hydrogen peroxide (\(H_2O_2\)), the hydroxyl radical (\(\bullet OH\)), and singlet oxygen (\(^1O_2\)) (Mudd, 1996). In an \textit{in vitro} analysis with different \(O_3\) concentrations, the pollen of different species displayed dissimilarities in the total soluble protein (TSP) content (Frank and Ernst, 2016). Increased allergen contents as a result of elevated \(O_3\) have also been revealed for other plant species (Eckl-Dorna et al., 2010; Masuch et al., 1997). However, additional studies on \textit{in vivo} and \textit{in vitro} fumigated ragweed pollen found contrary results regarding the allergen content of the major allergen \(Amb\ a\ 1\) (no alterations) (Kanter et al., 2013; Pasqualini et al., 2011).

### 4.1.4 Impact of NO\(_2\) exposure to the pollen grain

The influence of NO\(_2\) on pollen grain has been investigated in several studies. The study done on \textit{Betula pendula} showed decreased pollen viability (Shivanna et al., 1991) after exposure to NO\(_2\). Ragweed exhibited higher allergen levels under very high concentration of the pollutant (Ghiani et al., 2012; Zhao et al., 2016). PTM such as S-nitrosylation or the nitration of pollen proteins can result from exposure to air pollution such as NO\(_2\) (Frank and Ernst, 2016). Increased S-nitrosylation modification was observed following the fumigation of ragweed plants with raised NO\(_2\) concentrations during a cultivating period (Zhao et al., 2016). This happens on the key ragweed allergen \(Amb\ a\ 1\) proteins (Zhao et al., 2016). An \(O_3\)-dependent increase of nitration due to NO\(_2\) was reported to occur with aerosolized proteins (Shiraiwa et al., 2012b). Franze et al. (2005) reported that in the presence of NO\(_2\) and \(O_3\), major allergen (Bet v 1) from birch is effectively nitrated (Franze et al., 2005). However, the nitration degree was significantly lower when exposed to only NO\(_2\) which indicated that reactive species produced upon the interaction of \(O_3\) and
NO$_2$ performs an important function in the nitration process (Frank and Ernst, 2016). Conversely, nitration of Bet v 1 may modify the allergenic potential of the protein (Selzle et al., 2013).

4.2 Results and Discussion

Initially, in this research the intact birch pollen grain was first exposed to the gas phase and then proteins were extracted afterwards as described in Chapter 3 (section 3.2 & 3.3). Contrary to expectations, this study did not find a significant difference between the unexposed and gas-phase exposed samples in terms of modification. The nitrated modified form of the allergen protein was unable to be detected in the mass spectrum of intact pollen protein, which supports another work of Vrtala et al. (1993) that mentioned Bet v 1 is rapidly transported to the surface of the grain and released upon hydration (Vrtala et al., 1993). Vrtala et al. (1993) also mentioned that the allergen content of the pollen pellet fractions decreases after hydration (Vrtala et al., 1993). This was also observed in this research. Hence it appears, without hydration, PTM nitration of pollen is not likely to occur. In another study, it was mentioned that pollen grains are devised to defend their genetic material cargo that can be achieved via resilient to environmental pressures subjected to them during transport (Pope, 2010). Additionally, Pope (2010) also stated that the water composition of pollen is reliant on the atmospheric RH. That at high moisture the pollen grain swells by internal uptake of atmospheric water (Diehl et al., 2002; Dingle, 1966; Pope, 2010). Using the dot blot technique, the results further confirmed the absence of nitration as already indicated in the MS results. Subsequently, nitration seems to be present in some of the search results, where pollen was ruptured prior to exposure.
The samples were further subjected to quantification analysis using the tagging experiment and the enrichment strategy, which was supposed to allow quantification of the degree of nitration of the nitrated protein. Both non-enriched and enriched samples were analysed on the MS. At each stage of the tagging experimental step, aliquot amount of the sample was taken and checked on the MS. This helped in detecting any step that needed optimization. The results from MS/MS analysis, blotting techniques, in-gel digestion and TMT labelling will all be presented here.

### 4.2.1 Summary of technical and experimental replicates.

Experimental replicates are equivalent measurements of (biologically) separate samples and technical replicates are repeated measurements of the same sample that represent independent measurement (Blainey et al., 2014). Both experimental and technical replicates are necessary for accurate and consistent results. Technical replicates will aid identify errors caused by processing variation, while experimental replicates will help confirm that (biological) changes are real and not an irreproducible coincidence.

In this research, the technical replicates were employed in two ways; for the dot blot experiment, on single membrane, one sample is used once or in triplicates spots. The intensity measurement of spots under same experimental conditions were put together and the average reading was used for plotting graph. And for the MS analysis, experiments were done twice which are then combined and run as repeats on the MS, which then generates one or two data sets. For the experimental replicates (normally how many times the initial experiment was done), in total, the main experiments done with RH and NO2 only (on hydrated pollen) were 42 exposures with at least three exposures in a day. However, each experiment was conducted on three different days. Again, samples generated under same experimental conditions are sometimes combined and run on the
MS. The experimental replicates were also employed for the blot techniques.

4.2.2 Protein concentration

The protein concentration determined by the BCA method was ascertained in order to know the quantity of the protein in each sample. This is a requirement for determining the adequate amount of sample needed in µg/ml for each experiment such as the dot blotting (5 µg/ml) and MS/MS analysis (from 20 µg/ml upwards depending on the protocol). The result showed that all samples generated contained protein where some samples were more or less than 2 mg/ml (the standard of the BCA assay). Those above 2 mg/ml were diluted prior to the experimental procedure to enable quantification against the BCA standard. The supernatant acquired before addition of the second extraction buffer was reported to have the highest level of protein concentration. In an attempt to understand the reason behind this result, all pellet and supernatant generated before and after the addition of the second extraction buffer were ran on SDS-gel, which is discussed in a section.

4.2.3 SDS-PAGE gel separation

SDS-PAGE is a powerful method that separates proteins according to their molecular weight (measured in kilo Daltons, kDa) using protein molecular markers as indicators (also known as protein ladder) to estimate the size of the resolved protein by gel electrophoresis. SDS-PAGE was employed to determine the best (sample extract that has the most protein content) end product to be used: the pellet, supernatant or combination of both. The naming of the different pellet and supernatant samples are provided below, the methodology used to generate the various samples is given in Chapter 3 under section 3.3, which also explained how the pollen grain are being ruptured after exposure to NO₂:
• Pellet 1 (P1) obtained before addition of 2nd extraction buffer that contains SDS.
• Supernatant 1 (SN1) obtained before addition of 2nd extraction buffer that contains SDS.
• Pellet 2 (P2) obtained after addition of 2nd extraction buffer that contains SDS.
• Supernatant 2 (SN2) obtained after addition of 2nd extraction buffer that contains SDS.

Figure 4.3 shows the flow chart of the different pollen samples generated as explained above. Prior to running the gel, the protein concentration results from the BCA analysis showed that a greater amount of total soluble protein is present before the addition of second (2nd) extraction buffer. Hence, the addition of buffer containing SDS results in the loss of proteins instead of the expected gain. The reason behind the loss of protein is not understood. Regarding the proteins bands on the gel (Figure 4.4 A), the SN2 and P1 samples possess near identical protein bands. SN1 had the most concentrated protein bands, as diagnosed by visual inspection. Whilst, P2 appears to have the least amount of protein compared to SN1, SN2, and P1, again by visual inspection. As a result of the observations gained by the SDS-PAGE results, all subsequent work was carried out using the supernatant SN1 samples. The SDS addition step was not used in the extraction buffer.
Figure 4.3: Flow chart of the different pollen extract generated for the experiments. The pollen samples were ruptured mechanically (using precellys tube with metal beads) after exposure to NO$_2$ and different type of samples produced. This was termed SN1, P1, SN2 and P2 obtained before and after addition of SDS. Pellet 1 (P1) obtained before addition of 2$^{nd}$ extraction buffer that contains SDS. Supernatant 1 (SN1) obtained before addition of 2$^{nd}$ extraction buffer that contains SDS. Pellet 2 (P2) obtained after addition of 2$^{nd}$ extraction buffer that contains SDS. Supernatant 2 (SN2) obtained after addition of 2$^{nd}$ extraction buffer that contains SDS. 1$^{st}$ extraction buffer-50 mM Tris-HCl pH6.8, 10% sucrose and inhibitory proteases; 2$^{nd}$ extraction buffer-1st extraction buffer with additional 2% SDS.

Figure 4.4: SDS-PAGE gel results of unexposed sample and NO$_2$ exposed sample to 10ppm respectively (A & B). A- From left to right, pre stained protein ladder at 10µl and all sample extracts in equal proportions, 20µl. B- From left to right: pre stained protein ladder at 10 µl and all sample extracts in equal proportions, 20 µl. There is no visually detectable difference between the two gels. Pellet 1 (P1) obtained before addition of 2$^{nd}$ extraction buffer that contains SDS. Supernatant 1 (SN1) obtained before addition of 2$^{nd}$ extraction buffer that contains SDS. Pellet 2 (P2) obtained after addition of 2$^{nd}$ extraction buffer that contains SDS. Supernatant 2 (SN2) obtained after addition of 2$^{nd}$ extraction buffer that contains SDS. 1$^{st}$ extraction buffer-50 mM Tris-HCl pH6.8, 10% sucrose and inhibitory proteases; 2$^{nd}$ extraction buffer- 1$^{st}$ extraction buffer with additional 2% SDS.
Figure 4.4 (A & B) displays the gels of unexposed sample and NO$_2$ exposed sample to 10 ppm respectively. The polypeptide profiles, revealed by the SDS-PAGE analysis, of the pollen extracts (Figure 4.4 A & B) reveal no discernible differences among the unexposed and exposed pollen samples of SN1, P1, SN2 and P2 extracts. All electrophoretic profiles have several bands ranging from 130 to 15kDa in common, however, the density of the bands are lower in some of the samples. This indicates that the exposure of pollen to the NO$_2$ did not significantly modify the physical properties of the pollen extracts. To be precise, it did not affect the molecular weight of the soluble and insoluble proteins within the selectivity and sensitivity of the SDS-PAGE method (Cuinica et al., 2013). This is expected as the technique does not have the capability of detecting definite modification but is able to reveal the molecular weight of soluble and insoluble protein. Earlier study reported the presence of inestimable changes between the polypeptide profiles of pollen extracts exposed and not-exposed to atmospheric contaminants (Rezanejad, 2007), which is in agreement with this research findings. Irrespective of the experimental condition, the fact still remains that SN1 had the most concentrated protein bands.

**4.2.4 Trypsin digestion**

Digesting the proteins with trypsin enzyme converted the proteins into peptide. Even though the mass spectrometer can analyse the intact proteins, peptides are preferred during proteomics analysis since proteins can be problematic to work with and might not all be soluble under the equivalent conditions (Steen and Mann, 2004).
4.2.5 Protein/Peptide identification

Several procedures have been established to statistically measure peptide identification results from database search programs (Zhang et al., 2013). The obstacle of bottom-up proteomics is the requirement to assign peptide identifications back to their respective proteins, which becomes difficult when redundant entries or protein isoforms exist (Zhang et al., 2013).

Tables 4.1-4.4 below show the list of identified proteins from the MS analysis of different conditions/treatments of some of the samples. Each sample analyzed had greater than or equal to 5 identified proteins in each sample submitted (either pellet or supernatant). Each protein sample contained its own unique set of protein where some of the samples share one or two same type of protein while others were distinct. Every identified protein in the list is the master protein in the protein group, which is ranked according to the number of peptide sequences, the number of PSMs (peptide spectrum matches), their protein scores (sum of the ion scores of all peptides that were identified), and the protein sequence coverage. PSM is the total number of identified peptide sequence matched for the protein and its value may be higher than the number of peptides identified for high-scoring proteins because peptides may be identified repeatedly. PSM is one of the determinant of peptide confidence that is used in determining and influences protein confidence (Zhang et al., 2015). Protein coverage is the coverage of the identified protein calculated in percent by dividing the number of amino acids in all found peptides by the total number of amino acids in the entire protein sequence; while PSM is the total number of identified peptide sequences for the protein, including those redundantly identified. Both when higher indicates high protein confidence identification of the protein in question.
Normally, numerous amounts of proteins are identified and available for each sample when searched against the UniProt database. Using the high confident peptide filter, only the master proteins (the software identifies as high confident peptide) are displayed on the proteins page. By default, the top-ranking protein of a group becomes the master protein of that set. So, this could be one of the reasons why the different experimental conditions do not have the same identified protein because the score of a set of peptides would be different and might not be recognized as high confident peptide in another sample. Therefore, the master protein would be different in the same protein group.

Looking at the chromatogram of Figure 3.13 (Chapter 3), again it can be seen that each sample condition/treatment has its own unique chromatogram even though all samples are from the same pollen. These graphical representations illustrate that each treatment/condition has a different impact on pollen (chromatogram) which additionally explains the reason for the presence of dissimilar protein within the samples generated under different treatment. The NO$_2$ exposed sample had denser chromatogram followed by O$_3$ exposed sample and lastly, RH exposed sample. The reason for this is not clear but it may have something to do with the effect of gases on the pollen. “Even for a single cell, the proteome will change in response to different stimuli” (Han et al., 2008). Putting all the findings together, there is definitely an interaction between air pollutant and pollen grain.
### Control sample (not exposed)

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Protein description</th>
<th>Protein coverage</th>
<th>Peptide</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>P43186</td>
<td>Major pollen allergen Bet v 1-M/N</td>
<td>44.38</td>
<td>AFILDGDNLIPK, IVATPDGGSILK, KITFPEGSPFK, AVESYLLAHSDAYN, VAPQAISSEVAlEGNGGPITIKK, ITFPEGSPFK</td>
<td>N11 (Deamidated)</td>
</tr>
<tr>
<td>P43179</td>
<td>Major pollen allergen Bet v 1-F/I</td>
<td>44.38</td>
<td>AFILDGDNLFPK, IVATPnGGSILK, AVESYLLAHSDAYN, ISFPEGFPFK, VAPQAISSEVAlEGNGGPITIKK</td>
<td>N6 (Deamidated), N11 (Deamidated)</td>
</tr>
<tr>
<td>P43177</td>
<td>Major pollen allergen Bet v 1-D/H</td>
<td>36.88</td>
<td>AFILDGDNLVPK, AVESYLLAHSDAYN, INFPEGFPFK, VAPQAISSEVAlEGNGGPITIKK</td>
<td>N11 (Deamidated)</td>
</tr>
<tr>
<td>O49813</td>
<td>Olee1-like protein OS=Betula</td>
<td>19.28</td>
<td>LANPLGFMK, SIIRQAPALCFLsLLGFAYsESR</td>
<td>S13 (Phospho), S20 (Phospho)</td>
</tr>
<tr>
<td>P38500</td>
<td>Ferredoxin–nitrite reductase, chloroplastic</td>
<td>5.83</td>
<td>VEEREGYWLKEK, qVAVTRPVR, IGSDSHLGNLYK</td>
<td>Q1 (Deamidated)</td>
</tr>
</tbody>
</table>

Table 4.1: Identified tryptic peptides of birch pollen protein from the control sample (unexposed) (combination of 2 biological replicates run on MS, which is presented as 1 data). The protein description (protein name and description), coverage (the percentage of the protein sequence covered by identified peptides), peptide score (the sum of the highest ions score for each distinct sequence), sequence, modification and accession number (unique number for protein identification) are listed. In total, 5 proteins were identified where only three are the allergen protein.
## RH exposed sample

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Protein description</th>
<th>Protein coverage</th>
<th>Peptide</th>
<th>Modification</th>
</tr>
</thead>
</table>
| O23746           | Pollen allergen, Betv1 (Fragment) | 55.35 | VAPQAISVENIEGNGGP GTIK     
VAPQAISVENIEGnGGGP GTIK  
AFILDGDNLIPK  
GVFNYESETTSVIPAAR  
KITFPEGSPFK  
VAPQAISVENIEGNGGPGTIKK  
GVFnYESETTSVIPAAR  
ITFPEGSPFK  
LVATPDGGSILK  
GVFNyESETTSVIPAAR  
GVFNyESETTSVIPAAR  
VAPQAISVENIEGnGGPGTIKK  
VAPqAISSVENIEGnGGPGTIKK  
AVESYLLAHSDAYN | N15(Deamidated) |
| O23747           | Pollen allergen, Betv1 (Fragment) | 41.51 | VAPQAISVENIEGNGGP GTIK     
VAPQAISVENIEGnGGGP GTIK  
AFILDGDNLVPK  
GVFNyEETTSVIPAAR  
KITFPEGSPFK  
VAPQAISVENIEGnGGPGTIKK  
VAPQAISVENIEGnGGPGTIKK  
VAPqAISSVENIEGnGGPGTIKK  
AVESYLLAHSDAYN | N15(Deamidated); Y5(Phospho) |
| QOQLV6           | Major allergen Bet v 1.02A (Fragment) | 42.00 | VAPQAISVENIEGNGGP GTIK     
VAPQAISVENIEGnGGPGTIKK  
AFILDGDNLIPK  
KITFPEGSPFK  
VAPQAISVENIEGnGGPGTIKK  
ITFPEGSPFK  
VAPQAISVENIEGnGGPGTIKK  
VAPqAISSVENIEGnGGPGTIKK  | N11(Deamidated); Q4(Deamidated); N15(Deamidated); N4(Deamidated) |
<table>
<thead>
<tr>
<th>Accession</th>
<th>Protein Description</th>
<th>Peptide Score</th>
<th>Sequence</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q0QLW0</td>
<td>Major allergen Bet v 1.01C</td>
<td>23.33</td>
<td>IcNEIKLVAtPDGGstLK C2(Nitrosyl); T10(Phospho); S15(Phospho); T16(Phospho)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Fragment)</td>
<td></td>
<td>VAPQAISSVKnIEGnGGPGTIKK VAPqAISSVKnIEGnGGPGTIKK IVATpHGSILK</td>
<td>N11(Deamidated); N15(Deamidated)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VAPqAISSVKnIEGnGGPGTIKK VAPQAISSVKnIEGnGGPGTIKK</td>
<td>N4(Deamidated); N11(Deamidated); N15(Deamidated)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N6(Deamidated)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Q4(Deamidated); N11(Deamidated); N15(Deamidated)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N11(Deamidated); N15(Deamidated)</td>
</tr>
<tr>
<td>C0VV3</td>
<td>PR-10 protein (Fragment))</td>
<td>28.67</td>
<td>AFILDGDNLIPK KITFPEGSPFK ITFPEGSPFK IVPAPGGSILKISnKyHTK</td>
<td>S9(Phospho); N15(Deamidated); Y17(Nitro)</td>
</tr>
<tr>
<td>A0A096XCA4</td>
<td>Glutathione S-transferase</td>
<td>4.22</td>
<td>VIGESDLIK</td>
<td>NA</td>
</tr>
<tr>
<td>O49813</td>
<td>Oleo1-like protein</td>
<td>6.63</td>
<td>ELGmNPDDVIQ</td>
<td>M4(Oxidation)</td>
</tr>
<tr>
<td>Q39419</td>
<td>Polcalcin Bet v 4</td>
<td>16.47</td>
<td>MADDHPqDKAERER</td>
<td>Q7(Deamidated)</td>
</tr>
</tbody>
</table>

Table 4.2: Identified tryptic peptides of birch pollen protein from the RH exposed sample (combination of 2 biological replicates run on MS, which is presented as 1 data). The protein description (protein name and description), coverage (the percentage of the protein sequence covered by identified peptides), peptide score (the sum of the highest ions score for each distinct sequence), sequence, modification and accession number (unique number for protein identification) are listed. In total, 8 proteins were identified where only four are the allergen proteins.
### NO$_2$ exposed sample

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Protein description</th>
<th>Protein coverage</th>
<th>Peptide</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>O23747</td>
<td>Pollen allergen, Betv1</td>
<td>55.35</td>
<td>VAPQAISSVENIEGNGGPGTIK</td>
<td>N15(Deamidated)</td>
</tr>
<tr>
<td></td>
<td>(Fragment)</td>
<td></td>
<td>VAPQAISSVENIEGnGGPGTIK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GVFNYESETTSVIPAAR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AFILDGDNLIPK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GVFnYESETTSVIPAAR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GVFNyeSETTSVIPAAR</td>
<td>N4(Deamidated)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LVATPDGGSILK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AVESYLLAHISDAYN</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ITFPEGSPFK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GVFnYESETTSVIPAAR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>KITFPEGSPFK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VAPQAISSVENIEGNGGPGTIKK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VAPQAISSVENIEGnGGPGTIKK</td>
<td></td>
</tr>
<tr>
<td>O23746</td>
<td>Pollen allergen, Betv1</td>
<td>41.51</td>
<td>GVFNYEIEETTSVIPAAR</td>
<td>N15(Deamidated)</td>
</tr>
<tr>
<td></td>
<td>(Fragment)</td>
<td></td>
<td>VAPQAISSVENIEGNGGPGTIK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VAPQAISSVENIEGnGGPGTIK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AFILDGDNLVPK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AVESYLLAHISDAYN</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ITFPEGSPFK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GVFnYESETTSVIPAAR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>KITFPEGSPFK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VAPQAISSVENIEGNGGPGTIKK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VAPQAISSVENIEGnGGPGTIKK</td>
<td></td>
</tr>
<tr>
<td>Q546V0</td>
<td>Pollen allergen, Betv1</td>
<td>49.06</td>
<td>VAPQAISSVENIEGNGGPGTIK</td>
<td>N15(Deamidated)</td>
</tr>
<tr>
<td></td>
<td>(Fragment)</td>
<td></td>
<td>VAPQAISSVENIEGnGGPGTIK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AFILDGDNLFPK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GVFNYTEETTSVIPAAR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GVFnYTEETTSVIPAAR</td>
<td>N4(Deamidated)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IVATPDGGSILK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AVESYLLAHISDAYN</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VAPQAISSVENIEGNGGPGTIKK</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VAPQAISSVENIEGnGGPGTIKK</td>
<td></td>
</tr>
<tr>
<td>P43176</td>
<td>Major pollen allergen</td>
<td>44.38</td>
<td>VAPQAISSVENIEGNGGPGTIK</td>
<td>N15(Deamidated)</td>
</tr>
<tr>
<td>Protein ID</td>
<td>Protein Description</td>
<td>Coverage</td>
<td>Peptide Score</td>
<td>Sequence</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------</td>
<td>----------</td>
<td>---------------</td>
<td>----------</td>
</tr>
<tr>
<td>Q0QLS9</td>
<td>Major allergen Bet v 1.01E (Fragment)</td>
<td>23.33</td>
<td>VAPQAISSVENIEGnGGPGTIKK</td>
<td>N15 (Deamidated)</td>
</tr>
<tr>
<td>Q0QLW0</td>
<td>Major allergen Bet v 1.01C (Fragment)</td>
<td>31.33</td>
<td>AFILGDGDLFPK</td>
<td>N6 (Deamidated); N11 (Deamidated); N15 (Deamidated)</td>
</tr>
<tr>
<td>O65002</td>
<td>Isoflavone reductase homolog Bet v 6.0101 (Fragment)</td>
<td>8.33</td>
<td>VVFGDGNAR</td>
<td>N8 (Deamidated); S16 (Phospho); S22 (Phospho); S29 (Phospho)</td>
</tr>
<tr>
<td>A0A096XCA4</td>
<td>Glutathione S-transferase</td>
<td>4.22</td>
<td>VIGESLDLIK</td>
<td>T1 (Phospho); T2 (Phospho); C19 (Nitrosyl)</td>
</tr>
<tr>
<td>O49813</td>
<td>Olectic-like protein</td>
<td>5.42</td>
<td>LANPLGFMK</td>
<td></td>
</tr>
<tr>
<td>Q64LH2</td>
<td>Profilin-2</td>
<td>19.08</td>
<td>tMAlIGIFDEPMAPGcNMIVER</td>
<td>T1 (Phospho); T2 (Phospho); C19 (Nitrosyl)</td>
</tr>
<tr>
<td>V5LU01</td>
<td>Cysteine protease</td>
<td>7.77</td>
<td>FSEqQVLDCDntnAGCDGGLMEPAFTYVIK</td>
<td>Q4 (Deamidated); M11 (Oxidation); T12 (Phospho); N13 (Deamidated)</td>
</tr>
<tr>
<td>E1XUL9</td>
<td>Pectate lyase (Fragment)</td>
<td>8.53</td>
<td>HGFVQVVnNNYERWGsYALGGsAGPTILsQGNR</td>
<td>N8 (Deamidated); S16 (Phospho); S22 (Phospho); S29 (Phospho)</td>
</tr>
<tr>
<td>Q64LH3</td>
<td>Profilin OS=Humulus scandens</td>
<td>19.08</td>
<td>tSQALHGYYDEPMtPGcNMIVER</td>
<td>T1 (Phospho); T15 (Phospho); Q18 (Deamidated); C19 (Nitrosyl)</td>
</tr>
</tbody>
</table>

Table 4.3: Identified tryptic peptides of birch pollen protein from the NO2 exposed sample (combination of 2 biological replicates run on MS, which is presented as 1 data). The protein description (protein name and description), coverage (the percentage of the protein sequence covered by identified peptides), peptide score (the sum of the highest ions score for each distinct sequence), sequence,
Modification and accession number (unique number for protein identification) are listed. In total, 13 proteins were identified where 6 are the allergen protein.

**O₃ NO₂ exposed sample**

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Protein description</th>
<th>Protein coverage</th>
<th>Peptide</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>O23747</td>
<td>Pollen allergen, Betv1 (Fragment)</td>
<td>55.35</td>
<td>GVFnYESETTSVIPAAR VAPQAISVENIEGNGPGTIKK VAPQAISVENIEGNGGPITIK VAPQAISVENIEGNGPGTIKK VAPqAISVENIEGnGPGTIK GVFnYESETTSVIPAAR AFILDGDNLIFK VAPQAISVENIEGNGPGTIKK GVFnYESETTSVIPAAR KITFPEGSPFK LVATPDGGSILK AVESYLLAHSDAYN ITFPEGSPFK</td>
<td>N11 (Deamidated) Q4 (Deamidated); N15 (Deamidated) N4 (Deamidated)</td>
</tr>
<tr>
<td>Q546V0</td>
<td>Pollen allergen, Betv1 (Fragment)</td>
<td>60.38</td>
<td>VAPQAISVENIEGNGPGTIKK VAPQAISVENIEGNGPGTIKK VAPQAISVENIEGNGPGTIKK GVFnYESETTSVIPAAR AFILDGDNLIFK VAPqAISVENIEGnGPGTIK GVFnYESETTSVIPAAR VAPQAISVENIEGNGPGTIKK IVATPDGGSILK AVESYLLAHSDAYN ISFPEGFPFK GVFnYESETTSVIPAAR EMGETLLR</td>
<td>N11 (Deamidated) Q4 (Deamidated); N15 (Deamidated) Y5 (Phospho) N11 (Deamidated) N4 (Deamidated)</td>
</tr>
<tr>
<td>Accession</td>
<td>Description</td>
<td>pI</td>
<td>Sequence (Fragment)</td>
<td>Post-translational Modifications</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------</td>
<td>--------</td>
<td>------------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>O23746</td>
<td>Pollen allergen, Betv1 (Fragment)</td>
<td>52.83</td>
<td>GVFNYEIEITTSVIPAAR VAPQAISSVENIEGNGGPGTI IKK VAPQAISSVENIEGNGGPGTI IK VAPQAISSVENIEGNGGPGTI K AFILGDNLVPK VAPqAISSVENIEGNGGPGTI K KINFPEGFPFK VAPQAISSVENIEGNGGPGTI KK GVFaYEIEITTSVIPAAR AVESYLLAHSDAYN GVFnYeIEITTSVIPAAR GVFNyIEITTSVIPAAR EMGETLLR</td>
<td>N11 (Deamidated) Q4 (Deamidated); N15 (Deamidated)</td>
</tr>
<tr>
<td>Q9SCH5</td>
<td>Pollen allergen Betv1, isoform at7</td>
<td>41.88</td>
<td>VAPQAISSVENIEGNGGPGTIKK VAPQAISSVENIEGNGGPGTI KK VAPQAISSVENIEGNGGPGTI KK VAPqAISSVENIEGNGGPGTI KK VAPQAISSVENIEGNGGPGTIKK AFILGDNLIPK KITFPEGSPFK AVESYLLAHSDAYN ITFPEGSPFK EMGETLLR</td>
<td>N11 (Deamidated) Q4 (Deamidated); N15 (Deamidated) N11 (Deamidated)</td>
</tr>
<tr>
<td>Q0QLS9</td>
<td>Major allergen Bet v 1.01E (Fragment)</td>
<td>35.33</td>
<td>VAPQAISSVQnIEGnGGPGTIKK AFILGDNLIPK VAPqAISSVQnIEGnGGPGTIKK KINFPEGFP FK VAPqAISSVQnIEGnGGPGTIKK EMGETLLR VAPQAISSVQnIEGnGGPGTIKK</td>
<td>N11 (Deamidated); N15 (Deamidated) Q4 (Deamidated); N11 (Deamidated); N15 (Deamidated) Q4 (Deamidated) N15 (Deamidated)</td>
</tr>
<tr>
<td>Accession Number</td>
<td>Protein Description</td>
<td>Coverage</td>
<td>Peptide Score</td>
<td>Sequence</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------</td>
<td>----------</td>
<td>---------------</td>
<td>----------</td>
</tr>
<tr>
<td>Q0QLW0</td>
<td>Major allergen Bet v 1.01C (Fragment)</td>
<td>43.33</td>
<td></td>
<td>VAPQAISSVKnIEGrGGPTIK VAPqAISSVKnIEGrGGPTIK AFILGDNLFPK IVATPnGGSILK ISFPEGFPFK VAPqAISSVKnIEGrGGPTIKK EMGETLLR</td>
</tr>
<tr>
<td>O65002</td>
<td>Isoflavone reductase homolog Bet v 6.0101 (Fragment)</td>
<td>10.67</td>
<td></td>
<td>VVIFGDGNAR AVFNKEDDIGTYTIR IYVPEEK</td>
</tr>
<tr>
<td>A0A096XCA4</td>
<td>Glutathione S-transferase</td>
<td>4.22</td>
<td></td>
<td>VIGESLDLIK</td>
</tr>
<tr>
<td>O49813</td>
<td>Olec1-like protein</td>
<td>5.42</td>
<td></td>
<td>LANPLGFmK</td>
</tr>
</tbody>
</table>

Table 4.4: Identified tryptic peptides of birch pollen protein from the O₃ / NO₂ exposed sample (combination of 2 biological replicates run on MS, which is presented as one data). The protein description (protein name and description), coverage (the percentage of the protein sequence covered by identified peptides), peptide score (the sum of the highest ions score for each distinct sequence), sequence, modification and accession number (unique number for protein identification) are listed. In total, 9 proteins were identified where 6 are the allergen protein.
The overall MS proteomics results indicated a good protein digestion and identification in some of the samples. Additionally, the NO$_2$-exposed pollen samples showed slightly higher protein content compared with the other samples depending on the exposure conditions. This is evident from the number of proteins identified in each sample. Previous literatures have reported both a decrease and increase in the protein content of different pollen species exposed to diverse pollutants with variation in concentration and exposure time (Bist et al., 2004; Majd et al., 2004; Parui et al., 1998; Rezanejad, 2009; Rezanejad et al., 2003; Rogerieux et al., 2007). It can then be assumed that the effects of the gases are concentration and species specific.

All three exposed samples had 5 proteins in common (highlighted in red) while only 1 protein was common (highlighted in blue) to all 4 samples. The proteins highlighted in green are only present and common to NO$_2$ and O$_3$/NO$_2$ exposed samples. Higher protein content was seen in the sample exposed to NO$_2$ having 13 identified proteins (6 allergen proteins) as compared to RH (9 protein where 4 are allergen proteins) and O$_3$/NO$_2$ (9 protein where 6 are allergen proteins) exposed samples with fewer proteins from the unexposed pollen sample (5 protein where 3 are allergen proteins). Although higher protein identification was found in NO$_2$ exposed sample, the sequence coverage in the O$_3$/NO$_2$ exposed sample was found in comparison with the other samples to have higher coverage in most of the protein. Contrary to expectation, there was not much difference between NO$_2$ and O$_3$/NO$_2$ exposed sample as earlier studies mentioned the increased uptake of NO$_2$ in the presence of O$_3$. Both samples had the same number of allergen protein content, however none was nitrated as indicated on the MS analysis results (Tables 4.3 and 4.4). This technique clearly identifies the birch allergen protein, Bet v 1. All samples undergone similar PTMs as seen in the Tables (4.1-4.4).
4.2.6 Pollen rupture and Gas exposure

To further understand the effects of the gases on the pollen grain, different pollen rupturing techniques were used prior to the gas exposure. Figure 4.5 illustrates the flow chart of pollen rupture techniques and gas exposures at different NO₂ concentration. The TSP (total soluble protein) concentration of the pollen extract was quantified using the BCA protein assay. From the MS analysis results, hydrated pollen in rainwater showed higher allergen protein content in comparison to 24-hours-exposed pollen, however, the combination of crushed pollen and hydrated in rainwater gave the highest protein content. The contact of birch pollen grain with rainwater releases allergens by a mechanisms termed “abortive germination” that is described by development of short pollen tubes, which rupture at their tips and discharge particles containing allergen protein (Grote et al., 2003). Viewing the samples under scanning electron microscope (described in a Chapter) verified the reason behind the results obtained.

Figure 4.5: Flow chart illustrating pollen rupture techniques and gas exposures. All NO₂ exposures were done within 60 minutes. Note that the ppm values in the figure refer to the NO₂ concentration.
Table 4.5 below shows some of identified Bet v 1 protein and a comparison between peptide sequence coverage of 9 samples subjected to different conditions/treatments; control is intact pollen without exposure to RH and NO₂, RW is the hydrated pollen in rainwater, CR is crushed pollen and hydrated in rainwater, and 24 hrs pollen is exposed to high RH for 24 hours. Note, all generated samples were exposed to NO₂ at different levels of concentrations (2 ppm, 5 ppm and 10 ppm) with the exception of the control and RH samples. The columns highlighted in green were common to all samples generated. Again, when comparing the different isoforms and fragments within the samples, it was found that not all proteins are common to the 9 samples and each treatment/condition has its own unique combination of proteins at different percentage of peptide coverage.

This diversification amongst the identified proteins in each sample might be due to incomplete trypsin digestion and/or effect of the different treatment/condition on the sample. This result showed that pollen of same species had dissimilar tolerance to different pollutant concentrations.
<table>
<thead>
<tr>
<th>Description</th>
<th>Control Coverage</th>
<th>RH Coverage</th>
<th>RW 2ppm Coverage</th>
<th>RW 5ppm Coverage</th>
<th>RW 10ppm Coverage</th>
<th>CR 2ppm Coverage</th>
<th>CR 5ppm Coverage</th>
<th>CR 10ppm Coverage</th>
<th>24hrs Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione S-transferase - [A0A096XCA4_BETPN]</td>
<td>76.79</td>
<td>83.54</td>
<td>82.7</td>
<td>80.17</td>
<td>71.31</td>
<td>57.81</td>
<td>60.76</td>
<td>81.86</td>
<td>81.01</td>
</tr>
<tr>
<td>Pollen allergen Bet v1, isoform [Q9SCH5_BETPN]</td>
<td>71.25</td>
<td>0</td>
<td>63.75</td>
<td>0</td>
<td>67.5</td>
<td>63.75</td>
<td>75.63</td>
<td>76.25</td>
<td>71.25</td>
</tr>
<tr>
<td>Pollen allergen, Betv1 (Fragment) [O23746_BETPN]</td>
<td>76.1</td>
<td>74.84</td>
<td>74.84</td>
<td>80.5</td>
<td>78.62</td>
<td>0</td>
<td>0</td>
<td>74.84</td>
<td>76.1</td>
</tr>
<tr>
<td>Pollen allergen, Betv1 (Fragment) [Q546V0_BETPN]</td>
<td>69.18</td>
<td>86.16</td>
<td>78.62</td>
<td>65.41</td>
<td>71.07</td>
<td>74.84</td>
<td>86.79</td>
<td>74.84</td>
<td>82.39</td>
</tr>
<tr>
<td>Pollen allergen Betv1, isoform [Q9Z588_BETPN]</td>
<td>69.38</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>71.25</td>
<td>0</td>
<td>77.5</td>
<td>74.38</td>
<td>69.38</td>
</tr>
<tr>
<td>Major allergen Bet v 1.02B (Fragment) [QOQLV5_BETPN]</td>
<td>64.67</td>
<td>64.67</td>
<td>64.67</td>
<td>0</td>
<td>64.67</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Oleo1-like protein [OLEE1_BETPN]</td>
<td>16.27</td>
<td>16.27</td>
<td>5.42</td>
<td>16.27</td>
<td>5.42</td>
<td>0</td>
<td>0</td>
<td>16.27</td>
<td>19.88</td>
</tr>
<tr>
<td>Major pollen allergen Bet v 1-G [BEVI1G_BETPN]</td>
<td>0</td>
<td>63.75</td>
<td>63.75</td>
<td>69.38</td>
<td>71.25</td>
<td>63.75</td>
<td>75.63</td>
<td>63.75</td>
<td>0</td>
</tr>
<tr>
<td>Pollen allergen, Betv1 (Fragment) [O23749_BETPN]</td>
<td>0</td>
<td>78.62</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>94.34</td>
<td>0</td>
<td>80.5</td>
</tr>
<tr>
<td>Pollen allergen Betv1, isoform [Q9SCH9_BETPN]</td>
<td>0</td>
<td>0</td>
<td>67.5</td>
<td>54.37</td>
<td>71.25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pollen allergen Betv1, isoform [Q9SCH6_BETPN]</td>
<td>0</td>
<td>0</td>
<td>63.75</td>
<td>0</td>
<td>0</td>
<td>56.88</td>
<td>0</td>
<td>0</td>
<td>63.75</td>
</tr>
<tr>
<td>Pollen allergen Bet v 1 [Q96365_BETPN]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>63.75</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pollen allergen Betv1 (Q23752_BETPN)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>63.75</td>
<td>0</td>
<td>71.25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Major pollen allergen Bet v 1-K [BEVI1K_BETPN]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>83.13</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pollen allergen Bet v 1 - [Q96366_BETPN]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>71.25</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.5: Some of identified Bet v 1 protein and a comparison between peptide sequence coverage of the 9 samples subjected to different conditions/treatments; control is intact pollen without exposure, RW is the hydrated pollen in rainwater, CR is crushed pollen and hydrated in rainwater, and 24 hrs pollen is exposed to high RH for 24 hours. Note, all generated samples were exposed to NO2 at different levels of concentrations (2 ppm, 5 ppm and 10 ppm) with the exception of the control and RH samples. The columns highlighted in green were common to all samples generated. This data is representative of one of the replicates from the triplicates used in the above data.
Those proteins that share the similar set of peptide hits are grouped together for a more convenient report on the Table 4.5. 10 and above proteins were identified in each sample. Each protein sample contained its own unique set of protein but sometimes have two or more similar protein in common to another sample. Every identified protein, is ranked according to the number of peptide sequences, the number of PSMs, their protein scores, and the sequence coverage. Table 4.5 results demonstrated that an increase in the concentration of NO2 exposure results in the increased release of allergen protein present in the sample, signifying that the NO2 concentration has an impact on the pollen protein movement. There are two particular proteins highlighted in green (Table 4.5) that are common to all samples; this may suggest the importance of the protein and might be linked to a vital function of the pollen and allergenicity. However, this is observation that requires further research to verify which was beyond the scope of this study.

4.2.7 Dot blot
The dot blot technique was applied to assess the presence of nitration in the research samples. Antibody specific for nitration modifications, anti-nitrotyrosine antibody was used to determine the presence of the nitration in first set of exposed sample obtained (NO2 exposed but intact pollen) alongside nitrated BSA as the standard indicator. Even though dot-blotting technique (uses antibodies to detect specific proteins) is more sensitive than MS, it lacks the ability to identify and quantify the exact position of the modification. Very low levels of nitration were observed but the nitration signal was only marginally more than the background signal, which made definitive detection impossible, this was surprising. The applicability of the technique to distinguish nitrated proteins was confirmed by observing the positive dot generated by nitrated BSA. Figure 4.6 represents coloured (A) and grey scale (B)
images of the dot blot results of unexposed sample, 3 samples exposed at different NO₂ concentration (2, 5, and 10 ppm respectively) and nitrated BSA. Figure 4.7 represents the semi-quantification of degree of nitration from dot blot analysis (Figure 4.6) showing estimated Nitrated BSA concentration against unexposed and NO₂ exposed samples at different concentration. Hence, the lack of a definitive observation of nitration in the exposed pollen could be due to the following possibilities:

1. No nitrated peptides were generated by exposure to the air pollutants

2. The laboratory protocol does not expose the pollen samples sufficiently to the air pollutants,

3. The nitration signal is too low to be observed.

Figure 4.6: A & B- Coloured and grey scale images of the dot blot. From top to bottom dots in blue circles include: unexposed, exposed at 2 ppm NO₂, exposed at 5 ppm NO₂, exposed at 10 ppm NO₂ and nitrated BSA. The grey scale image contains the same information as the coloured image but in black & white for ease of distinguishing the differences by human eyes.
Figure 4.7: Represents the semi-quantification of degree of nitration from coloured dot blot image (Figure 4.6) showing estimated Nitrated BSA concentration against unexposed and NO$_2$ exposed samples at different concentration (2, 5, and 10ppm). Note that the intact pollen (IP) was exposed prior to rupturing the pollen grain samples.

Results from semi-quantification of degree of nitration using the dot blot images shows a certain amount of nitration on the NBSA and less on generated samples exposed at 10 ppm and 5 ppm of NO$_2$ respectively prior to pollen rupture. The visual inspection of the membrane did not reveal this but the Odyssey imaging system has sufficient resolution to observe small changes not visible by eye.

Further dot blot analysis was performed on the samples after the pollen was ruptured and the protein extract was subsequently exposed to the air pollutant gas mixture. The outcome was remarkable, as the samples exposed at 10 ppm concentration of NO$_2$ have undergone significant nitration while samples exposure to 2-5 ppm revealed lower amounts but significant nitration. As previously mentioned, three different rupturing methods were
employed in this study where one method appeared to work better with the NO₂ exposure. Interestingly, it is the method that most closely mimics the real-world scenario of pollen hydration by rainwater (the pollen grains while still on the plant can be rained on and exposed to pollutants) that has the highest nitration. The dot blot results indicated stronger signal of nitration on the pollen hydrated in rainwater and crushed in comparison to 24hours RH-exposure sample. A reason could be that only certain specific proteins get easily nitrated that were not made accessible in the 24hours RH-exposure samples. Figure 4.8 illustrates the results of the nitration detected in different samples using the dot blot analysis. All samples (supernatant protein extract) were spotted in triplicates. Multiple droplets were added per dot to reach the desired protein concentration resulting in the concentric circles seen. The other tiny green dot dissimilar from the droplets of the sample is artefact (probably as a result of fluorescent dirt or impurity) and pollen pellet extract on the membrane.
Figure 4.8: Demonstrates results from dot blot analysis of nitrated BSA, unexposed (intact pollen grain), RH exposed and NO₂ exposed sample (protein extract) at 2, 5 and 10 ppm (duplicate) all spotted in triplicates. Multiple droplets were added per dot to reach the desired protein concentration resulting in the concentric circles seen. The other tiny green dots are artefact and contaminant of the pollen pellet on the membrane. All samples (supernatant protein extract) used for NO₂ exposures are either crushed, 24 hours RH exposure and ruptured in rainwater prior to the gas exposure.
Figure 4.9: Quantification of nitration from dot blot analysis showing estimated Nitrated BSA concentration against NO\textsubscript{2} exposed samples at different concentrations and conditions. The nitrated rainwater sample at 10ppm appears to have the highest concentration in comparison to the other samples. Error bars express potential errors in the data set. The error bars represent standard error of the mean. RW- Pollen ruptured in rainwater, then subjected to 2, 5, and 10 ppm NO\textsubscript{2}; RH- Pollen ruptured by exposure to high RH for 24 hours, then subjected to 10 ppm NO\textsubscript{2}; and CR- Pollen ruptured by crushing, then subjected to 10 ppm NO\textsubscript{2}.

Figure 4.9 shows the quantification of degree of nitration for each sample represented as a bar chart. This was calculated based on the circular dimension of the dot blot images beside confirmed nitrated BSA sample as standard. It can be evidently seen that exposure to higher NO\textsubscript{2} concentration yields greater nitrated proteins under certain experimental conditions (hydration of pollen in rain water before gas exposure).

It is difficult to compare the degree of nitration observed in this study compared to the work of Reinmuth-Selze et al. (2014) and Franze et al. (2005) who investigated the allergen Bet\textsubscript{v1} expressed in e-coli and the pure allergen, respectively. They also used different metrics to characterise the degree of nitration. To provide a metric of nitration for this study, which can
be subsequently compared to future studies, we benchmark the nitration observed in the pollen samples to the nitrated BSA signal on an equal protein weight basis. The relative degrees of nitration of the different experiments are given in Table 4.6.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Degree of pollen protein nitration referenced to the same weight of nitrated BSA</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RW Control</td>
<td>0.02</td>
<td>Pollen ruptured in rain water, but not exposed to NO₂</td>
</tr>
<tr>
<td>RH</td>
<td>0.02</td>
<td>Pollen not ruptured, then subjected to high RH condition</td>
</tr>
<tr>
<td>RW2ppm</td>
<td>0.04</td>
<td>Pollen ruptured in rain water, then subjected to 2 ppm NO₂</td>
</tr>
<tr>
<td>RW 5ppm</td>
<td>0.06</td>
<td>Pollen ruptured in rain water, then subjected to 5 ppm NO₂</td>
</tr>
<tr>
<td>RW 10ppm</td>
<td>0.27</td>
<td>Pollen ruptured in rain water, then subjected to 10 ppm NO₂</td>
</tr>
<tr>
<td>CR 10ppm</td>
<td>0.21</td>
<td>Pollen ruptured by crushing, then subjected to 10 ppm NO₂</td>
</tr>
<tr>
<td>24HRS 10ppm</td>
<td>0.12</td>
<td>Pollen ruptured by exposure to high RH for 24 hours, then subjected to 10 ppm NO₂</td>
</tr>
</tbody>
</table>

Table 4.6: Degree of nitration of the pollen samples, under different nitration conditions, references to equivalent weight of nitrated BSA protein.

From Table 4.6, it can be seen that the most effective pathway for nitration of birch pollen is via rupture in rainwater followed by exposure to the highest concentration of NO₂. The effect of NO₂ concentration on the degree of nitration in the rainwater ruptured pollen appears to be
non-linear which potentially suggests threshold effects for the degree of NO₂ required to cause nitration. It can be seen that the control and the RH only experiments results in a small degree of apparent nitration, even though no NO₂ exposure occurred. These values can be used as a limit of detection of the experiment. The results shown in Figure 4.8 all fall below this limit of detection. Hence within the detection limits of this experiment, we conclude that for pollen protein to be nitrated, the pollen grain has to be ruptured in some manner.

4.2.8 Western blot

The technique was established in this research to identify and separate the nitrated proteins within the complex mixture of total proteins. The samples were electrophoresed on SDS-PAGE gel alongside nitrated BSA and subjected to probing with anti-nitrotyrosine primary and secondary antibodies. The transfer time was kept minimal to avoid losing proteins. A PVDF (Polyvinylidene difluoride) membrane was used, which allows for detection of low abundance proteins due to its greater binding capacity.

Nitration was detected in both nitrated BSA and NO₂ exposed rainwater (RW) samples (10 ppm). The level of nitration in the pollen samples is clearly less than that of the nitrated BSA (Figure 4.10). Probably due to low levels of nitrotyrosine residues present or only weakly nitrated protein were accessible to the antibody binding. The signal was only apparent in the pollen sample exposed to the greatest NO₂ concentration (10 ppm) as no obvious bands were seen in the other samples (Figure 4.10). The band relating to the nitration detection was situated around the 17-kDa marker of the molecular weight ladder. This experiment confirmed the dot blot results as revealed in section 4.2.6 (Figure 4.8). The benefit of the blots approach is the sensitivity because is antibody-based technique so it acknowledges
specificity. The obtained results were not used for semi-quantification of degree of nitration because the signals from the bands were not very strong.

Figure 4.10. Western blot result showing signals from nitrated proteins in nitrated BSA and NO₂ exposed samples (sample is from rainwater) pointed with blue and maroon arrows respectively. There was no signal from the unexposed sample pointed with green arrow. Samples were ran on SDS-gel, transferred onto the membrane and probed with anti-nitrotyrosine antibodies. The same 10 ppm band spot on a separate gel was used for the in-gel digestion analysis. The tiny green dots (pointed with yellow arrow) are artefact on the membrane.

4.2.9 MS analysis

A variety of chemical modifications can affect the structure and biological functions of proteins and peptides which are susceptible, both in vivo and in vitro (Yang and Zubarev, 2010). Major types of modification identified after several experimental exposures includes: Oxidation, deamidation (a spontaneous non-enzymatic reaction), carbamidomethyl, phosphorylation and in some instance nitration.
The MS analysis was unable to detect nitrated peptides from the exposed pollen samples at a high degree of confidence. However, the nitrated peptides of BSA were detected using the same approach. The reason for this is not entirely clear since the dot blot and western blot results provided clear evidence that nitration occurred in the pollen samples albeit at lower concentrations to the nitrated BSA samples. Most likely the negative pollen result is due to the lower abundance of the peptides of interest compared to the nitrated BSA samples. Subsequently, the pollen samples were further subjected to advanced investigation, in-gel digestion technique, and again ran on the MS. Figure 4.11 shows a recap of the basic processing steps for MS analysis.

![Diagram](image)

**Figure 4.11:** Basic processing steps used for the MS data analysis. Raw data files generated on the MS where searched on proteome discover software using the search engine Sequest HT against UniProt protein database to identify proteins and modifications.
4.2.10 In-gel digestion

Following the MS analysis of the samples and observation of nitration using the blot methods, additional efforts were made to see if the modification of interest could be detected on the MS in a more reproducible manner. The samples were also ran on SDS-gel and stained to reveal all the detected protein bands in the sample. The specific band (17 kDa) of interest was cut (Figure 4.12), digested and subjected to MS analysis to characterise and locate the nitrated peptide present in the sample. In doing so, the likelihood of detecting the modification was increased because the complexity of the sample was reduced and the protein of interest isolated. The sample is a highly complex mixture of different proteins that can be more sufficiently analysed when separated into fractions and digested separately, which lessen protein mixtures complexity.

Figure 4.12: Illustrates in-gel digestion gel with excised bands (circled in the black circle).
The in-gel digestion analysis was successful, as it allowed for detection of the nitration on the Bet v 1 and other proteins which were present in the digested band (Table 4.7). This was not reported in earlier literature studies done. The “informative ions are generated by fragmentation at the amide bond between amino acids and the resulting ions are called b-ions if charge is retained by the amino-terminal part of the peptide and y-ions if retained by the carboxy-terminal part” (Steen and Mann, 2004). Even though the mass increment of ~ 45 Da was observed on the product ion mass of suggested nitrated peptides, the exact modified fragment ion of interest Y13-Nitro could not be assigned within the b and y ion sequencing (Figure 4.13). Thus, these results cannot be considered completely robust. The incorrect assignment of the y and b ions could be due to low fragment ion signal and high levels of noise in the mass spectrum. Sometimes peptides are too short to be confident of assignment (m/z <620), the spectra not good enough to assign, or a wrongly assigned charge not a peptide (Chalkley et al., 2005).

To further and more conclusively validate the observed protein modification; de novo sequencing (using mass differences between two fragment ions to determine mass of an amino acid residue on the tryptic spectra) could be employed, which is outside the scope of this investigation. Figure 4.14 describes the obtained MS/MS product ion spectra of tryptic Bet v 1 peptide. The tryptic peptides usually become doubly protonated (peptides comprising more than 15 amino acid or basic amino acids such as histidine can have higher charge) and designated as (M + 2H)^2+ where M and H^+ represents the masses of the peptide and a proton respectively (Steen and Mann, 2004).
<table>
<thead>
<tr>
<th>Accession number</th>
<th>Protein description</th>
<th>Protein coverage</th>
<th>Sequence</th>
<th>Modification</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q9SCH7</td>
<td>Pollen allergen Betv1, isoform a87</td>
<td>10.00</td>
<td>AFILDGDNLFPK</td>
<td></td>
<td>19.39</td>
</tr>
<tr>
<td>O49813</td>
<td>Ole1-like protein</td>
<td>22.29</td>
<td>EGGLTLySSDSETDKSgyTRIPVDGDHEEIKeLALK</td>
<td>Y7 (Nitro); Y19 (Nitro); C32 (Carbamidomethyl)</td>
<td>31.05</td>
</tr>
<tr>
<td>O23747</td>
<td>Pollen allergen, Betv1 (Fragment)</td>
<td>13.84</td>
<td>EKGETLLKAYSyILLAHSDAYN</td>
<td>Y13 (Nitro)</td>
<td>21.55</td>
</tr>
<tr>
<td>Q0QLT4</td>
<td>PR-10 protein (Fragment)</td>
<td>8.67</td>
<td>GAGLFKAVENyLV</td>
<td>Y11 (Nitro)</td>
<td>11.24</td>
</tr>
<tr>
<td>E1XUM0</td>
<td>Pectate lyase</td>
<td>5.29</td>
<td>dMYILQQEMVVTSDKIDGR</td>
<td>N-Term (Acetyl); K16 (Acetyl)</td>
<td>19.73</td>
</tr>
</tbody>
</table>

Table 4.7: List and information of identified proteins from in-gel digested sample. Modified residues are highlighted in red. Bet v 1 nitrated protein information highlighted in green, Olee 1-like protein highlighted in purple and PR-10 protein highlighted in blue.

Figure 4.13: Peptide identification details showing peptide summary, fragment matches and sequence. The table illustrates some of the main characteristic of the detected Y13-nitrated peptide. It also shows the absence of b and y ions fragments on the ion series of the nitrated peptide (Y-Nitro) residue.
Figure 4.14: MS/MS product ion spectra of tryptic Bet v 1 peptide identified in the in-gel analysed sample. The fragment ions are labeled with their computer assigned mass-to-charge ratios. Each peptide fragment in the series is supposed to differ from its neighbor by one amino acid that will make it possible to determine the amino acid sequence by considering the mass difference between neighboring peaks in a series as indicated above. The black traces represent the ion chromatogram of the peptide.

Because the probability software can result in mistakes, there is need for careful validation of individual MS/MS spectra in order to eliminate false positive assignments (Li et al., 2011). This can include the identification of Immonium ions that are charged amino acids, which have no carboxylic acid group, but only some amino acids produce high intensity immonium ions like tyrosine, phenylalanine, histidine, and tryptophan (Falick et al., 1993; Papayannopoulos, 1995). Typically, the occurrence of a 3NT immonium ion at \( m/z \) 181.1 (Danielson et al., 2009) in the MS/MS spectra gives a clear sign of nitration, which is an essential benchmark for validation (Li et al., 2011). “Immonium ions are MS/MS fragment ions generated from a peptide by multiple internal cleavages and is a faithful signature for individual amino acids when observed in the MS/MS spectrum” (Falick et al., 1993). The immonium ion of tyrosine, which is 136 Da, will have a further 45 Da added if nitrated
making it 181 Da. Identifying y & b ions sequencing are also good pointers of the existence of a modified peptide; however, these are the simplest way of validating results for non-MS/MS expert.

### 4.2.11 TMT labelling experiment outcome

Tyrosine nitration occurs typically as low-abundance PTM that requires suitable enrichment methods prior to proteomic analyses (Prokai-Tatrai et al., 2011). The tagging protocol was expected to enrich the number of nitrated peptides present with in the complex samples, which in the end, only the tagged nitrated peptides will be available for analysis on the MS. This should allow quantification of nitration. The tagging experiment involved five major steps (Chapter 3, Figure 3.8). It started with sample digestion, N-terminal blockage, followed by reducing the nitrotyrosine to amino tyrosine by addition of sodium dithionite. The reduced peptides were tagged and enriched then subjected to MS analysis. “Reduction of 3-nitrotyrosine to 3-aminotyrosine by dithiothreitol (DTT) in proteins such as rat and bovine serum albumin (BSA) has been reported to be incomplete and this could result in an underestimation of the nitration level as measured by 3-nitrotyrosine immunoblotting” (Söderling et al., 2007).

The strategy was tested using nitrated BSA as standard since it was known to be nitrated and contains 21 tyrosine sites that can potentially be nitrated in vitro (Zhang et al., 2007) and in another study it contains 19 tyrosine residues (Peters, 1985). For Bet v 1, 7 tyrosine residues are present per molecule (Franze et al., 2005). 20 of nitrated tyrosine residues were observed in the standard nitrated BSA (Abcam) used after digestion and analysis on MS (Table 4.8).
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4 - [ALBU_BOVIN]

<table>
<thead>
<tr>
<th>Description and Coverage</th>
<th>Sequence</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4 - [ALBU_BOVIN]</td>
<td>RHPyFAPELLyYANK</td>
<td>Y6(Nitro); Y12(Nitro)</td>
</tr>
<tr>
<td>89.79</td>
<td>LGEyGQPQALINR</td>
<td>Y4(Nitro)</td>
</tr>
<tr>
<td></td>
<td>MPcTEDyLSLILNR</td>
<td>C3(Carbamidomethyl); Y7(Nitro)</td>
</tr>
<tr>
<td></td>
<td>RHPyFAPELLyyYANK</td>
<td>Y4(Nitro)</td>
</tr>
<tr>
<td></td>
<td>HPyFAPELLyyYANK</td>
<td>Y3(Nitro)</td>
</tr>
<tr>
<td></td>
<td>RHPyEAVSLLR</td>
<td>Y5(Nitro)</td>
</tr>
<tr>
<td></td>
<td>mPcTEDyLSLILNR</td>
<td>M1(Oxidation); C3(Carbamidomethyl); Y7(Nitro)</td>
</tr>
<tr>
<td></td>
<td>GLVLIaFSQyLQQePFDEHVK</td>
<td>Y10(Nitro); C14(Carbamidomethyl)</td>
</tr>
<tr>
<td></td>
<td>DAFLGFLYEySR</td>
<td>Y11(Nitro)</td>
</tr>
<tr>
<td></td>
<td>yNGVFQEcQAEyDK</td>
<td>Y1(Nitro); C8(Carbamidomethyl); C9(Carbamidomethyl)</td>
</tr>
<tr>
<td></td>
<td>RPyFSALTPEyVPK</td>
<td>C3(Carbamidomethyl); Y13(Nitro)</td>
</tr>
<tr>
<td></td>
<td>dAFLGFLYEySR</td>
<td>N-Term(Acetyl); Y11(Nitro)</td>
</tr>
<tr>
<td></td>
<td>yLcDNQDTISSK</td>
<td>Y1(Nitro); C3(Carbamidomethyl)</td>
</tr>
<tr>
<td></td>
<td>yLyeEIAR</td>
<td>Y1(Nitro); Y3(Nitro)</td>
</tr>
<tr>
<td></td>
<td>yLYEIAyR</td>
<td>Y1(Nitro)</td>
</tr>
<tr>
<td></td>
<td>HPYFYAEPLLyyYANK</td>
<td>Y11(Nitro); Y12(Nitro)</td>
</tr>
<tr>
<td></td>
<td>HPYFYAEPLLyyYANK</td>
<td>N-Term(Acetyl); Y11(Nitro)</td>
</tr>
</tbody>
</table>

Table 4.8: 20 Nitrated tyrosine residues of NBSA sample (Abcam) that were identified after digestion and MS analysis.

The results obtain from MS analysis proved that the protocol was working appropriately up to the stage of amino conversion (reduction of nitrotyrosine to aminotyrosine) also verified using dot blot experiment (Figure 4.15), however, after enrichment, the proteins seem to be lost completely. All tagged samples were supposed to bind the TMT resin while the non-tagged are discarded during the cleaning session. Results from analysis done on non-enriched samples indicated the presence of untagged proteins. A possible explanation is that the tagging was done incorrectly, reduction was incomplete or, due to low abundance of the 3NT, it might be difficult for the tags to bind the nitrated peptides thus, cannot be detected by the MS as earlier mention in similar research. The enrichment protocol used here might not be selective enough to enrich the low nitrotyrosine-containing proteins from the complex sample matrices. Furthermore, if the conversion of nitrotyrosine to aminotyrosine of a specific residue is incomplete, the complexities of the analytical task will be increased.
Figure 4.15: Result obtained from reduction of 3-nitrotyrosine to 3-aminotyrosine after addition of sodium dithionite and subjected to dot blot analysis. The dot within the green circle is the nitrated BSA sample prior to the addition of sodium dithionite, while the area within the blue circle is after reduction has occurred. The tiny green dots (pointed with orange arrow) are artefact on the membrane.

Optimization that was looked into included increasing the starting material; making sure the TMT tags were at correct pH and skipping the enrichment step. Table 4.9 shows the number of peptides (all untagged) present before enrichment after increasing the starting material. Increasing the amount of starting materials yielded peptides after enrichment but untagged peptide (Table 4.10). The findings were contrary to expectation even though at low confident peptide level, large amount of tagged proteins were been detected.

For future work, it is noted that Boersema et al. (2009) concludes that online stable isotope labeling is the more efficient method for the labeling of small quantity of sample, because sample loss is reduced by combining sample clean-up and labeling and performing MS analysis directly after labeling (Boersema et al., 2009). It is worth noting that nitration happens at randomly selected sides.
### Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4 - [ALBU_BOVIN]

<table>
<thead>
<tr>
<th>Sequence</th>
<th># PSMs</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>nEcFSLHKDDSPDLPK</td>
<td>1</td>
<td>N-Term(Acetyl); C3(Carbamidomethyl); K8(Acetyl)</td>
</tr>
<tr>
<td>ccAADDKEAcFAVEGPK</td>
<td>2</td>
<td>N-Term(Acetyl); C1(Carbamidomethyl); C2(Carbamidomethyl); C10(Carbamidomethyl)</td>
</tr>
<tr>
<td>IFTHADlclLDPTEK</td>
<td>4</td>
<td>N-Term(Acetyl); C9(Carbamidomethyl)</td>
</tr>
<tr>
<td>nEcFSLHKDDSPDLPK</td>
<td>1</td>
<td>C3(Carbamidomethyl)</td>
</tr>
<tr>
<td>ccAADDKEAcFAVEGPK</td>
<td>1</td>
<td>C1(Carbamidomethyl); C2(Carbamidomethyl); C10(Carbamidomethyl)</td>
</tr>
<tr>
<td>eccHGDLLecADDR</td>
<td>2</td>
<td>N-Term(Acetyl); C2(Carbamidomethyl); C3(Carbamidomethyl); C10(Carbamidomethyl)</td>
</tr>
<tr>
<td>vHKeccHGDLLecADDR</td>
<td>2</td>
<td>N-Term(Acetyl); C5(Carbamidomethyl); C6(Carbamidomethyl); C13(Carbamidomethyl)</td>
</tr>
<tr>
<td>rHPYFYAPELLYYANK</td>
<td>1</td>
<td>N-Term(Acetyl)</td>
</tr>
<tr>
<td>hPYFYAPELLYYANK</td>
<td>2</td>
<td>N-Term(Acetyl)</td>
</tr>
<tr>
<td>aEFVEVTKLVTDLTK</td>
<td>2</td>
<td>N-Term(Acetyl)</td>
</tr>
<tr>
<td>rHPYFYAPELLYYANK</td>
<td>1</td>
<td>N-Term(Acetyl); K16(Acetyl)</td>
</tr>
<tr>
<td>slHTLFGDElcK</td>
<td>2</td>
<td>N-Term(Acetyl); C11(Carbamidomethyl)</td>
</tr>
<tr>
<td>nEcFSLHKDDSPDLPK</td>
<td>1</td>
<td>N-Term(Acetyl); C3(Carbamidomethyl)</td>
</tr>
<tr>
<td>rHPYEAVSVLLR</td>
<td>3</td>
<td>N-Term(Acetyl)</td>
</tr>
<tr>
<td>MPtTEDYLSSLILNR</td>
<td>1</td>
<td>C3(Carbamidomethyl)</td>
</tr>
<tr>
<td>LcVLHElTPVSEK</td>
<td>1</td>
<td>C2(Carbamidomethyl); K7(Acetyl)</td>
</tr>
<tr>
<td>lkEccDKPLElEk</td>
<td>4</td>
<td>N-Term(Acetyl); C4(Carbamidomethyl); C5(Carbamidomethyl)</td>
</tr>
<tr>
<td>lkPDPNTlcDEFKADek</td>
<td>3</td>
<td>N-Term(Acetyl); C9(Carbamidomethyl); K17(Acetyl)</td>
</tr>
<tr>
<td>IGEYGFQNALIVR</td>
<td>2</td>
<td>N-Term(Acetyl)</td>
</tr>
<tr>
<td>LkEccDKPLElEk</td>
<td>2</td>
<td>C4(Carbamidomethyl); C5(Carbamidomethyl)</td>
</tr>
<tr>
<td>lhVDEPQNLIK</td>
<td>1</td>
<td>N-Term(Acetyl)</td>
</tr>
<tr>
<td>dDPHAcYSTVFDK</td>
<td>1</td>
<td>N-Term(Acetyl); C6(Carbamidomethyl)</td>
</tr>
<tr>
<td>slHTLFGDElcVASLR</td>
<td>1</td>
<td>N-Term(Acetyl); C11(Carbamidomethyl); K12(Acetyl)</td>
</tr>
<tr>
<td>KQTALVELLK</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>RHPYFYAPELLYYANK</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>SLHTLFGDElcK</td>
<td>1</td>
<td>C11(Carbamidomethyl)</td>
</tr>
<tr>
<td>yNGVFQeccQAEDK</td>
<td>1</td>
<td>N-Term(Acetyl); C8(Carbamidomethyl); C9(Carbamidomethyl)</td>
</tr>
<tr>
<td>eccDKPLElEk</td>
<td>1</td>
<td>N-Term(Acetyl); C2(Carbamidomethyl); C3(Carbamidomethyl)</td>
</tr>
<tr>
<td>LGGEYGQNALIVR</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>mPtTEDYLSLILNR</td>
<td>1</td>
<td>M1(Oxidation); C3(Carbamidomethyl)</td>
</tr>
<tr>
<td>HLVDEPQNLIK</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>lkPDPNTlcDEFK</td>
<td>1</td>
<td>N-Term(Acetyl); K2(Acetyl); C9(Carbamidomethyl)</td>
</tr>
<tr>
<td>Sequence</td>
<td>Modifications</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
<td></td>
</tr>
<tr>
<td>HPEYAVSVLLR</td>
<td>1 C21(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>HPYFAYPELLYYANK</td>
<td>2 C6(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>DAIPENLPLLTADFADKDVC</td>
<td>1 C21(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>DDPHAcYSTVFDK</td>
<td>2 C6(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>lKPDNPNTLCDEFK</td>
<td>4 N-Term(Acetyl); C9(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>lEccDKPLLEK</td>
<td>4 N-Term(Acetyl); K2(Acetyl); C4(Carbamidomethyl); C5(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>KVPQVSTPTLVEVSR</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DAIPENLPLLTADFADK</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>tVMENFVAFVFDK</td>
<td>2 N-Term(Acetyl)</td>
<td></td>
</tr>
<tr>
<td>LKPDPNTLCDEFK</td>
<td>2 C9(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>HPEYAVSVD</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>EccHGDLLEcADDR</td>
<td>1 C2(Carbamidomethyl); C3(Carbamidomethyl); C10(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>DAFGLSLYFYEY</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>tTWHSEIAH</td>
<td>2 N-Term(Acetyl); K4(Acetyl)</td>
<td></td>
</tr>
<tr>
<td>tcVADESHAGcE</td>
<td>1 N-Term(Acetyl); C2(Carbamidomethyl); C11(Carbamidomethyl); K13(Acetyl)</td>
<td></td>
</tr>
<tr>
<td>RPcFSALTPDETVPK</td>
<td>1 C3(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>kQTALVE</td>
<td>2 N-Term(Acetyl)</td>
<td></td>
</tr>
<tr>
<td>ShcIAEVEK</td>
<td>1 C3(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>TVMENFVAFVDK</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LAKEYEATLIEeccAK</td>
<td>1 C12(Carbamidomethyl); C13(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>hpPEYAVSVLLR</td>
<td>4 N-Term(Acetyl)</td>
<td></td>
</tr>
<tr>
<td>eYEATLEEeccAK</td>
<td>2 N-Term(Acetyl); C9(Carbamidomethyl); C10(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>YNGVFQEcQueryEDK</td>
<td>1 C8(Carbamidomethyl); C9(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>AEFVEVTKLVTD</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>tVnenFVAFV</td>
<td>1 N-Term(Acetyl); M3(Oxidation)</td>
<td></td>
</tr>
<tr>
<td>LFTFADLcTLPDTEK</td>
<td>2 C9(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>TVenFVAFV</td>
<td>1 M3(Oxidation)</td>
<td></td>
</tr>
<tr>
<td>kVPQVSTPTLVEVSR</td>
<td>6 N-Term(Acetyl)</td>
<td></td>
</tr>
<tr>
<td>LNNElfEF</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>qTALVEL</td>
<td>3 N-Term(Acetyl)</td>
<td></td>
</tr>
<tr>
<td>teVADESHAGcE</td>
<td>1 N-Term(Acetyl); C2(Carbamidomethyl); C11(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>EYEATLEEccAK</td>
<td>1 C9(Carbamidomethyl); C10(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>YlcDNGQTIS</td>
<td>1 C3(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>QTALVE</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>eAcFAVEGPK</td>
<td>1 N-Term(Acetyl); C3(Carbamidomethyl)</td>
<td></td>
</tr>
<tr>
<td>Peptide</td>
<td>Charge</td>
<td>Modifications</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------</td>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>fDLGEEHFk</td>
<td>1</td>
<td>N-Term(Acetyl); K2(Acetyl); K10(Acetyl)</td>
</tr>
<tr>
<td>VPOVSTPTLVEVSR</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>lAKEYEATLEcckAK</td>
<td>1</td>
<td>N-Term(Acetyl); C12(Carbamidomethyl); C13(Carbamidomethyl)</td>
</tr>
<tr>
<td>EceDKPLEEK</td>
<td>1</td>
<td>C2(Carbamidomethyl); C3(Carbamidomethyl)</td>
</tr>
<tr>
<td>fDLGEEHFk</td>
<td>3</td>
<td>N-Term(Acetyl); K2(Acetyl)</td>
</tr>
<tr>
<td>lVNEETEFAK</td>
<td>2</td>
<td>N-Term(Acetyl)</td>
</tr>
<tr>
<td>ccTKPESER</td>
<td>1</td>
<td>N-Term(Acetyl); C1(Carbamidomethyl); C2(Carbamidomethyl)</td>
</tr>
<tr>
<td>ETYGDMDAEckEKL</td>
<td>1</td>
<td>C9(Carbamidomethyl); C10(Carbamidomethyl)</td>
</tr>
<tr>
<td>fDLGEEHFk</td>
<td>3</td>
<td>K2(Acetyl)</td>
</tr>
<tr>
<td>EAcFAVEGPK</td>
<td>1</td>
<td>C3(Carbamidomethyl)</td>
</tr>
<tr>
<td>aEFVEVTk</td>
<td>1</td>
<td>N-Term(Acetyl)</td>
</tr>
<tr>
<td>ALkAWSVAR</td>
<td>1</td>
<td>K3(Acetyl)</td>
</tr>
<tr>
<td>ccTkPESER</td>
<td>1</td>
<td>N-Term(Acetyl); C1(Carbamidomethyl); C2(Carbamidomethyl); K4(Acetyl)</td>
</tr>
<tr>
<td>dlGEEHFk</td>
<td>1</td>
<td>N-Term(Acetyl); K8(Acetyl)</td>
</tr>
<tr>
<td>lcVLHEK</td>
<td>1</td>
<td>N-Term(Acetyl); C2(Carbamidomethyl)</td>
</tr>
</tbody>
</table>

Table 4.9: Identified peptide after increasing the starting material before enrichment.
Table 4.10: Identified peptide after increasing the starting material and enrichment

Evidently, from the above two Tables (4.9 & 4.10) there was a huge loss of peptides after the enrichment strategy, which was the main essence of the step, to remove untagged peptide. However, in this case even though the peptides have diminished, but none was tagged. In another analysis of unenriched in vitro nitrated bovine serum albumin (BSA) homogenate sample, 5.9% of nitrotyrosine-containing peptides were identified, while the enriched samples had 35%, respectively (Zhang et al., 2007).

Finally, after using 5 viral tubes of 100 µg at 0.76 mg/ml of nitrated BSA, few tagged peptides were identified before and after enrichment. Table 4.11 shows the identified peptide in different produced samples after increasing the starting material. Sample A- whole nitrated BSA tagged with TMT zero, C1-concentrate sample from spin column that was enrichment and tagged with TMT10 and C2- concentrate sample from spin column without enrichment and tagged with TMT10. The spin column is used for quick and easy cleanup of DNA or protein samples. The spectra of the identified tagged peptides were not favorable in reinforcing the earlier outcome (identified tagged peptides on product ion). As such, the...
The quantification of degree of nitration could not be done using the tagging technique, however, the protein nitration was earlier identified by blot and in-gel experiments and semi-quantified by the dot blot technique (section 4.2.6).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Confidence</th>
<th>Sequence</th>
<th>Search Engine</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>High</td>
<td>YYPMGPASVHL_FLADRFQGFLIKHHATNLAVSk</td>
<td>Sequest HT</td>
<td>Y13 (3AT TMT); K35 (Acetyl)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mLLPVPLLGLGLAAADPTV_FK</td>
<td>Sequest HT</td>
<td>N-Term (Acetyl); Y22 (3AT TMT); N-Term (Acetyl); Y21 (3AT TMT)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IQLTAAFFLATLLIGAVRL_YGSR</td>
<td>Sequest HT</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>High</td>
<td>VISVL_TVIVPFLPNPAI_cLRNK</td>
<td>Sequest HT</td>
<td>Y6 (3AT TMT); Y18 (3AT TMT); C19 (Carbamidomethyl)</td>
</tr>
</tbody>
</table>

Table 4.11: Identified tagged peptides (after increasing the starting material by 100%) before and after enrichment. The modification is highlighted in red and the tyrosine residue on the sequence in blue. Sample A- whole nitrated BSA tagged with TMT zero, C1-concentrate sample from spin column that was enrichment and tagged with TMT10 and C2- concentrate sample from spin column without enrichment and tagged with TMT10.
4.3 Conclusion

The main objective of this chapter was to report on the investigation of the effect of gas phase NO\textsubscript{2} on birch pollen grains. The experimental results from the initial part of this study demonstrated that the allergen proteins of interest are not greatly affected by air pollution exposure unless the pollen grains are ruptured. The types and abundance of the proteins identified appears to be dependent on the environmental conditions to which the pollen samples were exposed as revealed in the Polypeptide profiles by SDS-PAGE gel and MS analysis. Particularly, exposure to NO\textsubscript{2} without crushing the pollen generated more detectable protein content as compared to RH exposure sample alone. Previously reported work in the literature using expressed and endogenous protein observed significant amounts of nitration compared to the intact pollen results from this work. Thus, we hypothesized that nitration of the pollen proteins is significantly more difficult when the pollen grain is intact and not ruptured. We believe our experimental approach of using the whole pollen to be more representative of the real-world situation. From a biological point of view, this makes sense. The pollen protects its genetic cargo from biochemical transformations. The results at this level have shown clearly that once the pollen grain gets in contact with the environment, pollen particles are released. These findings raise intriguing questions regarding the extent of how these gases impact the pollen.

The second stage was to rupture the pollen using different methods. At this level, the ruptured pollen grains exposed to NO\textsubscript{2} at atmospherically relevant concentration levels were found to be significantly more nitration compared to the intact pollen grains. The rupturing of the pollen grains in rainwater, thereby mimicking the real-world situation where pollen still on the catkins likely interacts with rain, appeared to cause the greatest amount of nitration.
The lack of definitive detection of nitrated peptide on MS in the samples generated was anticipated because of previously reported studies (Nuriel et al., 2008; Petersson et al., 2001). However, for the commercially purchased nitrated BSA, the nitrated peptides were detected after digestion and MS analysis with no further analysis. Thereby confirming the validity of the approach. Identification and characterization of the other modified peptides with MS methods demonstrated that mass spectrometry is a useful analytical tool for biological samples. The pollen protein nitration was observed using dot and western blot techniques and with MS analysis using in-gel digested sample. The benefit of the blot approach is the sensitivity because it is antibody based technique so it acknowledges specificity. The detection of nitrotyrosine on the MS after in-gel digestion method suggested that the technique was sufficiently optimized for the analysis. Possibly, because of the low abundance of 3NT peptides, it was very challenging to identify modified proteins, which leads to the need for highly selective and sensitive analytical techniques such as enrichment approaches that rely on antibodies or chemically tagged protocols. As expected, the determination of 3-nitrotyrosine peptide on MS was much more demanding particularly within diverse complex proteins present in the generated samples. Even though the nitration was certain, which was verified using different techniques (dot and western blotting), determining exactly which peptide within any sample that has been nitrated needed a bit of detective work.

The last stage was to attempt the quantification of the degree of the nitration using TMT tags. Nitrated BSA was used as protein that is known to be nitrated. Unfortunately, a protocol could not be developed to successfully quantify the nitration. Various optimization steps were attempted such as increasing the amount of starting material. The technique identified a large number of tagged proteins at low confident peptide level, so it is possible the protocol was close to working but we cannot state this confidently. To summarize, a protocol has been developed that shows some promise but requires further optimization. Due to time
constraints, no further expansion and initial testing was performed in this study.

This study provides the first evidence of nitration of birch pollen protein directly from real pollen grains and their complex mixtures of proteins just as nature provided. Previous detection in earlier studies showed nitration of the Bet v 1 protein that was expressed in ecoli or in the pure isolated Bet v 1 protein. Neither of which are biological realistic in the real world. The protocols developed will be applicable for future bioaerosol allergenicity research.
Pollen grain Imaging

This chapter describes the imaging of birch tree pollen grain. It also examines the effects of rainwater, RH and NO₂ on the morphology and release of allergen proteins of birch.

Figure 5.1: Flow diagram of the graphical Abstract

The main objective of this chapter was to investigate the morphological changes on birch pollen grain surface upon interaction with elevated RH, NO₂ and rainwater. Figure 5.1 shows a graphical abstract of this chapter. The preceding chapter has shown that the interaction with these parameters has significant influence on the degree of pollen nitration observed. The Scanning Electron Microscope (SEM) facility at the School of Dentistry and the Birmingham Advance Light Microscopy (BALM) facility both at the University of Birmingham carried out the imaging and scanning shown in this chapter, respectively. The samples were prepared and handed over to BALM and School of
Dentistry for the fluorescence microscopy imaging and SEM scanning analysis, respectively. However, the results gathered were analyzed independently.

5.0 Synopsis

Environmental factors, such as RH and air pollutants have been shown to influence allergen discharge as well as morphological changes on pollen surface in a complex manner. The induced changes on the autofluorescence spectra and morphology of intact birch pollen have been observed using fluorescence microscopy and scanning electron microscope regarding exposure to NO\textsubscript{2} and RH and after subjecting it to different rupturing conditions. Exposure to air or pollutants and hydration or crushing damaged the pollen grains. The experimental results demonstrated that the use of fluorescence microscopy (FM) and scanning electron microscope (SEM) could provide additional information regarding morphological modifications on pollen grain upon the interaction of the pollen with atmospheric gases, rainwater and RH.

5.1 Objectives

In the preceding Chapter of this thesis, we looked at the effects of pollutants on these pollen grains at proteome level. The pollen was commercially purchased and artificially exposure to air pollutants in the highly equipped laboratory. However, in this chapter, the objectives are to:

- Investigate the visual changes of the treated and untreated pollen sample via imaging.
- Examine the effect of different rupturing protocol morphologically on the pollen grain surface.
5.2 Introduction

Air pollution and environmental conditions can impact on pollen grain affecting pollen morphology, the pollen cell wall, its protein content or protein release (Frank and Ernst, 2016). Birch is a widespread Northern European family of tree species that produces pollen, which can travel up to 1000s of km but typically the distance is a lot less during dispersal (Pope, 2010). Also, its highest concentrations have been measured as high as 2–3000 pollen grains m$^{-3}$ (Sofiev et al., 2006). Birch is the second most common of Britain’s broad-leaved trees and there are two native species in the UK - Silver Birch ($Betula pendula$) and Downy Birch ($Betula pubescens$) -which often hybridize (Savill, 2013). In this study, $B. pendula$ is the species of interest. The major allergen of $B. pendula$, Bet v 1 represent the protein allergens with molecular weight of ~17kDa, which has been categorized as major allergens because more than 90% of sensitized patients react to the allergen (Jarolim et al., 1989). Bet v 1 is the main cause of the tree pollen allergy in humans (Vrtala et al., 2001) affecting 100 million people worldwide (Mogensen et al., 2002). It is has a three-dimensional structure and is the most extensively studied allergen but yet the biological and physiological function remains elusive (Mogensen et al., 2002; von Loetzen et al., 2014). However, it is related to a group of pathogenesis-related proteins, PR-10, which are expressed through illness and stress situations (Van Loon and Van Strien, 1999) and seems to be abundant in plants (Breiteneder and Ebner, 2000).

Certain pollen species from trees are among the most important allergen causes, where birch pollen is one of the well-known significant aeroallergen dispersed by wind. Pollens dispersed by wind are dry and require hydration to stimulate germination (Songnuan, 2013). When dry pollen is exposed to water, there will be increase in osmotic potential that can lead to pollen damage (Taylor and Hepler, 1997). Thus, environmental conditions such as high humidity, rain and air pollution can influence the release of protein content.
within pollen and once released and suspended in the air are able to trigger allergy (Schappi et al., 1999).

5.2.1 Auto fluorescence

“Autofluorescence is the natural emission of cellular components due to a contribution of fluorescent compounds located in different cellular compartments” (Roshchina, 2012). The luminescence microscope is often used to observe visible (400–700 nm) autofluorescence of intact living cells excited by ultraviolet, violet, or blue light (Andersson et al., 1998; Bright et al., 1989). Fluorescence is the emission of visible light by a molecule at a precise wavelength when illuminated by light of a shorter wavelength (Hibbs, 2004). In another definition, it is a spontaneous radioactive decay process where molecular excited state is deactivated by photon emission (Atkin and de Paula, 2006). Different types of molecules will absorb light at particular wavelength. The fluorescence spectra and the emission intensity of an object of interest can also be measured due to special features of microscopes (Roshchina, 2012).

Chlorophyll is one of the many possible known fluorophore used for environmental monitoring with a maximum at wavelengths 675–680nm in plant cells (Agati, 1998). The excitation and emission maxima of fluorophores in solution are about 255 and 282 nm for phenylalanine; 275 and 303 nm for tyrosine, and 280 and 348nm for tryptophan (Huang et al., 2011). It is usual for absorbed energy by phenylalanine and tyrosine to be transferred to tryptophan, and emit fluorescence around 350 nm in proteins that contain tryptophan (Pan, 2015). The excitation and emission maxima from biological materials are due to the fact that fluorescence of the principal biological fluorophores is dependent on their indigenous environment, such as the pH, certain ion concentrations, temperature, RH, and the relative connection with other different molecules (Pan, 2015). “In a protein, different...
tyrosine or tryptophan molecules can exhibit different fluorescence depending upon their proximity to other amino acids in the same or other protein molecules” (Lakowicz, 2013). When looking only at the individual fluorophores, the sum of deviation in fluorescence of mixtures can be much greater than expected (Hill et al., 2013; Lakowicz, 2013). This is because the local environmental effect from dissimilar biological materials are not certainly the same in different forms, as the aerosols are often dry or somewhat dry (Hill et al., 2013). The nitration of amino acids (as observed in proteomics section, Chapter 4) might be expected to change the fluorescence ability of the protein. Hence, this study to see if the nitration could be observed by measuring the fluorescence.

Figure 5.2 demonstrates the single-shot fluorescence spectra from individual dried particles measured at 266nm excitation. Phenylalanine, tyrosine, tryptophan and NADH fluorescence emission peaks at 280nm, 310nm, 340nm and 450 nm respectively while the riboflavin emission from dry particles peaks near 560 nm and in an aqueous solution peaks near 520 nm (Pan, 2015). Phenylalanine, tyrosine, and tryptophan are aromatic amino acids and account for ~ 8.4% of all amino acids in proteins (Meadows et al., 2001). Several additional fluorescent molecules do exist in numerous biological systems, such as the chlorophylls present in plants but their influence is not that relevant in aerosols (Pan et al., 2007). A typical example is the huge quantity of chlorophyll in the environment, however, it displays slight fluorescence in atmospheric aerosols (Pan, 2015). Fluorescence microscopy (FM) has been used in studying the auto fluorescence of primary biological aerosols particles (PBAP) (Pöhlker et al., 2012).
5.2.2 Fluorescence microscopy (FM)

Fluorescence microscopy is the most widely used tools for observing the fluorescence and is an essential tool in modern biology studies (Gustafsson, 1999). FM is one of the most sensitive instruments available for morphological analysis of biological material (Fulcher, 1982) that allows sharp imaging details of a molecule. The technique also permits the observation of various biological structures not resolvable in usual microscopy (Huang et al., 2009). Three-dimensional (3D) structures imaging, measurement of interactions by multicolor colocalization, and dynamic processes recording in living cells at the nanometer scale are all now achievable due to new advances in these techniques (Huang et al., 2009).
5.2.3 Scanning Electron Microscope (SEM)

The SEM is a microscope that works by scanning a focused beam of electrons on a sample of interest generating high-resolution imaging of a surface. It uses electrons instead of light to generate image. The beam scanning enables information about a defined area on the sample to be collected. As a result of the electron-sample interaction, a number of signals are produced that are then detected by appropriate detectors. All the information regarding SEM operation was retrieved from http://www.nanoscience.com/technology/sem-technology/how-sem-works/.

![Schematic of a scanning electron microscope (SEM).](www.nanoscience.com)

Figure 5.3: Schematic of a scanning electron microscope (SEM). Adopted from (www.nanoscience.com)

5.3 Methodology

5.3.1 Materials

The Pollen was purchased from ALK-Abello laboratory and stored dry at ambient temperature (~23-25 degrees) and used without further processing. Synthetic Air and nitrogen dioxide (50 ppm) were purchased from BOC (UK) respectively.
5.3.2 Methods

There are various methods involve in imaging and scanning aerosols (Toner, 2007). The imaging of the treated and untreated sample under fluorescence electron microscopy allows for measuring and analyzing the absorption and excitation of various wavelengths of light within the sample while scanning the sample under scanning electron microscope generates high-resolution images of the sample surface.

5.3.3 Pollen imaging procedures

5.3.3.1 Light microscopy

Pollen protein often shows visible autofluorescence when excited by ultra violet or violet light (Roshchina, 2012). This fluorescence is the ability of an atom and molecules to absorb light at a specific wavelength and to subsequently emit light of longer wavelength after a brief interval (Valeur and Berberan-Santos, 2012). The “molecules responsible for most of the fluorescence in most biological cells are amino acids, nucleic acids, and some of the coenzymes” (Hill et al., 2013). The amino acids of the pollen protein that might become affected upon exposure to the atmospheric pollutants are either tryptophan or tyrosine residue since there is evidence of them becoming nitrated in both the preceding chapter and literature. In other to identify if the modifications that occurred on the pollen after exposure to these pollutants can also be spotted via physical change of the pollen grain, the treated and untreated pollen were viewed under Fluorescence microscopy. For the analysis, the pollen samples were imaged in two ways:

1. Imaging during real-time exposure to air and NO₂ on separate samples respectively, and
2. Imaging immediately after exposure to air and NO₂ respectively.
In order to carry out real time imaging while exposing the pollen to NO$_2$ and RH at BALM laboratory which does not have a fume cupboard; a protocol to contain and remove NO$_2$ from the air steam was required. This was done so as not to risk exposure of the researchers to a toxic gas during the experiment. During the exposure, the gas was passed at a flow of 1 L min$^{-1}$ for 60 minutes through a scrubber filled with 120 g of soda lime as described in the research of Ishebi et al (Ishibe et al., 1995). The gas concentration was continuously monitored throughout the experiment using an NO$_2$ analyser (previously described in Chapter 3). The NO$_X$, NO and NO$_2$ concentrations were monitored using the NO$_X$ analyzer. The soda lime was replaced with a clean batch after each experimental run. Figure 5.4 describes the experimental setup for testing the soda lime as an eliminator of NO$_2$.

![Diagram of the experimental setup for testing soda lime as an eliminator of NO$_2$.](image)

**Figure 5.4:** Diagram of the experimental setup for testing soda lime as an eliminator of NO$_2$. 

169
5.3.3.2 Sample preparation

The sample preparation protocol is slightly different for the two experiments:

- Real time exposure imaging; the pollen grain samples were randomly distributed across the surface of the cover-slide. The cover-slides with the sample and without sample were attached onto the bottom and top of a custom-made stainless steel chamber respectively (dimensions: 50x25x20 mm, Figure 5.6) using high vacuum grease. The samples were then handed over to BALM for fluorescence imaging.

- Pre-exposed sample imaging; the samples were first exposed to NO₂ and RH for 60 minutes each respectively, using the methodology described in Chapter 3 section 3.3, the samples were then subsequently placed on a microscope glass slide. The glass slide was covered with cover-slide using nail polish to prevent contamination during transportation between the two laboratories and to avoid movement of cover-slide during analysis. Samples were then handed over to BALM for fluorescence imaging.

Figure 5.5 below displays the apparatus used for the real-time imaging while the already exposed samples were just placed on glass slide and mounted onto specimen holder of the microscopy already covered with the cover slide. After sample preparation, it was handed over to the BALM facility and the microscopy investigation was initiated automatically for 30 minutes per session. The exposure of the samples to the UV light was minimized as much as possible to reduce the possibility of photo-bleaching on the samples.
5.3.3.3 Imaging instrument and analysis

Fluorescence microscopy is a powerful tool for investigating spatial and temporal changes in biological systems. Fluorescence images of the pollen grains were acquired before, during and after processing with NO$_2$ and high RH conditions.

Multi-channel confocal images were acquired at BALM. In order to allow a proper comparison between different time points, the same optical configuration (digital zoom, PMT, laser power, Galvano scanner and pinhole) were employed in all the acquisitions. Samples were photo-excited using a 458 nm laser (Ar/ArKr), 633 nm laser (He/Ne). The microscope used was an upright Leica SP8 laser scanning confocal system with 63x oil objective. ICY software was used for the post-imaging analyses.

5.3.4 SEM

5.3.4.1 Pollen sample preparation for scanning

For SEM, the samples were subjected to different conditions/treatments as discussed previously in Chapter 3 section 3.3 (subsection 3.4.3). Subsequent to processing, the samples were air-dried on a petri dish (if initially in liquid form). Samples were submitted
to the SEM service dry because the SEM utilizes vacuum conditions, so all water must be
removed from the samples to avoid water vaporization in the vacuum. In total, 9 different
samples were generated as detailed below:

- Undamaged dry pollen grain (intact pollen grain as provided by supplier)
- Undamaged dry pollen grain hydrated in rainwater over night (~22 hrs)
- Undamaged dry pollen grain exposed to high RH (~95%) for 24hrs
- Undamaged dry pollen grain exposed to high RH (~95%) for 1hr
- Crushed pollen grain (mechanically crushed dry using metal bead)
- Crushed dry pollen grain hydrated in rainwater
- Exposed (NO₂) pollen grain sample in extraction buffer
- Undamaged dry pollen grain exposed to NO₂ for 1hr
- Hydrated pollen grain exposed to NO₂ for 1hr

5.4 Results and Discussion

The SEM investigation showed that the pollen is roughly triangular in shape with a
diameter of approximately 20 µm and has noticeable pores upon the grain surface. These
morphological details are to be expected and provide the key evidence for identifying
birch pollen in field studies. The SEM work also revealed that pollen grains do suffer
structural damage by different treatment/condition, which likely enhances the release of
pollen proteins as presented in Chapter 4.
In the second section, fluorescence microscopy imaging provided fluorescence intensity values that illustrated the changes in the fluorescence intensity of the pollen after it was treated to both enhanced NO$_2$ and high RH.

### 5.4.1 Fluorescence microscopy (FM) results

From images A and B of Figure 5.6 below, it is clear that birch pollen fluoresces. The fluorescence was detected between 461-693 nm wavelengths. The yellow images were acquired with a CFP (Cyan Fluorescent Protein) detector (461 nm/515 nm wavelengths) that captures a portion of the emission from tryptophan and unfortunately only tiny amounts of the tyrosine emissions. The BALM laboratory does not have the laser capability to go to a lower excitation wavelength to cause significant tyrosine emission. The red images were acquired with a ChA (chlorophyll A) detector (640 nm/693 nm wavelengths). While the merge image provides the combination of both detectors. The tryptophan emission region fluoresced more strongly compared to the chlorophyll region. Previously, it has been reported that the chlorophylls present in plants does not have much influence on aerosols and it displays slight fluorescence in atmospheric aerosols. In other reported research, some characteristic damages were shown on the autofluorescence spectra of many pigmented and colorless pollens following long treatments with O$_3$ in various concentrations (Roshchina and Mel'nikova, 2001; Roshchina, 1999; V. V. Roshchina and V. N. Karnaukhov, 1999).
Figure 5.6: Fluorescence intensity of birch pollen grains; untreated (unexposed) and treated (exposed to NO$_2$). A – untreated pollen (as provided by supplier). B – pollen sample exposed to NO$_2$ at 10 ppm for 1 hr. CFP (Cyan Fluorescent Protein) is a detector that is excited at 461 nm/515 nm wavelength and also captures a portion of the emission from tryptophan. The ChA images (red) were acquired with a ChA (chlorophyll A) detector that is excited at 640 nm/693 nm wavelength. The merge images provide the combination of both detectors.

Figure 5.6 (images A & B) appear to show a distinctive variation in the fluorescence spectra of the untreated and treated pollen grains. Where the untreated pollen grains appear sharper with clearer images, and the pollen treated with NO$_2$ appears more smudged with blurry images acquired. The reason for this is unknown but may simply be speculated that room vibration led to less clear images in the NO$_2$ treated samples.

Different effects of pollution on external surface of exine (outer layer of pollen cover consisting of sporopollenin) have been demonstrated in previous research reported in the literature. Deep physical modification of pollen exine with artificial pollution was reported by some researchers, while others found no differences between polluted and non-polluted pollen (Sénéchal et al., 2015). The results of this study agrees with Ruffin et al. (1983) research who found no significant pollen morphologic changes despite the use.
of very high doses of pollutants (1% of NO$_2$, SO$_2$, or CO) (Ruffin et al., 1983). Additionally, in another study no physical modification of ragweed pollen was found from plants after being exposed to 80 ppb of O$_3$ during the entire vegetation period (Kanter et al., 2013), which disagrees with the results of Cerceau et al. (1996) (Cerceau-Larrival et al., 1996). The source of these results inconsistencies is probably due to the use of different pollen species and gas pollutant exposures (Sénéchal et al., 2015).

In this study, it is assumed that NO$_2$ will nitrate the amino acid (tyrosine) residue as shown in Chapter 4 and to a lesser extent the tryptophan residues. Since tyrosine and tryptophan are fluorescent, it was hypothesized that the nitration should be observable in time resolved fluorescence experiments. Tyrosine and tryptophan are the two amino acids with the most significant fluorescence. The peak fluorescence emissions of tryptophan and tyrosine are relatively close, with peak emissions differing by ca. 50 nm.

Time resolved measurements signified that pollen autofluorescence intensity, in both investigated emission channels, decreases upon fluorescence measurement. The fluorescence intensity values used for plotting graphs (not shown) are averages of multiple fluorescence intensity images of pollen grains (ca. 50-100 per sample). Each pollen fluorescence intensity image values were normalized and then the mean of the normalized values was calculated. Figures 5.7 & 5.8 show the time resolved average and normalized fluorescence signals of the pollen subjected to different environmental conditions. Figure 5.7 shows the fluorescence intensity observed in the tryptophan emission region, and Figure 5.8 shows the fluorescence intensity observed in the chlorophyll emission region. It is observed that whatever the environmental conditions, the intensity in all samples degrade over time to the same degree. This reduction in intensity can be assumed to be bulk photobleaching of the sample. Photobleaching occurs
when a variety of easily oxidizable components, such as proteins, nucleic acids, lipids and fluorophores react with ROS thereby losing their fluorescence signal (Dixit and Cyr, 2003; Martin et al., 2005). ROS production is mainly dependent on the photochemical properties of the fluorophore (Sugden, 2004) and the dose of excitation light (Bernas et al., 2004; Foyer et al., 1994).

Figure 5.7: Comparison between pollen grains fluorescence intensity of 3 different samples within the tryptophan region of the pollen grain analysed over a period of time (n=3). The control (untreated), air treated and NO2 treated are represented in blue, red and green respectively. The signals were normalized to account for the absolute differences in intensity. RH and NO2 appear to have more noticeable impact on the pollen fluorescence. Note that blurry images have not been used, hence the absence of data for frame 6.
Figure 5.8: Comparison between pollen grains fluorescence intensity of 3 different samples within the chlorophyll region of the pollen grain analysed over a period of time (n=3). The untreated, air treated and NO₂ treated are represented in blue, red and green respectively. The signals were normalized to account for the absolute differences in intensity. RH and NO₂ appear to have noticeable impact on the pollen fluorescence. Note that blurry images have not been used, hence the absence of data for frame 7.

Because of the bulk photobleaching during imaging, it was impossible to determine conclusively whether the environmental conditions affected the ability of the pollen to fluoresce. All measurements fell within the error bars of the other measurements conducted under different environmental conditions. Note, Microscope focus was lost (images became blurred) in some of the later runs which indicates that the sample moved and hence the data is not used, thus the lack of data for frames 6 and 7 of Figures 5.7 and 5.8 respectively.
5.4.2 SEM results

In this section, the study investigated the effects of different rupturing procedures on pollen grain morphology. The rupturing mechanisms investigated were 1. pollen grain immersion in rainwater overnight, 2. mechanically crushed pollen under dry conditions, using metal beads, and 3. exposure to high RH for 24 hours. The effect of NO₂ exposure on the morphological feature of the pollen grain was also investigated. Under atmospheric situations, aerosols comprise a broad range of moisture content, from very dry particles under low RH conditions, to extremely wet conditions for example pollen grains suspended in cloud water droplets (Selvam, 2010). In Chapter 4, it was shown that pollen proteins, such as the major birch pollen allergen Bet v I, were released in significantly greater amounts if they had been ruptured by the methods 1-3. Pollen allergens are integral pollen constituents mainly confined within the pollen grain in certain patterns of distribution (Behrendt and Becker, 2001). These allergens have to be released during a process of activation in order to become bioavailable (Sénéchal et al., 2015). The SEM images of different treated birch pollen samples showed that after hydration in rainwater, migration of some pollen material had occurred from inside the pollen onto the surface of intact pollen grains in comparison to the dry pollen case.

Below more details on the individual experiments are provided.

**Contact with rainwater** - After hydrating the pollen in rainwater and air-drying, it was examined using SEM. At the three different magnifications used, the pollen grains were found ruptured at their germination pores and surrounded by submicronic particles shown to be of high intensity in the SEM images. Previously, these pollen granules have been identified to contain the allergenic proteins of the pollen grain; and this process is referred to as abortive germination (Grote et al., 2003) (Figure 5.9 B). The finding is consistent
with the observations of Grote et al. (2003) and provides a causal reason to the elevated levels of nitrated allergen found in the rainwater processed pollen, as shown in Chapter 4.

**Crushed pollen**- In dry pollen grain, Bet v 1 are found predominantly in the cytoplasm and not within the pollen wall (Grote et al., 2003). However, after crushing it in dry state, there seems to be the movement of some particles probably allergen proteins from the intrinsic part of the pollen grain onto the cell wall (Figure 5.9 D). This result agrees to another research, which mentioned that the inner subparticles from birch pollen grains were shown to be released upon impaction on a solid surface at wind speed of about 3 m/s (Sénéchal et al., 2015; Visez et al., 2013).

**Exposed to high RH**- The RH is the ratio of the partial pressure of water at a given temperature (Seinfeld and Pandis, 2016). The RH in the troposphere is variable, depending on factors such as changing temperatures, meteorology and distribution of water sources. Water-soluble compounds are ubiquitous in atmospheric aerosol (Jimenez et al., 2009), therefore it is expected that water vapor will interact with the particle phase. Here, the pollen was humidified for 24 hours via exposure to high RH (~95%) with in a chamber. The germination pores have been ruptured open and no or lesser quantity of particles were also released as compared to the latter two methods (Figure 5.9 H).

Contrary to Taylor et al. (2004) research, the commercially purchased pollen used in this study did rupture upon hydration. This experiment on birch pollen grains, under various conditions showed that the emission of heterogeneous small particles or internal granules happens when the pollen source was humidified or crushed (Figure 5.9 images B, C, D and F). Grote et al. (2003) also showed that allergenic proteins are released from the birch pollen tubes predominantly upon rupture. Taylor et al. (2004), reported that fresh pollen ruptures in water after 3 hours (Taylor et al., 2004); however, this research could not
verify this statement because non-fresh (commercial) pollen was used in this study.

### 5.4.3 Impacts of different conditions/treatment on the surface of the pollen grain

**A- Undamaged pollen grain rehydrated in rainwater (~22 hrs)**

**B- Undamaged pollen grain**

**C- Crushed dry pollen grain hydrated in rainwater**
D-Crushed dry pollen grain
E- Undamaged pollen grain exposed to NO$_2$ at 10 ppm for 1 hr

F- Hydrated pollen grain exposed to NO$_2$ at 10 ppm for 1 hr
G-Undamaged pollen grain exposed to 1 hr RH

H-Undamaged pollen grain exposed to 24 hrs RH
Figure 5.9: Picture gallery of pollen characteristics after being subjected to different condition/treatment. Starting from top to bottom (A-I) A (control)- Undamaged dry pollen grain (intact pollen grain from storage container as provided by supplier) without any treatment. B- Undamaged dry pollen grain hydrated in rainwater over night (~22 hrs). C- Crushed pollen grain (mechanically crushed dry using metal bead). D- Crushed dry pollen grain hydrated in rainwater. E- Undamaged dry pollen grain exposed to NO\textsubscript{2} for 1hr. F- Hydrated pollen grain exposed to NO\textsubscript{2} at 10 ppm for 1 hr. G- Undamaged pollen grain exposed to 1 hr RH. H-Undamaged pollen grain exposed to 24 hrs RH. I- Pollen grain exposed to NO\textsubscript{2} at 10 ppm and mechanically crushed in precellys tube then extracted in extraction buffer. The blue arrow is pointed at ruptured grain after being crushed, while the green arrow is pointed at the particles expelled via germination pores following hydration in rainwater (rain-induced mechanisms of allergen particles release) and red arrow is pointed at the tiny white patches hypothesized to be induced by NO\textsubscript{2} exposure.

Figure 5.9: A-I provide an image gallery of birch pollen grains characteristic under different conditions/treatments. After hydration in rainwater and dry crushing the pollen, most examined pollen grains released particles through the germination pores and exine respectively (Figure 5.9 images B, C, D, E, and F). In the released particles, the most conspicuous materials are the starchy granules, however, smaller particles and amorphous materials were also expelled (Grote et al., 2001). Comparing the cytoplasmic release
mechanism of birch pollen to other pollens, particularly sweet grasses, birch pollen exine does not burst on hydration which could be due to its thick walls that might not favour bursting (Schäppi et al., 1999). However, birch pollen does rupture when crushed (Figure 5.9 image D, ruptured grain pointed with blue arrow) and also expel particles via germination pore upon hydration (Figure 5.9 image B, expelled particles pointed with green arrow). Once the pores are filled with water then the rest of the surface is supposed to be wetted as revealed in the research of Pope (2010). Looking at Figure 5.9 image A, the pollen pores are closed and the exine shrivelled, however, once subjected to some of the conditions mentioned above, the get ruptured open at their germination pores and swollen (Figure 5.9 image B) as compared to the undamaged (intact) pollen (Figure 5.9 image A). It can be seen that between Figure 5.9 images B, C, F and I that the pollen grain swells internally (Pope, 2010), but there is no obvious sign of water uptake upon the pollen surface. This is expected in the low pressure conditions of the SEM. Figure 5.9 images C and D are subjected to slightly altered conditions, however, both released particles onto the outer part of the pollen. For Figure 5.9 images E and F, it cannot be established that the tiny white patches pointed with red arrow and particles were induced by NO2 exposure. Exposure to the ambient air pollution increased the fragility of exine that is the most prominent results shown in many experiments (Majd et al., 2004; Rezanejad, 2009 Sénéchal et al., 2015). According to the initial fragility of a specific external pollen membrane, it causes collapse and numerous cracks on its surface (Sénéchal et al., 2015). In this study, no effect of the exposure to NO2 at 10 ppm for 1 hour was observed morphological on birch pollen grain. Another experiment strongly suggests that NO2 is able to strip off orbicules (small acellular structures of sporopollenin) from pollen grains and thus release them as free subparticles in the atmosphere (Shahali, 2011). For Figure 5.9 image G, is marginally less swollen in comparison to image H
because it was exposed to 1 hr RH as opposed to 24 hrs, it shows the swollen of the pollen grain with increased humidity exposure. This suggests that the opening of pollen germination pores can take place roughly within an hour. Finally, Figure 5.9 image I appeared to be coated with artefacts or substances contained in the extraction buffer (50 mM Tris-HCl pH6.8, 10% sucrose and inhibitory proteases). Hence, this indicates that extra care must be taken during sample preparation and processing so as not to introduce other foreign material into the sample before SEM imaging.

Taken together, these results conclude that pollen grains emit smaller particles after processing by rainwater immersion, high RH conditions, and mechanical action. This provides a mechanism through which the allergens travel to the surface of the pollen grain. From the pollen surface, it will be more facile for gaseous air pollutants to interact with the allergens and hence become more easily nitrated. It is worth noting that all the above-mentioned mechanisms of the pollen subparticles release are induced and do not depict the natural birch pollen germination.

5.5 Conclusion

In this chapter, the FM technique was used to try to observe the effect of nitration of birch pollen though use of the inherent autofluorescence of certain amino acids. It is shown clearly, that the pollen grains fluoresce in both the tryptophan and chlorophyll fluorescence emission channels. Furthermore, both channels show distinct photobleaching in time-resolved measurements. This photobleaching effect dominates any change in fluorescence that may be caused by the nitration of the amino acids found in the pollen protein. A possible future avenue of research that may be more revealing is the use of a lower wavelength laser to probe tyrosine fluorescence.
The SEM technique was used to observe morphological changes on pollen grain after subjecting it to different treatments and conditions. The SEM examination revealed that the pollen grain changes from shriveled to swollen upon hydrated with rainwater and exposure to high RH and showed that only certain conditions lead to smaller sub-pollen particle release from the pollen grain. As far as dry pollen is concerned, no evidence of the release of the sub-pollen particles onto the surface of dry pollen was observed. However, when in contact with moisture like mucosa of the upper respiratory tract, the allergen proteins elute within minutes and can induce local allergic reactions (Grote et al., 2003).

In conclusion, the SEM results suggest that the exposure of pollen to different environmentally relevant treatments and conditions provide pathways for the release of allergen-bearing subcellular particles from the birch pollen grains. However, all the mentioned mechanisms of the pollen subparticles release in this study was induced. This release of particles from the inside to the outside of the pollen is what allows significant nitrilation to occur. This is most likely why hydrated pollen in rainwater showed the highest nitrated allergen protein content within Chapter 4. This result may explain further why nitration was not detected prior to rupturing and provided added support for the hypothesis that ‘pollen does not get nitrated undamaged but it does when it is ruptured open’.
CHAPTER 6

Effect of pollen count on respiratory related ambulance callouts

This chapter investigates whether there is statistical relationship between birch pollen counts with temperature, NO\textsubscript{2} and various callout categories of the London Ambulance Service.

6.0 Synopsis

Exposure to pollen can contribute to several medical conditions and rise in hospital admissions. The laboratory results in Chapter 4 revealed an interaction between pollutants and birch pollen, in particular, NO\textsubscript{2} was able to nitrate various proteins. Such a mechanism may cause an increase in the allergenicity of pollen. This study examines the association between birch pollen count and allergic related illnesses using the London Ambulance Service callouts, that are discussed in Chapter 2, as the metric of illness intensity. The callouts were adjusted for temperature and NO\textsubscript{2} concentration. The analysis using odds ratio supports an increasing trend in the number of callout rates for some illnesses with respect to airborne pollen concentration. However, no significantly robust results were identified. Finally, the reasons for these null results are discussed.

6.1 Objective

In the preceding chapters, in the laboratory, the link between air pollutants and pollen-protein-specific modification at the proteome level as well as morphological changes after
exposure to NO₂, RH and rainwater were demonstrated. Additionally, the basic relationship between air mean temperature, and to a lesser degree RH, with certain medical conditions have been demonstrated using the ambulance callout rates as a proxy for illness. In this chapter, a comparison of allergic related illness measured by the London Ambulance Service will be compared to birch pollen counts recorded in Highbury, London. Note, a clear deficiency in this study is that the ambulance callouts are for the whole of London whilst the birch pollen data is just for Highbury.

6.2 Introduction

In order to strengthen investigations of seasonal allergic reaction such as asthma in humans, time series analysis of airborne pollen data for various urban locations have been conducted (Haberle et al., 2014). Development of predictive models between the variables of interest was possible due to the availability of the large data sets (Emberlin et al., 2002; Rodriguez-Rajo et al., 2003; Schäppi et al., 1998; Sofiev et al., 2013).

There are several factors that may provoke allergic reactions and asthma based on individual’s condition. This includes pollen, house dust mites, pollutant, climate, exercise, tobacco smoke, emotional issues, to mention a few (Osborne et al., 2017). Asthma exacerbation as a result of pollen effects is gradually understood (Osborne and Eggen, 2015). For example, a significant connection between grass pollen exposure and hospital admittance for asthma has been observed: in Australia both in adults (Erbas et al., 2007) and children (Erbas et al., 2012); in France (Huynh et al., 2010); in the UK (Lewis et al., 2000); in Spain (Altzibar et al., 2015; Tobias et al., 2004); in Italy (Ruffoni et al., 2013); in Hungary (Makra et al., 2012); and in the USA (Darrow et al., 2012; Gleason et al., 2014; Jariwala et al., 2014). Previously associated health outcomes with severe pollen
contacts include allergic rhinitis, food allergy (Datema et al., 2015), cardiovascular incidents (Brunekreef et al., 2000), preterm childbirths (Lavigne et al., 2017), and psychological conditions (Qin et al., 2013). Evidences suggested that the levels (Negrini et al., 2011; Ziello et al., 2012) and allergenicity of pollen are growing with time, probably in connection with worldwide weather alteration (Singer et al., 2005; Vardoulakis and Heaviside, 2012) and air pollution as well. Clearly there is a rise in the burden of allergic respiratory diseases (Beggs, 2004; Beggs and Bambrick, 2006; D'amato and Cecchi, 2008; D’amato et al., 2007), however; the reasons behind it are still not entirely understood (Reid and Gamble, 2009). Due to the lack of data suitably resolved over time and space, pollen concentration at present is mostly unclear (Schultz and Wang, 2006). An essential factor for estimating disease development and consequence can be based on knowledge of pollen concentration in the air (Schultz and Wang, 2006). Additionally, mapping the locations of allergenic plants in the UK can also provide the detail required for impact assessments (Mclnnes et al., 2017).

6.2.1 Pollen count

Pollen concentrations are highly variable daily and may travel lengthy distances partly because of weather conditions, thus, its concentration in the atmosphere (particles per m$^3$) is not just a confined occurrence (Osborne et al., 2017). It has been stated in some studies that pollen concentrations when measured, correlate across distances of 20 km (Erbas et al., 2007) and 41 km (Pashley et al., 2009), and yet there is possibility of the pollen to travel much further, including crossing a continent (Skjøth et al., 2007). Measuring the number of pollen grains in a given volume of air, using a pollen trap, generates a pollen count. A count of 70 pollen grains/m$^3$ or more is considered high in some cases (Kiotseridis et al., 2013). In another study, high pollen count is defined as 80 pollen
grains/m³ based on the details showing that 90% of patients with allergies express mild signs at this threshold (Skjøth et al., 2015). The risk posed by pollen counts to an individual depends on specific plant pollens the individual is allergic to. Essential knowledge on pollen count and concentration can significantly help to manage asthma and hay fever by providing the public with relevant information. Skjøth et al. (2009) reported that 90% of patients allergic to birch pollen show mild symptoms when the pollen count is above 80 grains/m³ at the start of the birch pollen season (Skjøth et al., 2009). However, 80% of patients do show indications of allergic reaction at a level below 30 grains/m³ during the late season (Emberlin 1997; Koivikko et al. 1986; Viander and Koivikko 1978).

The pollen count is simply the number of pollen spores per cubic metre of air. The UK pollen data is gathered using a network of pollen monitoring stations run by the Met Office. The stations all use the same type of device, the seven-day volumetric spore trap made by Burkard (Latalowa et al., 2002). Because collecting pollen from the ambient air on rooftops avoids measuring highly localized pollen concentrations, most stations are located on flat roofs of two or three story buildings. Figure 6.1 shows birch tree density (%) in broad-leaved forests and location of broad leaved forests in southern England and Wales (Skjøth et al. 2009) as well as location for pollen and air quality monitors, within UK.
Figure 6.1: Betula tree density (%) in broad-leaved forests and location of broad leaved forests in southern England and Wales (Skjøth et al. 2009). Also shown are the location of three pollen-monitoring sites (solid circles) and the meteorological stations (triangles) within UK.

“Analysis of long-term airborne pollen counts makes it possible not only to chart pollen-season trends but also to track changing patterns in flowering phenology” (García-Mozo et al., 2014) thus offering fundamental information to the public on how to prevent unnecessary exposure.
6.2.2 Pollen calendar

Depending on the time of year, the type of pollen in the air changes. Notably, weather conditions affect how much pollen is released and spread around. Previously, it has been stated that the principal sources of birch (*Betula spp*) pollen is the urban environment (Skjøth et al., 2012) due in part to the use of birch as ornamental trees. Pollen seasons are defined by four parameters namely: the start, duration, peak and the end (Haberle et al., 2014). Several other studies have reported that temperature and pollutant are influencing factors for longer pollen seasons (D’Amato et al., 2015; Reid and Gamble, 2009; Schmidt, 2016).

Figure 6.2 displays a generalized pollen calendar exhibiting when the main allergenic plants are in flower. Yearly, the exact of the pollen seasons will differ depending on the weather conditions and other natural and man-made factors. In the U.K, pollen from tree species are released typically in the mid-March to early June period, where birch has peak pollen release predominantly in April (Osborne et al., 2017). “Although the start of the season can vary by up to a one month and often occurs about two weeks later in Scotland compared to southern England” ([https://www.worcester.ac.uk/discover/nparu-pollen-types-birch.html](https://www.worcester.ac.uk/discover/nparu-pollen-types-birch.html)).

In Western Europe, the highpoint period of the pollen normally starts at the end of March, and in central and Eastern Europe, from the beginning to mid-April (Emberlin et al., 1990). For Northern Europe, the flowering season starts from late April to late May (depending on the latitude) (D’Amato, 1991). Pollen values peak between “1–3 weeks after the start of the season and the duration of the main season is remarkably dependent on temperature, thus varies from 2 to as much as 8 weeks” (D’amato et al., 2007). This statement implies that a relationship between Pollen season and temperature do exist.
Spieksma et al. (1995) reported that air temperature in the period prior pollen release is influential for the initial date of pollen season (Spieksma et al., 1995).

![Pollen calendar](https://www.worcester.ac.uk/pdfs/pollen-calendar.pdf)

**Figure 6.2**: Pollen calendar of different pollen types showing the start and end of their flowering seasons. Adopted from [https://www.worcester.ac.uk/pdfs/pollen-calendar.pdf](https://www.worcester.ac.uk/pdfs/pollen-calendar.pdf)

### 6.3 Methodology

#### 6.3.1 Data sets

Birch pollen count data of Highbury, London was provided by University of Worcester. LAS provided daily-anonymised ambulance data, which provided information on callout category for ambulances for the whole of London. Temperature was that of St James Park observatory, which occupies a central position in London. Data from government air pollution monitoring stations from the Automatic Urban and Rural Network ([https://uk-air.defra.gov.uk/data/](https://uk-air.defra.gov.uk/data/)) were used to calculate the daily concentration ($\mu g/m^3$) of air pollutants, nitrogen dioxide ($NO_2$). Data was that of urban background station, North
Kensington that had no missing data from the periods analyzed (01\textsuperscript{st} April to 31\textsuperscript{st} May 2005). North Kensington station was chosen because it represents background urban pollution levels and is known to be a site with good data coverage. The AURN site and pollen station are ~7 km distant to each other.

The data for the pollen count was recorded from every year from the 1\textsuperscript{st} of April to 31\textsuperscript{st} May (two months data) in the time period of 2005 to 2013 with no pollen record of the year 2012. Some years contain missing data within the pollen count season.

6.3.2 Methods

All 6 years (2005-2010) of data were combined and analyzed using only the dates inclusive of the 1\textsuperscript{st} April – 31\textsuperscript{st} May due to the availability of the pollen measurement data. It is noted, in some years, the birch pollen season has clearly started earlier than the 1\textsuperscript{st} April. Here, within the monitoring period (April and May), pollen season days are defined as those on which the recorded pollen count is greater than 30 pollen grains m\textsuperscript{-3}. If the pollen count is less than 30 pollen grains m\textsuperscript{-3} then these days are defined as off-season.

The data sets analyzed consist of 6 years pollen counts, illness codes, temperature and NO\textsubscript{2} (2005-2010). The pollen count record was interpolated to remove any missing data from the record. The temperature dependence and long-term trend taken from Chapter 2 was considered.

The statistical software R (version 3.0.2) was used for statistical analysis and, excel was used for data manipulation and generation of some plots.
6.3.2.2 Odds ratio

The odds ratio (OR) statistic measures relationship between an exposure and outcome (Szumilas, 2010). “Specifically, the OR measures the ratio of the odds that an event or result will occur to the odds of the event not happening” (McHugh, 2009).

The criteria of setting the limit values of high and low pollen day for calculating the OR was based on the information regarding pollen counts being high or low and the available data range. The set criteria number used were less and greater than 30 grains/m³. The odds ratio in the data sets were searched and identified, using the formula below. Results are expressed as odds ratios (OR) with their 95% confidence intervals designated as 95% CI.

\[
\text{OR} = \frac{a/c}{b/d} = \frac{ad}{bc}
\]

Where  
- \(a\) = Number of exposed cases
- \(b\) = Number of exposed non-cases
- \(c\) = Number of unexposed cases
- \(d\) = Number of unexposed non-cases (Szumilas, 2010)

In this study,

\(a\) = daily number of cases of call out category of interest with pollen count over set criteria

\(b\) = daily number of non-cases of call out category of interest with pollen count over set criteria

\(~\approx~\) population of London – daily number of cases of call out category of interest with pollen count over set criteria

\(c\) = daily number of cases of call out category of interest with pollen count below set criteria

\(d\) = daily number of non-cases of call out category of interest with pollen count below set criteria
criteria

= population of London – daily number of cases of call out category of interest with pollen count below set criteria

≈ population of London

n.b. set criteria = 30 grains/m³ of birch pollen count.

Because b ≈ d, the OR simplifies to a/c. Results are reported in section 6.4.3

6.4 Results and discussion

In an attempt to identify the causes of increased allergic illness, the correlation between trends in pollen counts and allergic related illness codes was evaluated. The time series analysis across the 6 years data found evidence of no obvious association between the pollen count of Highbury, London and LAS callouts. As shown in Figures 6.3 A and 6.4 A, there is little obvious evidence of correlation in the 2006 data set (data with highest pollen count) with RCI and Asthma illnesses, a similar lack of correlation is observed in all years and by combining all 6 years data (Figure 6.3 B and Figure 6.4 B). However, the lack of apparent correlation was for same day association between the variables as the model used did not investigate any time lag pollen exposure and illness indication.
Figure 6.3: Scatter plots of respiratory chest infection (RCI) versus pollen count of Highbury, London using single (2006), A and all year’s data (2005-2010), B. There is no obvious correlation between the variables.
In relation to weather, some ambulance callout categories have already been shown to be temperature dependent in Chapter 2, and in particular respiratory chest infection is found to be very temperature dependent. Figure 6.5 presents the scatter plot of respiratory chest

Figure 6.4: Scatter plots of Asthma versus pollen count of Highbury, London using single (2006), A and all year’s data (2005-2010), B. There is no obvious correlation between the variables.
infection (RCI) versus temperature for the investigated time period. A slight negative relationship between the two variables was observed but is less significant than the relationship shown in Chapter 2 due to the reduced data density of only using 2 months of data which span a smaller range of temperatures than what is observed if the whole year is analysed.

![Scatter plot of RCI (respiratory chest infection) versus temperature of St James Park, London. There is a slight negative relationship between the two variables.](chart.jpg)

**Figure 6.5: Scatter plots of RCI (respiratory chest infection) versus temperature of St James Park, London. There is a slight negative relationship between the two variables.**

### 6.4.1 Trends in pollen counts

Characteristically, birch pollen season begins with low pollen counts before the peak season starts. However, a large inconsistency in the pollen count trends is evident, indicated by the presence of high pollen days. To justify the reason of these trends is not within the scope of this study. Longer days of observation and comparison with environmental conditions might provide the reason behind the pollen count trends (Spieksma et al., 1995). The high pollen days may be caused by several factors such as
weather, and occurrences of long-range transport (Ziello et al., 2012). Figure 6.6 shows
the time series plot of the complete 6 years data investigated. There are no consistent
trends in the plots with respect to the 6 years data, yet, there is a common occurrence of
high pollen days either in mid or end of April. Some years show cyclic behaviour (Figure
6.7; cyclic behaviour and high pollen days indicated with blue arrow). In the peak period
of the birch season that occurs in April, the count can be very high because each birch
trees produce millions of wind-dispersed pollen grains

(https://www.worcester.ac.uk/discover/nparu-pollen-types-birch.html).

Figure 6.6: Shows the trend of the complete 6 years data investigated, 2005-2010.

Previous research indicates that per decade over the last thirty years, the birch pollen
seasons now starts five days earlier (Emberlin et al., 1997; and Emberlin et al., 2002).
Also, records on the start of birch pollen seasons monitored across three sites (Cardiff,
Derby and London) in the UK for forty-two years showed a trend for the pollen season to
begin earlier (Emberlin et al., 1997).
Birch pollen season has clearly started as indicated on the pollen calendar (Figure 6.1) and stated by the Met Office (Met Office, 2017) before measurements were recorded. Hence pollen exposure occurring before 1st April has been missed. This suggests data limitation and restriction of intensive analysis. Future longer-term studies that will incorporate all pollen season could hypothetically overcome these restrictions. In this study, the limitation was the lack of recorded data for days prior to the peak birch season. Typically, different tree pollen experience short peak periods of 2–4 weeks with lower concentrations, again restraining the statistical influence to assess their effects on diseases, unlike the combined grass pollen that naturally has prolonged flowering period.
and additional days of higher pollen concentrations (Osborne et al., 2017). Furthermore, establishing species-specific links using time series analysis may be difficult because tree pollen periods can overlap significantly (e.g. ash and birch) (Osborne et al., 2017).

### 6.4.2 Time series comparison between illness codes and pollen count

In some years, there appears to be a slight relationship between high pollen counts with asthma, respiratory chest infection, dyspnoea and allergic reactions. However, this observation is difficult to make statistically robust. This difficulty is almost certainly due to other influencing factors such as pollutant and weather change, precipitation, humidity, thunderstorms, wind, atmospheric blocking, heat and types of source vegetation (Osborne et al., 2017). “These may well be difficult to separate as individual factors, as they are often strongly linked (e.g. temperature and pollination in plants), and it can be difficult to identify sufficiently large data sets to perform the appropriate stratified analyses” (Osborne et al., 2017). Further in-depth analysis with variable data might provide more crucial and interesting information. Note that particularly strong pollen counts sometime seem to be associated with peaks in certain callout categories, as highlighted (white) in Figure 6.8.

It is reported in another study, exposure to pollen concentration of 20 pollen grains/m$^3$ of air showed noticeable symptoms in patients allergic to grass and at concentration of 65 pollen grains/m$^3$, the symptoms were intensified while at 120 pollen grains/m$^3$ (several hours exposure), it causes dyspnoea in some patients (Rapiejko et al., 2007). Comparable symptoms transpired after contact with birch pollen. Thus, it can then be established that clinical signs of allergic disease are dependent on the concentration and the kind of aeroallergen the individual was exposed to (Rapiejko et al., 2007) and the time of exposure.
Figure 6.8: Demonstrates the daily trend of birch pollen count and some of the illness codes using the 2006 birch pollen count (Pollen C) data (asthma, allergic rash reaction (ARR) and respiratory chest infection (RCI)). The highlighted section in white indicates rise in the illness codes during high pollen count days.

Figure 6.8 displays plots of the daily trend of birch pollen count (2006) and some of the illness codes using the 2006 data (asthma, allergic rash reaction and respiratory chest infection) where it can noticeably be seen that the illnesses data do have a cyclic up and down behaviour even in the nonappearance of pollen season possibility due to other influencing factors as mentioned earlier. Very few studies have narrated significant associations between pollen concentration and hospital admissions (Lierl and Hornung, 2003; Zhong et al., 2006), which might be due to geographic differences in allergen levels or the prevalence of allergies (Anderson et al., 1998). Some researchers have reported that pollen counts correlate weakly with symptoms (Agarwal et al., 1984; Buters et al., 2010;
Frenz, 2000; Marsh et al., 1987), since (i) the allergen exposure alone does not strictly represent the counts (Buters et al., 2010; Frenguelli et al., 2010; Galan et al., 2013), (ii) relationship between pollen and allergic symptoms is non-linear (Caillaud et al., 2014; Caillaud et al., 2012), and (iii) atmospheric conditions or air pollution may interact with pollens (Annesi-Maesano et al., 2012; Lubitz et al., 2010). “In general, pollen count and allergen in ambient air follow the same temporal trends. However, because a 10-fold difference can exist in allergen potency of birch pollen, symptoms might be difficult to correlate with pollen counts, but perhaps better with allergen exposure” (Buters et al., 2010).

Figure 6.9 displays scatter plots of pollen count data versus temperature and NO₂ respectively indicating no significant relationship with temperature and NO₂. In another study by Newnham et al. (2013) the start of the birch pollen season strongly correlated with March mean temperature, which reinforced previous findings that the timing of the birch pollen season in the UK is particularly sensitive to spring temperatures (Newnham et al., 2013). However, this study did not have the March pollen count data to verify previous literature findings.

Unfortunately, the pollen data set was not very dense and measured in a specific area of London whereas the ambulance callouts are representative of the whole of London. The environment where the pollen data was recorded may also influence results. For instance, in larger geographical areas, pollen samplers might be placed at different locations. Hence the levels of allergens in environment may vary quite significantly from the levels sensed by the sampler in comparison to a distance from the sampler where the patients may live. Again, all this comes down to where an individual gets exposed and illness data recorded. A future project could profitably investigate the ambulance callouts that are local to the
pollen counting measurements not more than 41km away. However, for this study we did not have geotagged ambulance data available.

Figure 6.9: Scatter plots displaying the relationship between year 2005 data of birch pollen count with temperature (A), and birch pollen count with NO$_2$ (B) respectively. There is no statistically significant relationship found between the variables. The data sets used are that of year 2005.
Putting all the results together, there were no clear identified patterns of association between pollen count, pollutant and analysed allergy related illness of the LAS when either individual or collective years’ data are considered.

6.4.3 Odd ratio outcome

Odds ratios associates the manifestation of an outcome of concern such disease after exposure to the variable of interest like pollen count (Szumilas, 2010). A ratio of 1.0 is called the null value and is interpreted to mean that there is no relationship between the disease and the exposure, above 1.0 indicates that the exposure increases the risk of disease, and below 1.0 indicates that the exposure protects from the disease.

Before conducting the odds ratio analysis, the data was corrected for long term and temperature dependent trends, as discussed in Chapter 2. Both the illness categories and pollen (birch) count used are of 2005-2010 dates ranging from 1st of April to 31st May.

<table>
<thead>
<tr>
<th>Illness codes (2005-2010)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma</td>
<td>1.012(0.812-1.261)</td>
</tr>
<tr>
<td>Allergic rash reaction</td>
<td>1.014(0.843-1.219)</td>
</tr>
<tr>
<td>COPD</td>
<td>1.037(0.8767-1.226)</td>
</tr>
<tr>
<td>Dyspnoea</td>
<td>0.998(0.8134-1.223)</td>
</tr>
<tr>
<td>Generally unwell</td>
<td>1.002(0.815-1.232)</td>
</tr>
<tr>
<td>Respiratory chest infection</td>
<td>0.984(0.810-1.194)</td>
</tr>
</tbody>
</table>

Table 6.1: Reports the odd ratio between pollen counts and allergic related illness codes. Both the illness categories and pollen (birch) count used are of 2005-2010 dates ranging from 1st of April to 31st May. The reported associations (OR above 1) are not statistically robust with the 95% confidence limits of all the ORs lying either side of unity. This indicates that even though the ORs results might imply increase risk due to exposure, they are not statistically significant.
Table 6.1 reports the OR and their 95% CI for pollen counts with respect to allergic related illness codes. Some of the analysed illnesses (Asthma, Allergic rash reaction, COPD and Generally unwell) show odd ratios that are greater than the null value of 1. This indicates that pollen counts over the set criteria (30 pollen grain/m$^3$) lead to an increased prevalence of illness categories. However, none of the reported associations are statistically robust with the 95% confidence limits of all of the ORs lying either side of unity. This indicates that even though the ORs results might imply increase risk due to exposure, they are not statistically significant.

In general, the lack of strong relationship and reduced risk on high pollen days could be linked to a reverse causation effect, due to increased medicine use such as antihistamines and enhanced managing of illnesses during high pollen season, as patients have increased knowledge of the situation (Osborne et al., 2017). The absence of any well-defined association between birch pollen and asthma is coherent with the recent Osborne et al. (2017) study. However, in New York City links between tree pollen including birch was established as opposed to previous work (Ito et al., 2015) and the finding from this research findings.

Figure 6.10 displays a forest plot of the OR results where the results of the different illnesses, with 95% CI, are shown in the plot. The forest plot is a graph that has one line representing results for different parameters in the same study (Petrie et al., 2003). It can be observed that the OR values were less than one in dyspnoea, respiratory chest infection and more than one in asthma, allergic rash reaction, COPD and generally unwell as shown in the plot (Figure 6.10). However, the 95% CI indicates that the results are not statistically robust. That is the presence of pollen does not increase the risk of the diseases with OR above 1 and prevents diseases below 1.
6.5 Conclusion

The chapter sets out to see if ambulance callout rates, for categories that might be linked to allergic diseases, could be linked to birch pollen counts and NO₂. Previously in Chapter 2 we have highlighted the importance of temperature on certain ambulance category callout rates. There were no consistently clear patterns of association between the illness codes and birch pollen count and NO₂ observed.

The odds ratio analysis suggests that birch pollen could be linked to certain callout categories, but none of the associations were strong and were all statistically insignificant. Nevertheless, this chapter does provide some evidence to suggest a pollen count threshold above which adverse effects are more likely to occur.
In conclusion, more research is needed in this area to understand further the relationship between pollen counts, pollutant concentration and weather in relation to human illnesses. Understanding this relationship may be the underlying answer why there are additional increase in pollen amounts, which in turn, leads to a larger exposure of humans to pollen allergens, with potentially severe concerns for public health. The colocation of health data with the pollen data would likely help generate data which is more amenable for statistical analysis. In future, it might be profitable to look at the grass pollen season. From a statistical standpoint, there is a better chance of finding a statistically robust result because of the longer flowing season which would allow for a long time series to be analysed.
CHAPTER 7

Summary

This chapter summarizes the different phases of the work carried out and described in this thesis. General overviews of the principal outcomes obtained from the various sections of the work are organized in this chapter.

The overall aim of this study was to establish the relationship between pollen, in particular birch pollen, with air pollutants and weather and to see if these relationships could be linked with increased allergenicity of pollen. If established, these relationships could provide rationale behind the general observation of increasing allergic diseases in westernized countries. It is important to remember that certain pollen species without modification are natural atmospheric environmental allergens that in some instances responsible for severe human health effects (Bosch-Cano et al., 2011; Cresti and Linskens, 2000; D'amato et al., 2010; Traidl-Hoffmann et al., 2009). In the atmosphere, pollen co-exists with air pollutants and weather. The combination of pollen with pollution and weather has the potential to increase the allergenicity of pollen. The summary of the results for each scenario is reported below.

- Chapter 2 explored the relationships between observed weather, in particular temperature, and a number of ambulance calls, incidents and response times. The data were studied using statistical models and specifically time series analysis. The time series data all showed a significant long-term increase in callout frequency, which were typically non-linear and category dependent due to increased ambulance usage in London. Certain categories show a clear seasonality and where present, is hypothetically driven by temperature. The findings show that
both cold and hot temperature influence different categories of illnesses, as earlier stated in other studies. The relationship of the top 20 call-outs and mean air temperature were inspected, where some illnesses have displayed lag effects indicating the onset of illness categories requires a period of incubation. Ambulance services are also shown to be affected due to increased demand by patients when there is extreme weather, in particular heat waves and cold waves were investigated. Finally, we highlighted that the identified relationship between weather and ambulance callout rates could be gainfully used to forecast ambulance callout rates and thereby improve the efficiency of the London Ambulance Services.

- This is the first study that has shown the effects of ambient temperature on London ambulance call-outs for specific categories (Mahmood et al., 2017). Given the lack of research into temperature effects on London ambulance callouts, the study will contribute to an understanding in this regard and may also serve as a firsthand information for ambulance services and hospital staff to plan and prepare way ahead of time. As ambulance callouts often occur for situations that do not need hospital admission, it may imply that ambulance callout data might be useful in surveillance systems since it can provide the facility to monitor health outcomes that would not ordinarily be captured during hospital admissions or in case of loss of life. The modelling approach for the prediction could serve as an early warning tool for health surveillance systems as well as ambulance services.

- Chapters 3 & 4 of this research was a laboratory study into post-translational modification of birch pollen protein. In particular, the study investigated the effect of NO₂ on protein of pollen grain responsible for allergies in humans. To do this, a
full methodology and laboratory protocol was devised both to expose the pollen to NO₂ and other environmental conditions, and subsequently to measure the effect at the proteomic level. The NO₂ concentrations used were between 2 ppm to 10 ppm and exposure time ranged from 60-120 minutes, which led to realistic life time exposures of pollen grains in the urban atmosphere. Similarly, realistic exposures of O₃ and RH were also used. The proteomics approach investigated the whole structure of the protein. Proteomic analysis of this post-translational modification was ascertained using mass spectrometry-based and dot blot techniques. The findings indicated that the interaction between gas phase pollutants and pollen can cause protein specific modifications; in particular, addition of a nitro group (–NO₂) to the phenolic ring of a tyrosine residue (Zhan and Desiderio, 2009), which is in agreement with some of the previously carried out researches. In a nutshell, a link between air pollutants and pollen protein specific modification was demonstrated. However, there were challenges in detecting and quantifying the degree of nitration using the MS analysis and TMT labeling tags respectively. This study provided the first evidence of nitration of pollen protein directly in real pollen, which contain a soup of complex protein mixtures held within the protective case of pollen walls. Previous studies demonstrated nitration of the allergen, but to do so, they had to isolate of the allergen protein Bet v 1 by expressing it in e-coli or directly isolated Bet v 1 protein.

- In the preceding Chapters (3 & 4), the changes that occur on a protein of birch pollen grain were assessed at the proteome level. Here (Chapter 5), the investigation was at morphological level upon contact with rainwater, RH and NO₂ as well. This study has also proven within pictorial evidence that birch pollen grain can rupture through its germination pores in high RH and moisture and also
contain fluorescence materials. The fluorescence intensity reduces over time mainly due to photobleaching. The finding that, under induced moist conditions, pollen grains release protein contents and can be nitrated, adds to our understanding of the relationship of pollen exposure and allergic sensitization. It may also help to improve the understanding of the reason behind increased pollen allergenicity. In line with previous investigations, this release mechanism may explain the recurrent observation that illnesses particularly asthma attacks commonly strike after episodes of heavy rainfall (Grote et al., 2000).

In the last empirical Chapter (6) of this study, again using a statistical model, the laboratory-derived results were supported with a time series and odd ratio examination of allergic related illness codes. This took into account the pollen count data from for Highbury in London (provide by the University of Worcester), temperature data of SJP and NO₂ data of North Kensington, London. Note that from the first experimental Chapter, we already know that temperature is a strong determinant. However, this was done to advance our understanding and begin to link the relationship between pollen and illnesses. That is to see if there would be a strong signal of increased allergic illnesses when both pollen count and pollutant concentrations are high. The study showed only a weak and non-statistically robust relationship between the variables. Nonetheless, from Chapter 2, we noted that temperature is a major determinant in respiratory illness although the lack of adequate data made it difficult to unpick the more subtle effects of pollen concentration to the temperature effects, and possibly other non-environmental effects. Undoubtedly, there are additional factors besides the absolute level of the pollen count that is influencing the occurrence of allergic
related illnesses since the trend of the illnesses during pollen and non-pollen season did not change in a statistically significant manner.

In a wider context of this thesis, we demonstrate that the relationships between temperature, and to a lesser extent RH, and some medical conditions do exist and have been established (Chapter 2). We show that the air pollutant NO₂, under certain realistic environmental conditions, can nitrate pollen grains (Chapter 4). Since protein nitration is often linked to diseases, this is an important link to have been established. It has also revealed that; RH and hydration affects pollen grain morphologically and enhances the release of its particles and also shows that pollen grain fluorescence (Chapter 5) where the intensity reduces largely as a result of photobleaching. Although no strong statistical correlations between pollen, pollution, and illness, as interpreted using ambulance callout rates, could be established (Chapter 6), It is possible that studies with a larger sample size may allow for obvious relationships to be observed (Carracedo-Martinez et al., 2008).

7.1 Concluding remarks

This thesis has demonstrated straightforward, reproducible techniques to advance understanding of the relationship between pollen, pollutant, weather and human health. Collectively, the overall finding of this study outlines the critical impact of weather, pollutant and bioaerosols on human health.
CHAPTER 8

Evidence based recommendation

Currently, understanding the effect of air pollutants on pollen and their allergenic potential is a critical scientific subject. The work in this thesis has made significant inroads into understanding the mechanisms of the interaction of air pollution with allergenic pollen. However, the effect of air pollutants on the allergenic potential of pollen is not yet completely clear.

8.1 Future directions

The outcomes of this research have shown that some issues remain unresolved and require further investigation. This study highlights some areas where further understanding of the interaction of air pollution, weather, and bioaerosols with human health is required. To develop a full picture of this relationship, additional studies are needed. Recommendations of future study areas to be further explored are identified and reported below:

- Even though the proteomic MS approach, detailed in this thesis, is currently one of the best available analytic technologies that is able to offer both qualitative and quantitative information about natural and post-translationally modified proteins, it has its limitations. MS does not guarantee valid data and must be used with other thoroughly validation methods for optimal sensitivity, selectivity, specificity, precise and accurate identification and quantification of protein of interest.

- Precise and accurate quantification of altered proteins is of vital significance to numerous areas of biology (Duncan et al., 2009; Stevens et al., 2008). However, it
is clear at this point that, perhaps due to very low amount of protein tyrosine nitration and less forceful protocol, the definite identification, and precise quantification of nitration partially remains unsolved and requires further investigation. Thus, there is the need for continuous strive to overcome the misidentifications and quantification that have troubled this and other studies with the hope that potential investigation will overcome these challenges. In future investigations, it might be possible to reuse the Tandem Mass Tag (TMT) system or a different advanced analytic and quantitative method in which determining the specific sites of this modification will not remain a challenge. Continuous efforts to avoid interference from non-nitrated peptides without losing recovered nitrated peptides are also an important issue for future research. Employing “de novo sequencing” is further recommended to properly validate modification found in the searches. Though, quantification of degree of nitration was not achieved using the tagging protocol, it was semi-quantified using dot blot technique. A successful tagging procedure would have provided precise degree of nitration in each sample that may help explain the effect of variable concentration on the pollen protein.

- The exposure of birch pollen to the air pollutant NO₂ at levels that can be pondered safe for human wellbeing protection, display adverse effects on the protein content of the exposed when compared with the control sample. Thus, further research should be undertaken to investigate the effects between pollen and higher air pollutants concentration.

- As already mentioned earlier, identified relationship between weather and ambulance callout rates could be beneficially used to forecast ambulance callout rates and thereby improve the efficiency of the London Ambulance Service.
Francis Pope is actively working with London Ambulance Service, West Midlands Ambulance Service and Scotland Ambulance Service to do just this. And the work presented in this thesis is the first step, towards better ambulance callout predictions.

- There has been a clear trend towards an increase in atmospheric pollen despite the absence of explicitly identified drivers as reported in previous studies. Various hypotheses abound: interactions between allergens and other inducing factors such as greenhouse gas CO$_2$ (Ziska et al., 2009), precipitation, humidity, thunderstorms, wind, atmospheric blocking, heat and types of source vegetation (Osborne et al., 2017) that intensify the development and symptoms of allergic disease. In this study, the trends of the pollen counts could not be attributed to either temperature nor pollutant concentration, but as suggested by other studies may be influenced by the anthropogenic increase of the greenhouse gas CO$_2$ (experimental) (Darbah et al., 2008; Rogers et al., 2006; Singer et al., 2005; Wayne et al., 2002; Ziska et al., 2009; Ziska et al., 2008). In this regards, more research is needed in this area because a further worldwide increase in atmospheric CO$_2$ is projected (Parry, 2007).

- It was not possible to investigate the significant relationships of pollen count (Highbury station) and other interested variables further because of data limitation. Larger data sets will likely help find statistically valid relationships. A more comprehensive model should be established to enable a better and complete forecasting. Thus, advanced and complete data collection and their incorporation into more comprehensive models are suggested. This might aid in determining exactly how pollen affects human health statistically.
• Investigation of other allergic pollen species such as grass pollen might be useful because of the greater counts and longer season as mentioned in another study (Osbourne et al., 2017). Data from other pollen stations might also be useful but we were unable to obtain them for this study.

• A further study with more focus on the water content of the pollen grain, which is very rarely studied, is also suggested.

• The laboratory and modelling protocols developed in this proposal can now be used to investigate the impact of gas phase pollutants, other than NO2, upon post-translational modification of pollen protein. In particular, this should also include exposure to nitrous acid (HONO), which is a source of the most important daytime radical, the hydroxyl radical (OH). HONO is highly surface active on aerosols and one might expect it to be so on bioaerosols. Different allergenic pollen species (such as grass) can also be investigated.

8.2 Importance of research replication

"Published research findings are sometimes refuted by subsequent evidence, with ensuing confusion and disappointment" (Ioannidis, 2005). Contradiction and disagreement is perceived through the range of research designs, from scientific trials and traditional epidemiological investigations (Ioannidis et al., 2001; Lawlor et al., 2004; Vandenbroucke, 2004) to the most modern molecular research (Ioannidis et al., 2001; Michiels et al., 2005). Incorrect discoveries may be the popular or even the huge majority of published research statements in modern study that is an increasing concern (Colhoun et al., 2003; Ioannidis, 2003; Ioannidis, 2005). Hence, this calls for need of research
Replications. Replications are an important part of scientific disciplines. It tests the credibility of original studies and has the potential to separate true outcomes from those that are unreliable. Both biological and technical replicates are necessary for accurate and consistent results. Technical replicates will aid identify errors caused by processing variation, while biological replicates will help confirm that biological changes are real and not an irreproducible coincidence.

Hence, we recommend that further tests are conducted by other groups to corroborate our findings.
References


Collins, N., 2012. Exhaust fumes are twice as deadly as roads, study claims.


Eckl-Dorna, J., Klein, B., Reichenauer, T.G., Niederberger, V. and Valenta, R., 2010. Exposure of rye (Secale cereale) cultivars to elevated ozone levels increases the allergen content in pollen. Journal of Allergy and Clinical Immunology, 126(6): 1315-1317.


El-Sharkawy, M.F., 2013. Assessment of Ambient Air Quality Level at Different Areas inside Dammam University, Case Study. Journal of King Abdulaziz University: Meteorology, Environment & Arid Land Agriculture Sciences, 24(2).


Ghiani, A., Aina, R., Asero, R., Bellotto, E. and Citterio, S., 2012. Ragweed pollen collected along high-traffic roads shows a higher allergenicity than pollen sampled in vegetated areas. Allergy, 67(7): 887-894.


Ipsen, H., & Løwenstein, H. (1983). Isolation and immunochemical characterization of
the major allergen of birch pollen (Betula verrucosa). *Journal of allergy and clinical immunology*, 72(2), 150-159.


Lubitz, S., Schober, W., Pusch, G., Effner, R., Klopp, N., Behrendt, H. and Buters, J.T., 2010. Polycyclic aromatic hydrocarbons from diesel emissions exert proallergic


Radauer, C. and Breiteneder, H., 2006. Pollen allergens are restricted to few protein families and show distinct patterns of species distribution. Journal of Allergy and Clinical Immunology, 117(1): 141-147.


Rogerieux, F., Godfrin, D., Senechal, H., Motta, A., Marliere, M., Peltre, G. and Lacroix, G., 2007. Modifications of Phleum pratense grass pollen allergens following artificial exposure to gaseous air pollutants (O3, NO2, SO2). International Archives of Allergy and Immunology, 143(2): 127-134.
Savill, P.S., 2013. The silviculture of trees used in British forestry. CABI.


Simon, R., 2010. "20th December 2010".


Viander M, Koivikko A (1978) Seasonal symptoms of hypo-sensitized and untreated hay-
fear patients in relation to birch pollen counts — correlations with nasal sensitivity, prick tests and RAST. Clin Allergy 8:387–396.


Who, 2014. WHO Ambient (outdoor) air quality and health


www.licor.com

www.nanoscience.com,

www.ons.gov.uk,

www.thermofisher.com,


