Study on the Biological Air Quality in Bellaterra (Barcelona) and Vitoria-Gasteiz: pollen and spores and their allergens

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PhD Thesis

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To obtain The PhD grade

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SUMMARY

Airborne allergens are major players between the airborne particles that have influence on respiratory human health. Allergy to Poaceae pollen and to *Betula* pollen are between the most important respiratory allergies in Europe.

Phl p 5 is the major allergen and one of the most representative of the pollen of the species of the Poaceae family. The Phl p 5 allergen is responsible of 88.4% of allergic cases in Europe.

Bet v1 is the major allergen and the most representative of the pollen of the *Betula* species. It Birch pollen provokes symptoms in 10-20% of allergic patients in North European countries.

Alt a 1 is the major and the most representative allergen of the spores of the fungal genus *Alternaria*. At a world level, the incidence of the allergy to *Alternaria* spores varies between 4 and 40 % of the atopic patients.

This study focuses on the detection and quantification on a daily basis, for the first time, of the allergens Phl p 5, Bet v 1 and Alt a 1 in Bellaterra (Barcelona) and Vitoria-Gasteiz, two localities in the North of Spain, for the years 2013-2015. It aims also to observe their correlation with the daily concentrations of corresponding pollen (Poaceae and *Betula*) and spores (*Alternari*a) types as well as with pollens presenting cross reactivity. The correlation with environmental factors is also undertaken, taking into consideration Temperature (maximum, minimum and mean), Precipitation, Relative Humidity and PM10.

The sampling method used for allergen detection, multi-vial cyclone sampler, has proven to be efficient for pollen allergen sampling but not for sampling the allergens from fungal spores. To sample Alt a 1 a test was done with a High volume Total Particle Sampler (TPS).

ELISA analyses was the method used to quantify Phl p 5 and Bet v 1. Alt a 1 could not be detected with this method with samples obtained with the cyclone sampler, but yes with those from the TPS sampler. Dot-Blotting was also tested with TPS samples to detect Alt a 1 and showed to be the most sensitive method for this allergen.

The total allergen Phl p 5 and Bet v 1 measured per year varied from one year to another as well as from one location to another. The Annual Allergen Integral (sum of daily concentrations) in year 2015 were 10 times lower than in 2013 and 2014, respectively.

Poaceae pollen was detected in days where no Phl p 5 was detected while Bet v 1 was detected in days where no *Betula* pollen was found. There was a positive significant correlation between daily concentrations of Phl p 5 and Poaceae pollen in Bellaterra (Barcelona), years 2013 and 2015. Also, a positive significant correlation was observed between daily concentrations of Bet v 1 and *Betula* pollen in Vitoria-Gasteiz, year 2015. But, no significant correlation was found between daily concentrations of Bet v 1 and *Betula* pollen in Bellaterra (Barcelona), years 2014 and 2015.

To explain the presence of Bet v 1 in the atmosphere outside the main pollination season of *Betula*, a correlation between the allergen and the pollen concentrations of the cross-reactive genera *Alnus*, *Castanea*, *Corylus* and *Quercus* has been undertaken. A correlation was found for *Quercus* pollen and Bet v 1 daily values in Bellaterra (Barcelona), year 2015, but not in year 2014 neither in Vitoria-Gasteiz 2015.

Phl p 5 and Bet v 1 daily concentrations did not show correlation with the meteorological parameters (Maximum, minimum and mean Temperature, Precipitation, Relative Humidity) but a positive correlation was found with the PM10 values from Montcada and the values in Bellaterra (Barcelona).

RESUMEN

Los alérgenos aerovagantes están entre las partículas atmosféricas con mayor influencia en la salud respiratoria humana. La alergia al polen de Poaceae y al polen de *Betula* se encuentran entre las alergias respiratorias más importantes en Europa.

Phl p 5 es el principal alérgeno y uno de los más representativos del polen de las especies de la familia Poaceae. Su alérgeno Phl p 5 es responsable del 88,4% de los casos de alergia en Europa.

Bet v1 es el principal alérgeno y el más representativo del polen de la especie *Betula*. El polen de abedul provoca síntomas en el 10-20% de los pacientes alérgicos en los países del norte de Europa.

Alt a 1 es el alérgeno mayor y el más representativo de las esporas del género *Alternaria*. A nivel mundial, su incidencia alérgica varía entre 4 y 40 % de los pacientes atópicos.

Este estudio se enfocó, por primera vez, en la detección y cuantificación en base a datos diarios de los alérgenos Phl p 5, Bet v 1 y Alt a 1 en Bellaterra (Barcelona) y Vitoria-Gasteiz, dos localidades en el Norte de España, en los años 2013-2015. Estableció también su correlación con las concentraciones diarias de los correspondientes tipos de polen (Poaceae y *Betula*) y esporas (*Alternaria*) así como con los pólenes con reactividad cruzada. Se ha hecho también la correlación con Temperatura (máxima, mínima y media), Precipitación, Humedad Relativa y PM10.

El método usado para la detección de alérgenos, captador multi-vial ciclón, se ha mostrado eficiente para capturar alérgenos de polen, pero no para muestrear alérgenos fúngicos. Para muestrear Alt a 1 se probó con un captador de partículas en suspensión de alto volumen (TPS).

Análisis ELISA fue el método usado para cuantificar Phl p 5 y Bet v 1. Alt a 1 no pudo ser identificado con este método en las muestras obtenidas del captador ciclón, pero sí en las obtenidas con el TPS. La detección de Alt a 1 en las muestras TPS usando Dot-Blotting resultó ser el método más sensible a la detección de este alérgeno.

La cantidad total anual de Phl p 5 y Bet v 1 variaron de un año a otro y de una localidad a otra. La Integral Anual de Alérgeno (suma de las concentraciones diarias) en 2015 fue 10 veces menor que en 2013 y 2014, respectivamente. El polen de Poaceae fue detectado

en días en que no se detectó Phl p 5 mientras que Bet v 1 fue detectado en días en que no había polen de *Betula*. Se encontró una correlación positiva entre las concentraciones diarias de Phl p 5 y el polen de Poaceae en Bellaterra (Barcelona), años 2013 y 2015. También, se observó una correlación positiva entre las concentraciones diarias de Bet v 1 y polen de *Betula* en Vitoria-Gasteiz, año 2015. Pero, no se encontró ninguna correlación entre las concentraciones diarias de Bet v 1 y el polen de *Betula* en Bellaterra (Barcelona), años 2014 y 2015.

Para explicar la presencia de Bet v 1 en la atmósfera fuera del período de polinización de *Betula*, se calcularon las correlaciones entre las concentraciones del alérgeno y las de los pólenes que presentan reactividad cruzada con ella: *Alnus*, *Castanea*, y *Corylus*. Se encontró correlación entre las concentraciones diarias de polen de *Quercus* y Bet v 1 en Bellaterra (Barcelona), año 2015, pero no en el 2014 ni en Vitoria-Gasteiz 2015.

Las concentraciones diarias de Phl p 5 y Bet v 1 no mostraron correlación con los parámetros meteorológicos (Temperatura máxima, mínima y media, Precipitación, Humedad Relativa) pero sí se observó una correlación positiva de éstos en Bellaterra (Barcelona) y las concentraciones de PM10 de Montcada.

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I. INTRODUCTION

1. Aerobiology

The atmosphere constitutes the primary trajectory for long-distance transportation of a wide range of organisms and compounds (Dingle, 1966). Anemophily is the mechanism of the plants and fungi to disseminate their reproductive cells (pollen grains, spores) using the travel paths of air masses as transport vector. This mechanism implies the production of a high quantity of biological particles to guarantee the survival of the species. Besides the benefits provided by the production, liberation and transport of these particles, which are paramount for the maintenance of life and biodiversity, they are known to cause health problems in humans and animals (allergy) as well as in plants (phytopathology).

Aerobiology (from Greek ήr , aēr, “air”; bíoV , bios, “life”; and - logía , -logia) is the scientific terminology for that studies concerned about organic particles, such as bacteria, fungal spores, pollen grains and viruses, which are transported by the air (Cecchi, 2013). The analysis of pollen and fungal spore contents in the atmosphere aims to inform on the phenology of anemophilous plants, the life cycle of fungi (Gage et al.., 1999), to forecast crop yields (Galán et al.., 2008), to establish preventive measures against potentially impending fungal plagues of crops (Viljanen-Rollinson et al.., 2007), and even to monitor and assess the impact of concurrent climate change on pollen and spore quantity and dynamics (García-Mozo et al.., 2006).

1.1. Airborne Particles

1.1.1. Pollen grains

Pollen is the biological structure of angiosperms and gymnosperms denominated male gametophyte (Webb and Webb, 1983) whose task is to fertilize the ovules. The transport of the pollen since the anther to the stigma of the flower (pollination) can be carried out by wind (anemophilous plants), animals (zoophilous plants), or water (hidrophilous plants). The pollen dispersed by the wind (anemophily) is an important portion of the airborne biological fraction and has a very important role to trigger allergic respiratory diseases.

Not all pollen grains cause allergy nor are all equally allergenic. In general, the distribution of airborne pollen taxa of allergological interest is related to vegetation areas (D'Amato et al.. 2007). In Europe, 12 pollen types originating from anemophilous plants are of interest due to their allergenic characteristics: ragweed (*Ambrosia*), alder (*Alnus*), mugwort (*Artemisia*), birch (*Betula*), goosefoots (Chenopodiaceae), hazel (*Corylus*), cypresses including yews (Cupressaceae/Taxaceae), olive (*Olea*), plantains (*Plantago*), plane tree (*Platanus*), grass (Poaceae), and wall pellitory (including stinging nettle) (*Urtica*/*Parietaria*)(D'Amato et al.., 2007) (D'Amato et al.. 2007). The pollen grains from this group are small, very light in mass, aerodynamic, with relatively thin wall and powdery, non-sticky surface (Moore et al.., 1991). Between these pollen types, two of the most important at a European level because of their allergenic capacity are *Betula* and Poaceae pollen grains.

1.1.1.1.*Betula* genus

Birch (*Betula* sp.) is a deciduous tree of the Betulaceae family. Birches are a typically rather short-lived pioneer species. *Betula* is widespread, although it only grows in the northern hemisphere; it is found across most of Europe where it is particularly common in northern and central regions but is scarce in the Mediterranean territories, especially in Spain, which northern regions constitute a southern border of the *Betula* distribution area (De Bolòs et al.., 1990). *Betula alba* L. and *Betula pendula* Roth. (Lobo et al.., 2001) are the two birch species found in northern Spain. Some authors (Ranta et al.., 2005) consider both species as members of the same taxonomic section (*Betula alba*). Birch may also form part of riparian forests, along with *Alnus glutinosa*, *Salix atrocinerea*, *Frangula alnus* and *Fraxinus angustifolia*, or even, *Betula pendula* is grown as an ornamental tree in some cities in the area. The number of pollen grains per inflorescence was estimated by Erdtman (Erdtman, 1943) as 6×10^6 . *Betula* pollen is therefore a major aeroallergen, common in the air of many European cities; a great deal of research has sought to ascertain airborne pollen seasons for this pollen type (Clot, 2001; Corden* et al.., 2000; Emberlin et al.., 1997; Hallsdóttir, 1999). For example, in Northern Europe, during the peak birch pollen season the daily average of airborne *Betula* pollen concentrations observed could reach ranging from 1000 to 10,000 pollen grains/m^3 (Ranta et al.., 2008). The highest *Betula* pollen concentrations in Spain are recorded in the northwest of the country, where daily mean pollen concentrations often

exceed 100 pollen grains/m^3 and annual values are greater than 1,000 pollen grains/m^3 (Aira et al.., 2005; Jato et al.., 1999).

Betula pollen is distributed by wind and impacts human health by causing seasonal hay fever, pollen-related asthma, and other allergic diseases (Müller- Germann et al.., 2015), being one of the most important causes of respiratory allergy in Northern and Central Europe (Emberlin et al.., 1993, 1997; Spieksma et al.., 1995; Heinzerling et al.., 2009).

Figure 1 *Betula* sp., branch showing a male inflorescence.

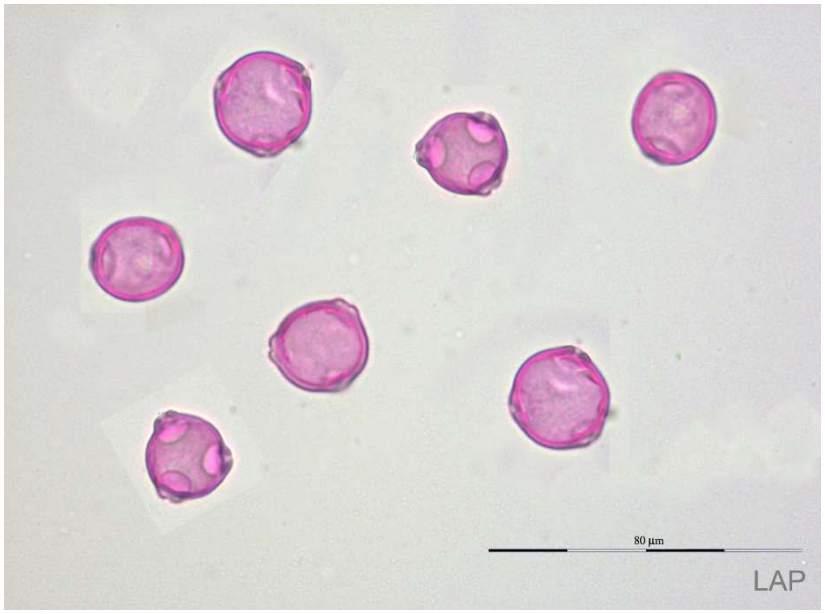


Figure 2 *Betula* sp. pollen grains seen underlight microscope.

1.1.1.2.Poaceae family

The Poaceae or Gramineae family is a large family of plants known popularly as grasses. This family includes the cereals, bamboos and the grasses of natural grassland and cultivated lawns and pastures. In general, they are herbaceous plants, mostly annual or biannual, with hollow stems, except at the nodes, and with narrow alternate leaves borne in two ranks. The grass family is widely distributed and abundant in all around the world, including the Antarctic Peninsula.. The pollen of most species of the Poaceae family share the same characteristics under the light microscopy (Driessen et al.. 1989). So, in the aerobiological studies, are considered that all grass species have the same pollen type. Prieto-Baena et al.., 2003, calculated the pollen production per inflorescence for several special, obtaining that they may range from 14,500 to more than 22 x 10^6 pollen grains, the amount being clearly higher in the perennial species.

The Poaceae family is well represented in the Iberian Peninsula, with daily airborne pollen concentrations during spring that in extreme or peak days can reach 800 grains/m^3 (Córdoba; Fernández-González et al.., 1999).

Figure 3 Poaceae plants from diverse species common in natural and cultivated grasslands.

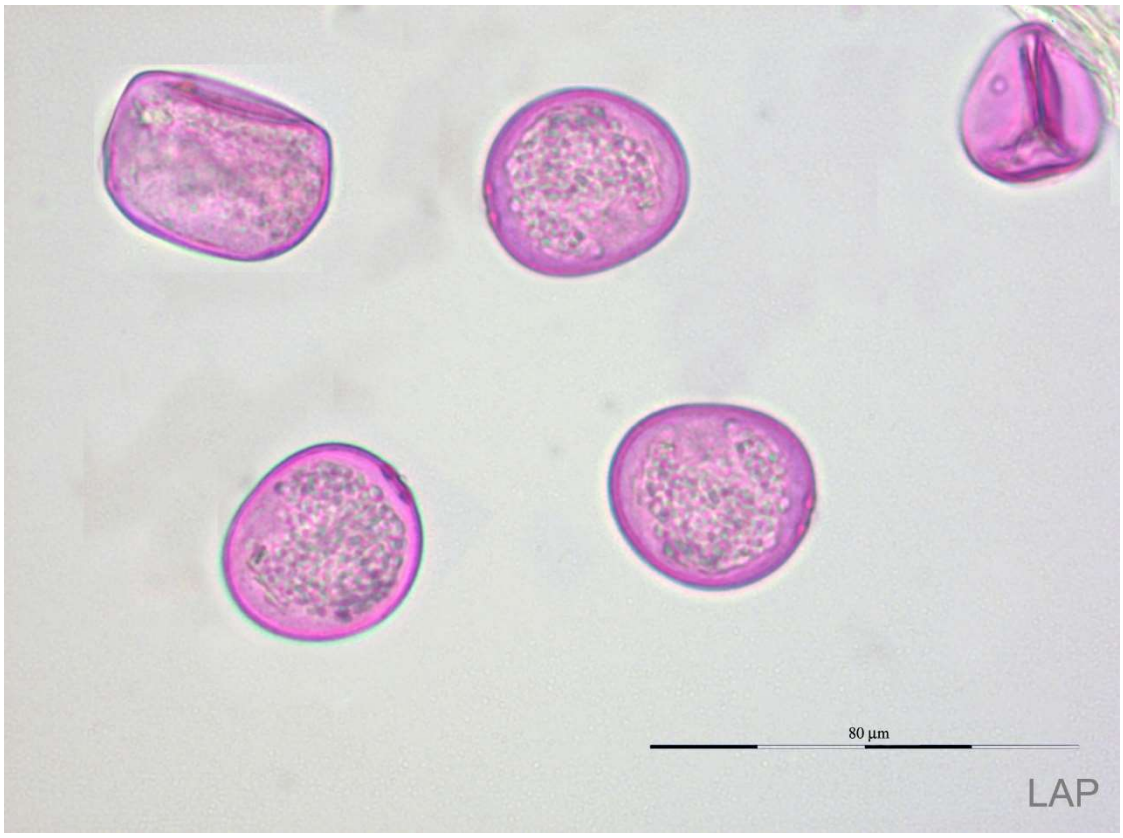


Figure 4. Poaceae pollen grains seen at the light microscope.

1.1.2. Pollen allergens

Pollen allergens are identified as substances (generally glycoproteins) from the pollen grain that trigger an allergic reaction in atopic people. The majority of the pollen allergens are from a limited number of protein families. Their biological functions are presumably related to the recognition, attachment, growth and development of the pollen tube on and within the pistil, i.e. to make the hydrolysis of proteins, polysaccharides, and lipids, to binding of metal ions and lipids, and to the cytoskeleton (Radauer et al.., 2008). Allergen activity can be detected, depending on the specific role of the protein in question, both before and after germination (Alché et al.., 2002; Buters et al.., 2010). These proteins were found in organelles, such as mitochondria and endoplasmatic reticulum , and in polysaccharide particles and starch granules m (Behrendt and Becker, 2001). Allergens are sometimes stored in the ectexine of the pollen wall. Rapid elution and water solubility is considered an important prerequisite for a protein to behave as a major allergen (Grote et al.., 2001; Gupta et al.., 1995).

Pollen releases several substances, such as lipid mediators and larger quantities of adenosine; factors which seem to influence the sensitization reaction of the immune system. Once sensitized, the allergen seems to govern the observed patient symptoms (environmental pollutants influence the extent of the immune reaction too. However, these compounds do not originate from pollen. (Lubitz et al.., 2010),

The Allergome database (http://www.allergome.org) listed 157 completely or partially sequenced pollen allergens (Mari and Riccioli, 2004). Comparison of their sequences with the Pfam protein family database (http://www.sanger.ac.uk/Software/Pfam) revealed that they contain only 29 protein families /(Bateman et al.., 2004) . whereas, the 137 plant food allergens were classified into 27 protein families. Ten of 29 pollen allergen families were also represented among food allergens. The prolamin family, which was the most abundant family of plant food allergens, was ranked 12th (of 29 protein families) among pollen allergens (Radauer and Breiteneder, 2006).

According to Radauer and Breiteneder (2006), about 25% of the allergenic pollen species were grass pollen (Poaceae, 43 species); other abundant families were Cupressaceae (cypress family, 16 species), Betulaceae (birch family, 12 species), Arecaceae (palms, 8 species), Asteraceae (ragweed family, 8 species), and Fabaceae (legumes, 7 species).

1.1.2.1.*Betula* pollen allergens

The order Fagales accounts for eight families, including Betulaceae, which can be divided into Betuloideae (mainly the genera birch and alder) and Coryloideae (mainly the genera hazel, hornbeam, hop-hornbeam, and *Ostryopsis*). The pollens of this Betulaceae family are a frequent cause of allergic rhinitis, mainly in northern and central Europe (Stevens and Davis 2001; Consortium 2010; Egger et al.. 2008).

Table 1 Radauer and Breiteneder (2006). Taxonomic distribution of pollen allergen families

Family	Number of allergens													
	Total	**Expansin C-term**	**Profilin**	**EF hand**	**Expansion N-term**	**Ole e 1-like**	**Pectate lyase**	**Ribonuclease**	**Bet v 1-like**	**Glycohydr 28**	**FAD binding**	**Thaumatin**	**Prolamin**	**Amb v 1**
Cupressaceae	20			1			7			3		6		
Arecaceae	1		1											
Poaceae	71	26	6	2	14	3		8		2	7			
Asteraceae	13		3				2						1	3
Plantaginaceae	1					1								
Oleaceae	12		1	3		4								
Chenopodiaceae	4		1	1		1								
Brassicaceae	8		2	4						1		1		
Betulaceae	13		2	3					5					
Fagaceae	3								3					
Platanaceae	2									1				
Cannabaceae	2		1											
Urticaceae	3		1										2	
Euphorbiaceae	2		2											

C-term, C-terminal domain; N-term, N-terminal domain; Glyco hydr 28, glycosyl hydrolase family 28; FAD binding, flavin adenine dinucleotide binding protein

The allergen of birch pollen belong to the Bet v family. So far, it has been possible to isolate, purify and characterize seven different allergens of *Betula* (available from http://www.allergome.com, accessed Nov 2018). The first one is the most important and significant. Bet v 1 is considered a major allergen since it affects more than 90% of patients sensitized to *Betula* (Moverare et al.., 2002). Bet v 1 was found only inside the pollen grain. So far this allergen has not been found in any plant parts studied with the only exception of one report on the presence of low levels of Bet v 1 in birch leaves (Grote and Fromme, 1986). However, Bet v 1 is a pathogenesis-related molecule (PR-10), Bet v 1 shares a molecular homology with many plants of the Betulaceae family (Jensen-Jarolim 2014). There is molecular homology between the Bet v 1 of Betulaceae pollens and the Bet v 1 homologs of hazelnut (Cor a 1) and of Rosaceae fruits, which include apple, peach, pear, apricot, and cherry. In effect, it has been demonstrated that Mal d 1, the major allergen of apple, shares sequence homology with Bet v 1 (Klinglmayr et al.. 2009). In addition, Mal d 1 may be considered a reliable diagnostic tool for birch pollen allergen–associated apple allergy (Kollmann et al.. 2013). Based on the information of Allergome data set (available from http://www.allergome.com, accessed Nov 2018), actually it is recognized that Bet v 1 shows cross reactivity with several allergens: Act d 11, **Aln g 1**, Api g 1, Ara h 8, Car b 1, **Cas s 1**, Cic i 1, **Cor a 1**, Dau c 1, Dio k 1, Mal d 1, Man i 14kD, **Que a 1.** The ones appearing in bold are airborne pollen allergens.

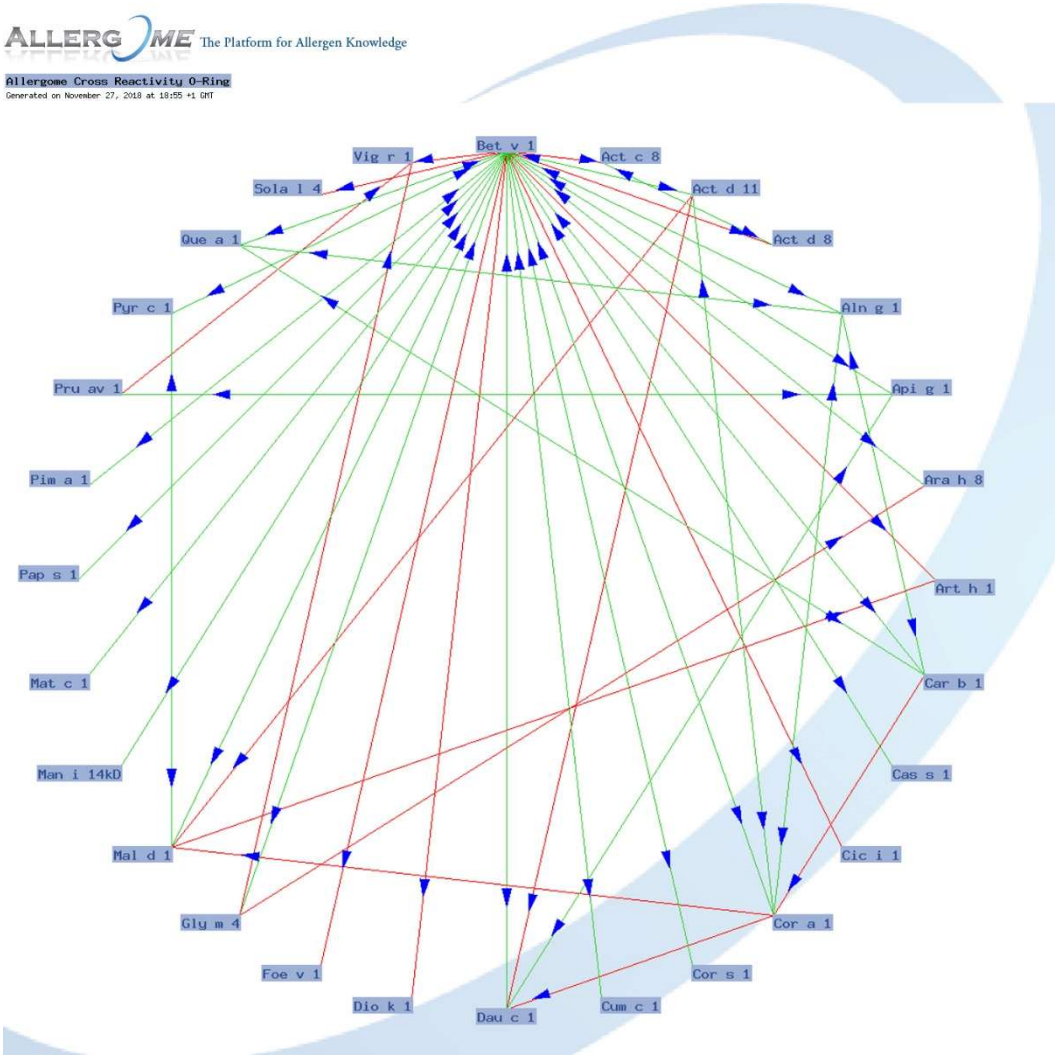


Figure 5 . Bet v 1 cross reactivity obtained of Allergome data set (from http://www.allergome.com, accessed on Nov 2018)

1.1.2.2.Poaceae pollen allergens

Several genera that belong to the subfamily Pooideae are considered to be the main sources of pollinosis, and are potentially the most allergenic. These genera are *Phleum*, *Dactylis* and *Lolium*. Up to 12 subfamilies of allergens have been identified, and the most widely studied belong to the species *Phleum pratense* and *Lolium perenne*. In *P. pratense*, 10 allergens have been identified to date (available from http://www.allergome.com, accessed Nov 2018). Phl p 5, a 29 kDa major allergen from timothy grass pollen, is one of the most reactive members of group 5 allergens of Poaceae family (Maglio et al.., 2002).

Based on the information of Allergome data set (available from http://www.allergome.com, accessed Nov 2018) actually it is recognized that Phl p 5 shows cross reactivity only with Poaceae allergens: Lol p 5, Phl p 6, Poa p 5 (Figure 6).

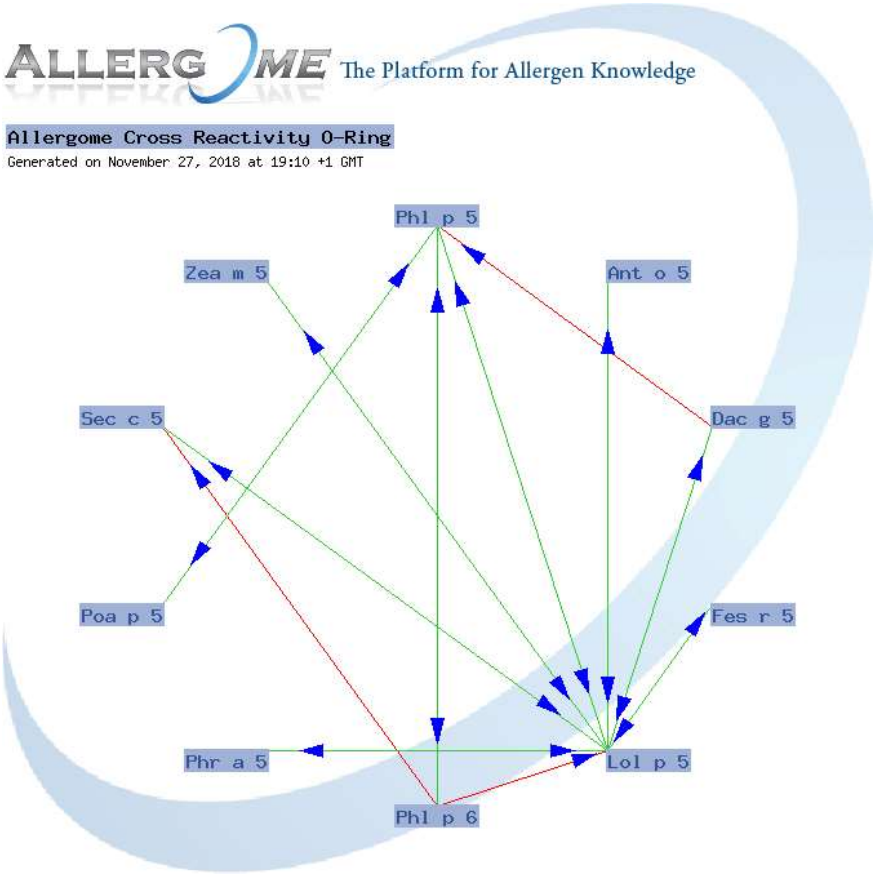


Figure 6 Phl p 5 cross reactivity obtained of Allergome data set (http://www.allergome.com accessed Nov 2018)

1.1.3. Fungal spores

Fungi are eukaryotic, non-chlorophyllous and heterotrophic organisms that live as saprophytes, parasites or symbionts of animals and plants under nearly all environmental conditions. These organisms are reproduced by spores that are sexually produced (zygospores, ascospores or basidiospores) or by asexual via (conidia).

Fungal spores are universal atmospheric components indoors and outdoors and are now generally recognized as important causes of respiratory allergies. Allergic reactions associated with fungi involve the lower respiratory tract more frequently than pollen

allergies do (Lehrer, Aukrust, and Salvaggio 1983). More than 80 genera of fungi have been associated with symptoms of respiratory tract allergy (Gravesen 1979; Burge, Solomon, and Muilenberg 1982; Knutsen et al.. 2012). Aerobiological studies have shown the majority of fungal spores in outdoor air to be from the phyla Ascomycota and Basidiomycota (Horner et al.. 1995). The outdoor spore concentration ranges from 230 x 10^6 spores/m^3 (Cole, 2012; D'Amato and Spieksma, 1995). Atmospheric fungal spore concentration exceeds mean pollen concentration 100–1,000 times (Burge, 1989). Spore concentration in the air varies substantially depending on climatic factors such as temperature, wind and moisture. The majority of the fungal species grow in the outdoor environment. Examples are *Alternaria*, *Cladosporium*, *Epicoccum* and *Ganoderma*.

Alternaria alternata is one of the most frequently encountered species, predominantly occurring in the outdoor environment and the fungal specie more allergenic (Simon-Nobbe et al.., 2008). *Alternaria* is a widespread opportunistic pathogen and ubiquitous saprophytic fungus (Mayser et al.., 2002; Kirk et al.., 2001). Economically, due to being a phytopathogen, causes serious impact on a large variety of crops , e.g. olive, small grain cereals, tobacco, cauliflower, broccoli, pepper, carrot, potato, and fruits like tomato, citrus, melon or apple (Kirk et al.., 2001; Logrieco et al.., 2003; Thomma, 2003). In Spain, *Alternaria* is responsible for important losses, regularly or sporadically, in tomato (Tello, 1999), citrus (Vicent et al.., 2000), olive fruit (Moral et al.., 2008) or potato (Iglesias et al.., 2007). *Alternaria* asexual reproductive structures are conidia, which are non-motile and wind dispersed spores. These conidia are commonly related to respiratory allergic diseases, through the activation of IgE-mediated antibodies producing rhinitis, asthma and atopic dermatitis (D'Amato et al.., 1997; Pereira et al.., 2006; Bartra et al.., 2009) and in food allergies in humans and animals (Barkai-Golan, 2008). *Alternaria* also have clinical significance for the production of toxic secondary metabolites involved in cutaneous, osteomyelitis, pulmonary infections and keratomycosis in humans (Singh et al.., 1990), cancer in mammals (Thomma, 2003), phaeohyphomycotic infection in cats (Miller, 2009) and horses (Genovese et al.., 2001) and mycotoxicoses in farms (Gruber-Schley and Thalmann, 1988), between others.

1.1.4. Fugal allergens

The exact prevalence of fungal allergens has not been established since reports of skin test reactivity to fungi range from 3 to 91%, depending upon the population studied, extracts used, and species tested (Beaumont et al.. 1985; Crameri et al.. 2006; Esch 2004).

The list of fungal allergens officially approved by the Nomenclature Subcommittee of the International Union of Immunological Societies (IUIS; www.allergen.org) spans 105 iso-allergens and variants from 25 fungal species belonging to the Ascomycota and Basidiomycota phyla (Agarwal and Gupta 2011). However, the number of fungal proteins able to elicit type I hypersensitivity reactions described in the literature is much longer, even if many of these allergens are poorly characterized. A recent catalogue of the fungal allergens described (Simon-Nobbe et al.. 2008) lists 174 allergens for the genus of the Ascomycota and 30 for the genus of the Basidiomycota. However, this list does not include many fungal allergens, which have been only partially characterized in terms of primary sequence.

14 *Alternaria* allergens have already been identified so far. The major allergen is Alt a 1, identified as a protein formed by a two-chain dimer linked by disulfide bonds with a molecular weight of approximately 30 kDa but the biological function of this protein has not been elucidated. (Vougue et al.., 1998).

1.2 Airborne particle dispersion.

Pollen dispersion in the atmosphere starts with the pollen release from the anthers. In many anemophilous trees, the anther's burst is a result of dehydration due to high temperature, solar exposure, low humidity and moderate wind (Cresti and Linskens, 2000; Helbig et al.., 2004). In other plants, floral parts, such as the filaments in grasses actively push the anthers into an exposed position, and anther's dehiscence may be a result of passive desiccation or active reabsorption (Kazlauskas et al.., 2006; Wahl and Puls, 1989).

Once the particles are released into the atmosphere, the spatial and temporal evolution they follow is determined to a large extent by meteorological factors, as well as by the presence / absence of obstacles (orography, valleys, oceans, civil works, etc.) that could facilitate or interfere with its dispersion. The atmospheric currents facilitate the displacement in height, while a very pronounced topography will limit its longitudinal dispersion. The pollen records from aerobiological monitoring sites have traditionally been interpreted as if the grains always originate from the local environment. But this perception is changing to the understand of the broader atmospheric particles movements. The recurrent evidences of pollen and spore long distance transport, including continental (Belmonte et al.. 2000; Belmonte et al.. 2008; Sofiev et al.. 2006; Siljamo, Sofiev, and Ranta 2007; Siljamo et al.. 2008; Skjøth et al.. 2009) and intercontinental scales (Prospero et al.. 2005; Kellogg and Griffin 2006; Rousseau et al.. 2008).

The large-scale dispersion of atmospheric constituents is controlled by synoptic-, continental-, or hemispheric- scale meteorological phenomena. In particular, it means that even combined observations at both source and receptor points cannot describe the transport conditions. In fact, in many cases the mere connection between the sources and receptors is difficult to establish due to complicated large-scale dispersion patterns. Under such constraints, the indication of the long range transport (LRT) can be either foreign pollen grains, which cannot be produced locally, or “wrong” time of appearance of the grains, which is significantly outside the local flowering period. (Nichols 1967; Ritchie and Lichti-Federovich 1967; Rousseau et al.. 2006; Porsbjerg, Rasmussen, and Backer 2003).

The life span of the particles in the atmosphere strongly depends on factors such as: deposition intensity, transformation and interaction with chemicals. Attending to the cause of the end of the stay of the particles in the atmosphere, the phenomenon receives the name "sedimentation" if it happens by phenomena of gravity, affecting mainly to the particles of great size and / or weight. The name is "Impaction" when they hit an obstacle, mainly affecting medium-sized particles, such as pollen that reaches the upper respiratory tract. The name is "diffusion" when these can be inhaled and exhaled repeatedly before deposition in bronchi and bronchioles; this type of phenomenon occurring in particles of very small size (less than 3 μm) that have low fall speed and reduced inertia.

Aerobiological processes are defined as follows: 1-emission, 2-dispersion, 3-transport and 4-deposition. Any change in these factors may affect the presence airborne biological particles.

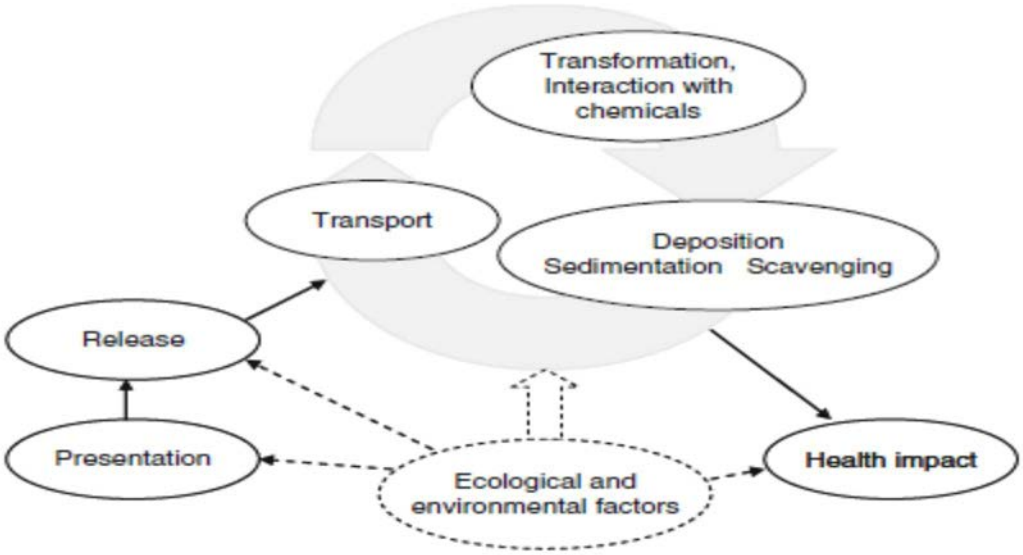


Figure 7. Phases of aerobiological processes related to transport of chemically inactive biogenic aerosol. Extended from *(Isard et al.., 2005)*.

Similar to the processes that ensure the presence of pollen in the air occur for the presence of fungal spores, and pollen and spores allergens.

1.3 Airborne particles concepts and parameters

Aerobiology is an interdisciplinary science where not only are studied the airborne biological particles but also are involved other sciences such as physics, mathematics, chemistry, etc. This science is rather recent, beginning by mid-20th Century, and with time and with growing number of aerobiologists at work, standardization of methodologies and terminology have been needed.

Recently, the European Aerobiology Society (EAS) and the International Association for Aerobiology (IAA) have published an article with the recommended terminology for aerobiological studies (Galan et al.., 2017). In this thesis, it is used this terminology

and, in the case we need a new term, it is always proposed following the logics of this paper and is indicated in the description.

Main concepts used related to pollen, spores and allergens are:

Daily concentration defined as the amount of pollen/spore/allergen per unit volume of air obtained for the whole day and expressed as pollen/m^3 , spore/m^3or ng/m^3.

Peak pollen/spore/allergen day (new term) defined as the day of the year (date) with the maximum pollen/spore/allergen daily concentration.

Annual Pollen/Spore Integral (APIn/ASIn) expressed as Pollen *day/m^3/ Spore *day/m^3. They are obtained by summing the average daily concentration over the year.

Annual Allergen Integral (AAIn) (new term) expressed as ng *day/m^3. It is obtained by summing the average daily concentration over the year.

Monthly Pollen/Spore/Allergen Integral (MPIn/MSIn/MAIn) (new term) defined as the total sum of the average daily concentration over the month. They are expressed as pollen/m^3, spore/m^3 or ng/m^3.

Pollen/Allergen presence (new term). This terminology is used in this thesis to determine the period when pollen/allergen is present in the air and takes into account the days with pollen/allergen measured plus the day before and the day after.

1.4 Atmospheric chemical and Physical interactions

At all spatial and temporal scales, pollen, spores and, if present in a free form in the air, allergens are subjected to chemical and physical interactions with other atmospheric constituents. Chemical pollution can stress both the pollinating plants and the pollen grain, which cause qualitative and quantitative changes of the pollen. The impact starts already during the pollen formation. For example, Aina et al.. (2010) found an increased amount of the allergenic proteins in the grass *Poa annua* if the plant is grown in soil contaminated with cadmium (Aina et al.., 2010). Similarly, the pollen vitality of *Parietaria judaica* was found maximal in soils enriched with heavy metals (Fotiou et al.., 2011). Processes taking place in the air during pollen transport are very poorly studied and one can only guess the type and intensity of the involved reactions. In an attempt to classify these processes, one can consider the physical transformations

(rupture of grains, phase transformations of allergen particles, coagulation with other aerosols, etc.); chemical transformations (oxidation and nitration), and biological transformations of the particles (loss of viability, germination). Some of these processes can lead to substantial changes in the atmospheric features of the particles affecting their life time and dispersion features, and some are of importance from medical and biological points of view.

1.4.1 Temperature

Temperature is the primary determinant of the metabolic rate of plants, and therefore it is an important determinant of phenology including that of allergenic plants (Linderholm, 2006).

Spieksma et al.. (1995b) examined atmospheric birch (*Betula*) pollen data from five European stations (Basel, Vienna, London, Leiden, and Stockholm) between 1961 and 1993 (records from 18 to 30 years). They found weakly rising trends of annual sums of daily concentrations at all stations. More recent European studies, in Denmark (Rasmussen, 2002) and Switzerland (Frei, 1998) found trends of increasing amounts of pollen over the latter decades of the 1900s that were related to climate change. Teranishi et al..(2000) studied the association between Japanese cedar (*Cryptomeria japonica*) pollen and temperature from 1983 to 1998 in Japan. They found a significant positive correlation between total pollen count in a year and temperature in July the previous year. In North America, Levetin (2001) has found statistically significant increases in a number of taxa, including *Juniperus* , *Quercus* , *Carya* , and *Betula* , since 1987. There is some evidence that these increases have been associated with increases in average winter temperature.

1.4.2 Precipitation

The precipitation influences the different phases of aerobiological processes, although the intensity of this will depend on the phenological state of the plants, the time of day, or the rain intensity. The precipitations recorded in phases prior to the pollen season intensify the pollen concentrations. During the release or dispersion stages, they block the release and exert a mechanical action on the airborne particles precipitating them to the ground, being the most effective atmospheric washing in spring-summer (Belmonte & Roure, 1985, Fornaciari et al.., 1997; Recio et al.., 1997). Soft and prolonged

precipitation is more effective than intense and short-lasting rainfall (Iglesias et al.., 1993).

1.4.3 Thunderstorms

Thunderstorms have been linked to asthma epidemics, especially during the pollen seasons. Grass pollen was reported by (D'Amato et al.., 2007; Suphioglu, 1998) as responsible for thunderstorm-associated asthma epidemics in 1987/1989 Melbourne and 1994 London. D'Amato, et al.. (2007) found out that a 50-fold increase in atmospheric concentration of Lol p 5 after rainfall. Taylor et al.. (2007) also suggested that thunderstorm asthma epidemics may be triggered by grass pollen rupture in the atmosphere and the entrainment of respirable-sized particles in the out flows of air masses at ground level.

Vaidyanathan et al.. (2006) reported that thunderstorms are responsible for a quicker release of pollen allergens than in normal rain or dry conditions exploring the effects of electric fields on fresh pollen grains collected from Bermuda grass flowers. Pollen did not rupture within 1 h of contact with water. Only after exposure to an electric field, they ruptured instantly upon immersion in water. The higher the voltage, the higher the percentage of rupture of the pollen. Electric fields, generated in the laboratory and of magnitude found during thunderstorms, affected the pollen after as little as a 5 seconds exposure.

1.4.4 Relative Humidity

This factor favors the development of the plant and facilitates flowering, but an increase above a specific threshold prevents the dehiscence of the anthers and decreases the levels of pollen in the air.

In general, when the humidity of the air decreases, the walls of the anther dehydrate, which favors its breaking and the exit of the pollen grains. The environmental dryness favors the dispersion of pollen grains, while its ability to absorb moisture, a phenomenon known as "harmomegatia", causes them to become heavier and sediment (Emberlin, 1994).

1.4.5 Wind

The wind is a basic factor for the particles to become airborne, be transported away from their source of emission and be depositing later in more or less remote places, however the study of the relationship between both parameters and interpretation through mathematical analysis is quite complex. To study this variable one must take into account direction and speed (Plaza 2017).

The wind direction has a clear influence on the pollen composition collected in the aerobiological samples. A plant community located in the course of the dominant wind will cause a pollen cloud composed of particles coming from it. The taxa that are not in the course of the wind will be underrepresented as long as it dominates a specific direction (Plaza 2017).

The speed, if strong, produces a dilution of the pollen content, so the collection efficiency is reduced, but if it is excessively low, it does not balance the anthers and prevents them from releasing the pollen, so the pollen dispersion slows down (Bricchi et al.., 1992).

On the other hand, the wind favors the reflotation of already settled pollen, incorporating into the atmosphere pollen types that have already completed their pollination period, which in certain occasions translates into a very long post-op period (Plaza 2017).

1.4.6 Insolation

Insolation is related to temperature and photoperiod. The extension of the photoperiod is intimately associated with the end of plant dormancy and with the activation of the synthesis of growth hormones (gibberellins and cytokinins) that regulate the onset of flowering. The increase in insolation favors the presence of airborne pollen, since it produces a dehydration of the tissues, facilitating dehiscence (Galán et al.., 1995). In addition, Molina et al.., (1996) indicate that the pollen production, number of inflorescences and flowers is proportional to the vegetal cover and solar radiation trapped by each taxon.

1.4.7 Atmospheric Pressure

Although only few studies on the importance of atmospheric pressure have been carried out, the effects of pressure on the threshold of pollen/spores levels can be very diverse.. The role of pressure is revealed in long-distance pollen transport. In connection with the NAO (North Atlantic oscillation) studies it has been shown that pressure is a much more

important factor causing changes in pollen concentrations than has been realized thus far (Smith et al.. 2009). Emberlin (1994) suggested that an increase in the strength of the Atlantic westerlies over north-west Europe would enhance the long-distance transport of pollen from northern and central Europe to Scandinavia. Although the role of climatic factors in exacerbating asthma is poorly understood (D' Amato et al.. 2001), high and low atmospheric pressures have been linked to asthma attacks (Garty et al.. 1998; Celenza, Fothergill, and Kupef 1997). There is evidence that changes in temperature, barometric pressure and relative humidity have some influence on the worsening of asthmatic symptoms (Hashimoto et al.. 2004). The variations in atmospheric pressure can cause remarkable error in the function of pollen trap; for instance, in Switzerland at an altitude of about 2,300 m at Gütsch where the mean air pressure is 770 hPa a 10% error compared to sampling in Basel where the air pressure is 979 hPa on an average (Gehrig and Peeters 2000).

1.4.8 CO_2

Higher CO_2 concentrations stimulate photosynthesis in many plants especially when enough nutrients such as nitrogen are available (Bazzaz, 1990). Therefore, increasing temperature and high CO_2 may affect the pollen load in a region.

Rising CO_2 concentrations may affect the amount of pollen produced per plant as well as the allergenic content of the pollen grains itself. Greenhouse experiments with ragweed plants showed that plants exposed to elevated levels of CO_2 increased their ragweed pollen production (Ziska and Caulfield, 2000) as well as the Amb a 1 content per pollen grain (Singer et al.., 2005).

1.4.9 Ozone

Exposure to ground O_3 causes not only increased risk of asthma exacerbation (Chen et al.. 2004; Guarnieri and Balmes 2014; Hernandez et al.. 2010) but also, has a priming effect on allergen induced responses as well as an intrinsic inflammatory effect in the airways of allergic asthmatics (Cakmak et al.., 2012; Cakmak et al.., 2012; Kehrl et al.., 1999). O_3 is thought to be lowering the threshold concentration of allergen able to induce symptoms; O_3 can enhance the airway responsiveness. Molfino et al.. (1991) reported that a 1-h exposure to 0.12 ppm O_3 while at rest caused a two-fold reduction in the

provocation concentration of inhaled antigen required to cause early bronchoconstriction in specifically sensitized asthmatic subjects.

The previous exposure to O_3 (and NO_2) on subsequent pollen allergen significantly increases the allergen-induced release of eosinophil cationic protein in nasal lavage of patients with seasonal allergic rhinitis (Devalia et al.., 1998). Rogerieux et al.. (2007) exposed timothy grass pollen to ozone (O_3), nitrogen dioxide (NO_2) and Sulphur dioxide (SO_2) alone or in combination.

1.4.10 Nitrogen Dioxide (NO_2) and Sulphur Dioxide (SO_2)

NO_2 is less oxidative than O_3 and thus the inhalation of NO_2 is usually not associated with significant changes in bronchial function of asthmatic patients (L. Jack Roger et al.., 1990). But combined inhalation of both NO_2 and allergens simultaneously was demonstrated to induce allergic bronchoconstriction (Tunnicliffe et al.., 1994).

SO_2 is generated primarily from burning of Sulphur-containing fossil fuel. It has been shown to induce bronchial obstruction very rapidly (within 2–5 min) in asthmatic patients at low concentrations (Li et al.., 2007; Park et al.., 2001; Riedel et al.., 1988). O_3, NO_2 and SO_2 have been found to interact with allergenic pollen in the ambient air to aggravate symptoms of asthma (Feo Brito et al.., 2007; Puc, 2011).

Rogerieux et al.. (2007) showed that exposure of grass pollen to NO_2 and SO_2 induced a decrease of grass allergen recognition by patients' sera. This decrease could be due to a mechanical loss of allergens from the altered pollen grains and/or post-translational modifications affecting allergen recognition by IgE.

1.4.11 Particulate Matter

Sources of particulate matter can be man-made or natural. Some particulates occur naturally, originating from volcanoes, dust storms, wild fires, living vegetation, and sea spray. Human activities, such as the burning of transportation, power plants and industry. Fine particles in the air are linked to health hazards such as heart disease, altered lung function and lung cancer (Fernvik et al.., 2002; Saunders et al.., 2010).

Epidemiological studies have shown that the manifestations of asthma are increased by air pollution in individuals who are already affected, e.g., the interaction between

ambient PM 10 and pollen in studies of short-term effects (Lierl and Hornung 2003; Feo Brito et al.. 2007; Skolnick 2004).

The investigation of the potential role of traffic particulate matter (TPM) or pure carbon core particles in the initiation and persistence of experimental allergic inflammation, show highlight the importance of the exposure to a combination of particulate matters and pollen allergens (Fernvik et al.. 2002).

2. Airborne sampling methodologies

2.1. Monitoring instruments for pollen, spores and allergens

The different sampling methods that have been developed in the field of Aerobiology are based on physical principles, such as impaction, suction, filtration, electrostatic precipitation or precipitation and gravitational deposition (Mandrioli et al.., 1998)..Each of the samplers presents a series of characteristics that make it specific when choosing the most appropriate method for the type of particles and analysis that one wants to perform. The collection methods must satisfy the objectives of the sampling program, be efficient in capturing particles and compatible with the required counts and analytical methods. At present, there is no effective prototype with a broad spectrum of airborne particles and specific analyzes, so each plot of Aerobiology has developed its own sampling methods (Mandrioli et al.., 1998).

2.1.1. Gravitational deposition methods

They are the simplest methods and are based on the existence of a horizontal surface on which the particles are deposited by the effect of gravity, being retained on the surface thanks to an adhesive. The efficiency of this type of collector varies very significantly depending on the size of the particles, speed and direction of the wind or atmospheric turbulence, and even with the concentrations in air. Another limitation is the inability to calculate the volume of air sampled so that the data cannot be expressed per unit volume (Lacey et al.., 1996). Among the gravitational deposition sensors are the Durham and the Tauber.

2.1.2. Impact methods

If the wind speed is greater than the specific gravitational force of fall for each particle, they will travel along a generally horizontal path. When a particle approaches an obstacle, the particle will hit the obstacle and remain impacted. The capture efficiency of a sampler of this type will depend on wind speed, morphological characteristics of the particle and size of the collector surface (Mandrioli et al.., 1998).

The most commonly used impaction samplers are Rotorod and Rotoslide. These have a good performance to sample pollens but their main disadvantage is that they cannot act for prolonged periods of time, so they usually operate intermittently (Mandrioly et al.., 1998).

2.1.3. Filtration methods

The air passes through a porous surface where the particles, depending on their size, are retained. Among these sensors is the McLeod modified later by Seoane and Suarez (1983) called the "airplane pickup" ("CAP"), and the devise developed by Cour (Cour, 1974). In the Cour pollen trap the filtering surface is a 20 x 20 cm surface prepared with 5 layers of hydrophilic gauze impregnated with silicone oil. Both traps counted with a vain to make the capturing surface always oriented to the dominant wind. The main drawback in these methods is that the efficiency varies with the wind speed and that it is not possible to know the daily variation of the pollen concentrations (Mandrioli et al.., 1998) unless the periodicity of changing the capture surfaces is changed to daily.

2.1.4. Methods of electrostatic and thermal precipitation

Both are often used to catch small particles. While in the electrostatic method trapped particles are electrically charged and attracted to an opposite charge electrode, in the thermal method the air flows into the collector and the particles are conducted from a hot surface to a cold one (Falagiani, 1989).

2.1.5. Liquid intrusion method

Liquid intrusion methods work by introducing air into a container of water. When the air rises in the form of bubbles, the particles are transferred to the liquid medium and retained in it (Cage et al.., 1996).

2.1.6. Suction methods

These methods are based on the absorption of the air where the particles to be sampled are contained by means of a vacuum pump. To retain these particles, several methods can be used, such as filtration, impaction, thermal and electrostatic precipitation and liquid intrusion. Among the suction sensors is the "Spore trap" by Hirst (1952), which was specially designed to collect fungal spores per unit of time and volume. Currently, there are models manufactured by Lanzoni srl (Lanzoni VPPS 2000, VPPS 2010 between the most used) and Burkard Manufacturing Co. Ltd. (Burkard 7-days recording between the most used). In these pollen and spore traps the particles penetrate through a hole and are deposited by impact on the surface of a transparent plastic tape covered with adhesive material that is moving at a speed of 2mm/h thanks to a clock mechanism. Among the advantages of these traps, we can emphasize its simplicity and that they allow to have a continuous record of the atmospheric content that can be analyzed in a daily and even hourly basis.

The detection of airborne allergens cannot be realized through the previous pollen sampling techniques. Therefore, other methods were developed for allergen sampling. The most common samplers used for the capture of allergenic particles are: Andersen (Andersen 1958), Burkard Cyclone (Emberlin and Baboonian 1995), Coriolis® Delta (Carvalho et al.. 2008), Chemvol ® (Demokritou et al.. 2002).

2.2 Pollen and spores counting methods

Pollen/spores identification requires knowledge of basic palynology (morphology), and it is conducted based on a comparison with reference microscopic slides or by using pollen/spores identification keys and atlases. Pollen grains and fungal spores are commonly analyzed under light microscope. The magnification is chosen so that pollen can be safely identified according to the characteristics specific for each taxon.

Because the pollen sampler of the Hirst type is the one used in this thesis, the methods of quantification will be described in the material and methods section.

In recent literature, several different methods for automated pollen detection have been described:

1. Systems that make use of multifocal optical microscopic images of air samples collected by a conventional Hirst-type pollen sampler. A first step in automated counting of the pollen is the discrimination of the pollen grains from other airborne material in the images (Bonton et al.., 2001; Landsmeer et al.., 2009). For subsequent identification of the pollen grain, several characteristic pollen features, including shape, statistical grey-level and specific pore/colpus features are extracted from the images by pattern recognition software tools (Boucher et al.., 2002; Chen et al.., 2006). These methods report various levels of success in identification of specific pollen types: 77% in samples from airborne pollen (Boucher et al.., 2002) or 97.2% in samples containing three allergenic pollen taxa (Chen et al.., 2006).

2. A fully integrated pollen sampling system that automatically collects, prepares and records by making use of a conventional light microscope (Ronneberger, 2007). The method developed for the recognition of the pollen employs digitized images, using the grey-value of each pixel (Ronneberger et al.., 2002). This system reached a recognition rate in “real world” samples of 84.3% (Ronneberger, 2007). Up until now, it was not developed beyond a stage of a prototype and it did not reach the stage of becoming commercially available.

3. Other systems do not make use of digitized images of pollen, but are based on the technology of particle counters by laser light.

- In the system described by Kawashima et al.. (Kawashima et al.., 2007) , pollen is characterized by the sideways and forward scattering of laser light. Air, containing the airborne particles, is passed through the optical system and irradiated by a laser beam. The scattering of light signals caused by the pollen grains is recorded in real time and processed by a computer. During a sampling period in late summer, pollen from nettle (*Urticaceae)*, ragweed and grass (*Poaceae)* could be separated well by different scattering patterns. For other European pollen taxa, the system has not been tested yet.
- In Japan, another real-time airborne pollen counter was developed by the company Kowa. The technology is based on a laser particle counter and on the characteristic distribution of pollen on the scattered diagram according to the

grain size versus the fluorescent hue. In Japan, this counter is used by the Tokyo pollen information network systems (Suzuki et al.., 2008).

- Recently, a new methodology was presented on the 9th International Congress of aerobiology: the WIBS 4 (Wide-Issue Bioaerosol Spectrometer). This instrument combined information from laser light scattering with 2D-spectroscopic measurements. The instrument was successfully used in an area with a low diversity of pollen (Sodeau and O'Connor, 2016).
- In the recent years, a new sampler and analyzer pollen monitoring method based on optical based on time-resolved scattering and fluorescence is being developed. Some results have already been obtained in Switzerland (Crouzy et al.., 2016) and the development goes on incorporating new sampling sites.

4. Another method is based on the Coulter counting principle (Zhang et al.., 2005). Pollen was suspended in a KCl aqueous suspension and passed through a microchannel. The changes in conductance, due to the passing of the pollen, were recorded and analyzed. In this system juniper (*Juniperus)* and grass pollen could be discriminated.

2.3 Allergens detection and quantification techniques

The great specificity that the antibodies possess and the ability of them to recognize an almost unlimited number of molecules, have led to the development of a multitude of immunological tests in the field of Immunology. These tests differ in their speed and sensitivity, being some of them strictly qualitative and others quantitative. In Table 2, the most commonly used and recognized techniques in Immunology are presented as a summary.

There are other techniques for detection of airborne allergens that combine immunological methods with different technologies that are listed below.

Electron microscope was also used to analyze diesel exhaust particles bound with airborne allergen using immunogold labelling with specific monoclonal antibodies and a high voltage transmission electron -microscopic imaging technique (Knox et al.. 1997)

Table 2. Comparative table on the most used immunological techniques. *the sensitivity depends both on the affinity of the antibody or on the density and distribution of the epitope (De Linares, 2007).

Test	**Sensitivity* (µg Ac/ml)**	**Technique**	**Applications**	**Method of analysis**	Features and applications
Fluid precipitation reaction	3-30	Quantitative	Experimental	Tubes with liquid media	
Precipitation reactions on gel					
Radial immunodiffusion (Manzini method)	0,2-1	Quantitative	Clinical	Variable gels with antibody	**Compete serum components**
Double immunodiffusion (Ouchterlony method)	3-20	Quantitative		Agar gel	**Free antigens in complex ratios**
Inmunoelectrophoresis	3-20	Quantitative	Clinical	Agar gel	**Serum proteins immuno-deficiency diseases)**
Agglutination reactions					
Direct	0,05		Clinical	Hematology	**Bacterial infections**
Passive agglutination	0,001-0,01		Clinical		
Inhibited agglutination	0,001-0,01	Quantitative	Clinical	Urine	**Drug usage and viral exposure)**
Radioinmunoessay (RIA)	0,0001-0,001	Quantitative	Clinical	Solid phase (tubes or plates)	**Insulin levels in diabetic and hepatic patients**
Test Radioalergosorbent (RAST)		Quantitative	Clinical	Cellulose disk	**Skin prick test substitute for aeroallergens**
Enzyme linked immunoessay (ELISA)	0,0000-0,001	Quantitative /qualitative	Clinical	Solid phase	**Allergenic activity in the atmosphere**
Inmunotransference (Western Blot)		Quantitative /qualitative	Clinical	Polyacrylamide gel	**Allergen detections in animals and plants**
Inmunofluorescence	**1**	**Quantitative /qualitative**	**Clinical**	**Fluorescent staining: fluorescing / rhodamine**	**Autoimmune diseases**

Real time QCM based immunosensor. Potential trial of real-time monitoring of airborne allergen using quartz crystal microbalance (QCM) technology. Monoclonal antibodies of cat allergen (Fel d 1) were immobilized onto a gold-coated quartz crystal using a cysteamine self-assembled monolayer (SAM). The trial resulted in antigen–antibody binding despite interference from dust particles (Morris et al.. 2014).

qPCR. Mohanty et al.. (2017) proposed that molecular techniques provide an alternative approach that is less labor intensive and enable identification of any species by its genetic fingerprint. by using quantitative polymerase chain reaction (qPCR) to evaluate pollen concentrations in air samples. The author concludes that the qPCR method is more accurate and sensitive than current pollen monitoring techniques and, therefore, has the potential to be used in various pollen monitoring stations (Mohanty, Buchheim, and Levetin 2017; J. S. West et al.. 2008)

3. Pollen databases

The different research groups working on aerobiology have developed the own pollen and spore databases through the time and they have also accorded to establish wider networks and common and shared databases. Also, the incorporation of computer science in the field of Aerobiology has allowed the quicker diffusion of the aerobiological information for the medical and the public health proposals. Regional, national and international Aerobiology associations and networks, as well as the different control stations, have Web pages where pollen or fungal data can be consulted, as well as information related to allergic processes. Some of the most important websites are:

Generals sites Spain scope

- REA [Red Española de Aerobiología]
  http://www.uco.es/rea/
- Comité de Aerobiología de la SEAIC [Sociedad Española de Alergología e Inmunología Clínica]
  https://www.polenes.com/
  http://www.seaic.org/

Other Spanish sites at a regional level

- AeroUEx - Información Aerobiológica en Extremadura
  http://www.aerouex.es/
- Aerobiologia de Andalucia Oriental
  http://www.ugr.es/~aerobio/
- Aerobiología Costa del Sol - Estaciones de la Universidad de Málaga
  http://webdeptos.uma.es/biolveg/02Aer/00HAer/01Aer.html
- Aerobiología en Andalucía Central - Grupo de Aerobiología de la Universidad de Córdoba
  http://www.uco.es/aerobiologia/
- Informació del Pol·len - Ajuntament de València
  http://www.valencia.es/polen
- Información polínica de Galicia
  http://www.sergas.es/Saude-publica/Informacion-polinica
- Portal de Sanidad de la Junta de Castilla y León
  http://www.sanidad.jcyl.es/polen
- Red Andaluza de Aerobiología (RAA)
  http://www.uco.es/raa/
- Red Palinológica de la Comunidad de Madrid
  http://www.madrid.org/polen/
- Xarxa Aerobiològica de Catalunya and Punt d'Informació Aerobiològica
- http://lap.uab.cat/aerobiologia
- Xarxa Balear d'Aerobiologia
  http://www.caib.es/sacmicrofront/contenido.do?idsite=297&lang=CA&cont=7198

Andorran site

- Xarxa pol·línica - Qualitat de l'aire a Andorra
  http://www.aire.ad/pollen.php

Generals sites Europe scope

- Polleninfo.org
  http://www.polleninfo.org/

Other international sites

- AFEDA [Association Française d'Etude des Ambroisies] (France)
  http://afeda.pagespro-orange.fr/
- AIA [Associazione Italiana di Aerobiologia] (Italy)
  http://www.ilpolline.it/
- Aerobiology International [The International Portal on Aerobiology]
  http://www.isac.cnr.it/aerobio/ai/index.shtml
- EAN [European Aeroallergen Network]
  https://ean.polleninfo.eu/Ean/
- ESA [European Aerobiology Society]
  http://www.eas-aerobiology.eu/
- IAA [International Association for Aerobiology]
  https://sites.google.com/site/aerobiologyinternational/
- NPARU [National Pollen and Aerobiology Research Unit]. University of Worcester (UK)
  http://www.pollenuk.co.uk/
- National Allergy Bureau
  http://pollen.aaaai.org/nab/
- PAAA [Pan-American Aerobiology Association]
  http://www.paaa.org/
- Palynologiska laboratoriet (Sweden)
  http://www.nrm.se/forskningochsamlingar/miljoforskningochovervakning/palynologiskalaboratoriet.7000.html
- RNSA [Réseau National de Surveillance Aérobiologique] (France)
  http://www.pollens.fr/
- Rede Portuguesa de Aerogiologia (Portugal)
  http://www.rpaerobiologia.com
- Witamy w Serwisie Alergologicznym (Poland)
  http://www.alergen.info.pl/

II. Justification, Hypothesis and Objectives

The evaluation of air quality is currently one of the lines of research that is being promoted in the field of sciences dealing with environmental quality, especially in relation to anthropogenic pollutants (nitrogen dioxide, sulfur dioxide, carbon monoxide, suspended particles and ozone) since they are one of the major triggers of asthmatic diseases in industrialized countries (D'Amato et al.. 2001). However, even though it has been established that a quarter of the total atmospheric solid particles have a biological origin (Knox 1993), there are few studies that relate air quality to pollen grains, spores, viruses or bacteria.

The aerobiological studies that are currently being carried out indicate that pollen from anemophilic plants is the main biological pollutant in the air (Baeza Ocariz et al.. 2002). This phenomenon is triggering the emergence of pollen allergies and other diseases of the respiratory system that, according to the World Health Organization, are classified among the six most common diseases internationally (WHO 2007).

Among the pollen grains and spores cataloged as potentially dangerous for health Poaceae pollen type is considered as the most harmful pollen in the world, affecting 80% of people allergic to pollen in Europe (D'Amato et al.., 2007). The Phl p 5 allergen is responsible of 88.4% of allergic cases in Europe (Heinzerling et al.. 2009) as well as in other parts of the world (Freidhoff et al.. 1986). On the other hand, Birch pollen is a major cause of allergic reactions in the Northern Hemisphere (Jarolim et al.. 1989) since these airborne pollen grains play a role in exacerbations of seasonal asthma (Frei 1999; Fanta and Christopher 2002). Birch pollen provokes symptoms in 10-20% of allergic patients in North European countries (Haahtela 1979, and Bousquet et al.. 2007) and 96% of all tree-pollen allergic patients possess specific IgE to Bet v 1 and 60% show reaction to this allergen (Jarolim et al.. 1989).

As showed by Simon-Nobbe et al.. (2008), the incidence of fungal spores is worldwide, being *Alternaria* the most important with an incidence of 4 to 40% of atopic patients. *A. alternata* is considered as one of the main source of Alt a 1 that is reported as a risk factor for developing asthma (Feo Brito et al.. 2012). Asthma caused by *A. alternata*

allergy is characterized by symptoms that are more persistent, greater disease severity, and a higher risk of fatal outcome.

Despite that pollen and fungi and allergens are well reported to be of impact to human health, the dynamics of these particles (specially of allergens) in the atmosphere and their interaction with meteorological and anthropogenic factors are not yet well reported. Some studies have shown airborne allergen dynamics during the main pollen or spore season, but very few have provided their dynamic along a whole year and through years.

The hypothesis of this thesis memory is to show the existence of seasonal allergenic activity in the atmosphere of Bellaterra (Barcelona) and Vitoria-Gasteiz, two cities of Northern Spain, in relation to the two important allergenic pollen types Poaceae and *Betula*, and to show that there is a parallel dynamic between the concentrations of pollen and allergenic proteins in the air.

The aim of this study is to detect, to measure and understand the origin of such allergens in the atmosphere and to map their daily and annual dynamics in Bellaterra (Barcelona) and Vitoria-Gasteiz, and to observe their correlation with other relevant atmospheric factors such as meteorological parameters and particulate matter. With it the present thesis aims to bridge the gap and to provide a complete annual result of the chosen allergens for more than one year in the same location of study and to compare it between the two localities.

In addition, the interest of this thesis is also on airborne fungal allergens that are not enough studied up to now. Due to their ubiquitous nature and the lack of information on the release mechanism, there are very few studies reporting reliable methodologies of sampling and analysing Alt a 1, the major allergen of *Alternaria*, in the atmosphere. This thesis investigates several sampling methods and several analysis methods in order to reach a concrete result and a better understanding of the atmospheric dynamics of this fungal allergen and its correlation with the airborne fungal spores concentration. For this, it is evaluated the adequacy of the methodology of the samplers and the immunological technique for the detection of airborne fungal allergenic particles for to determine which methodology offers the best results quickly and effectively.

The specific objectives are the following:

1) To study the temporal behavior (with a daily basis) of two pollen types (Poaceae and *Betula*) and their main allergens (Phl p 5 and Bet v 1, respectively). For this, it is realized:
    - A study of the aerodynamics of Poaceae pollen and Phl p 5 in Bellaterra (Barcelona) for the years 2013 and 2015.
    - A study of the aerodynamics of *Betula* pollen and Bet v 1 in Bellaterra (Barcelona) for the years 2014 and 2015.
    - A study of the relationships between pollen and allergens and environmental parameters (Temperature, Precipitation, Relative Humidity and PM10) through a statistical analysis of correlation.
    - In the case of *Betula* allergen, a study to observe if there is a relationship between Bet v 1 airborne dynamics and the dynamics of pollen types other than *Betula* with recognized c ross-reaction.
2) To study the temporal (with a daily basis) and the spatial behavior of *Betula* pollen and Bet v 1 in two cities of Northern Spain (Bellaterra and Vitoria-Gasteiz). For this, it is realized:
    - A comparative study between the aerodynamics of each biological particle in the two cities during 2014 through a statistical analysis of correlation.
    - A study to establish the relationships between pollen and allergens and the environmental parameters (Temperature, Precipitation, Relative Humidity and PM10) through a statistical analysis of correlation.
3) To detect the airborne fungal allergen Alt a 1, from *Alternaria alternata*, in the atmosphere of Bellaterra (Barcelona) and to quantify its daily load allergenic. For this, it is determined:
    - The best methodology for the collection of this fungal allergen
    - The best methodology for the detection and quantification of this fungal allergen.

III. Material and Methods

1. Sites under study

Catalonia is localized in the North-East of the Iberian Peninsula (Fig. 8). According to Allué-Andrade (1990), Bellaterra (Barcelona) can be described as fresh (13.6–15.2 ◦C) and humid (594–740mm) from the nain climatic parameters and then corresponding to the phytoclimate Fresh-Continental Oriental-semihumid. On the other hand, Vitoria-Gasteiz is situated in the Basque Country, in the North of the Iberian Peninsula, in the boundary between the Euro-Siberian and the Mediterranean regions, and is classified as having a Fresh-Atlantic-semihumid phytoclimate.

Table 3: Information about the 2 monitoring sites, including geographical location (altitude, latitude and longitude), and climatic characteristics (mean annual temperature and total annual rainfall). (1) Data from Observatori Fabra (Barcelona) period 1971-2000 elaborated by Meteocat and (2) Data from Vitoria-Gasteiz Airport period 1981-2010 from Aemet, elaborated by Ayuntamiento de Vitoria Gasteiz.

	Geographical characteristics			**Climatic characteristics** [1,2]	
Station	**Altitude (m.a.s.l)**	**Latitude**	**Longitude**	**Mean annual temperature (ºC)**	**Total annual precipitation (mm)**
Bellaterra (Barcelona)	245	41º 30' N	02º 06' E	14.8	652
Vitoria-Gasteiz	513	42º 21' N	02º 06' W	11.7	743

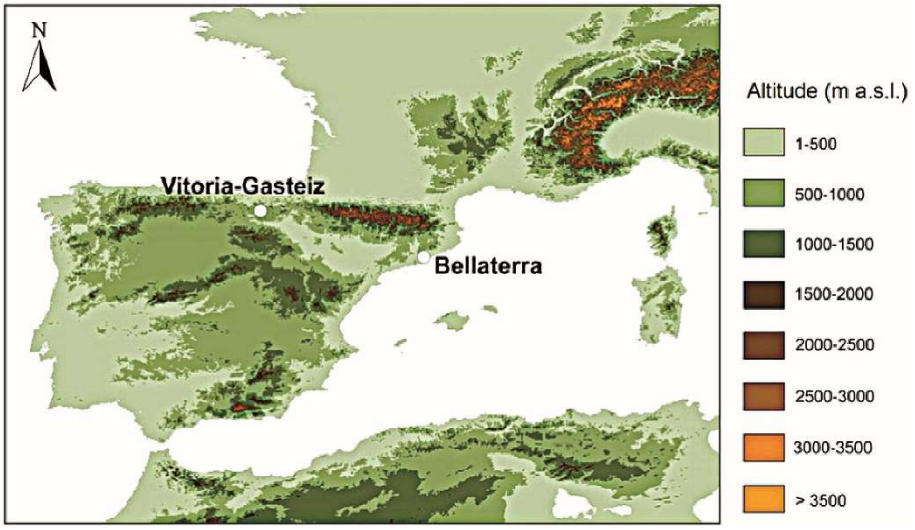


Figure 8. Location of the two aerobiological sampling stations. modified from Izquierdo et al.. (2017).

2. Airborne Pollen/Spores sampling

For the sampling and quantification of the airborne pollen, a Hirst (1952) volumetric collector Lanzoni VPPS 2000 (Italy) was used. This collector aspires air at a known rate of 10 L/min , and thanks to a vain, the air sampled is always from the predominant direction. When the air enters into the collector it impacts on a drum that has been prepared with a plastic surface coated with a siliconized adhesive substance that retains the pollen and spores. The drum is mounted on a clock system that makes it advance 2 mm per hour and the size of its surface makes possible to sample during one week. One has to annotate the time when the new sampling drum is installed in order to be able to well recognize the portion of the sample that will correspond to each day. Once a week the sample tape surface with the particles adhered is collected and brought to the laboratory, where it will be cut into the correspondent daily samples.

The Hirst sampler that has provided the pollen data for Bellaterra (Bellaterra) is operating since 1994 for the Catalan Network of Aerobiology (Xarxa Aerobiològica de Catalunya, XAC) and the one that offered the pollen data for Vitoria-Gasteiz is at work since 2004 for the Public Health Laboratory of Álava (Arabako Osasun Publikoko Laborategia).

Figure 9. Lanzoni VPP2000 air sampler at the C Building of the Universitat Autònoma de Barcelona, in Bellaterra.

Figure 10. Burkard Multi-vial Cyclone (in front) and Lanzoni VPP2000 air samplers at the C Building of the Universitat Autònoma de Barcelona, in Bellaterra.

3. Airborne Allergen sampling

The airborne allergen samples were collected using two samplers.

Multi-vial cyclone air sampler (Lipmann and Chan 1979) from Burkard Manufacturing Company Limited (England). This sampler aspires 16.6 L/min of air, and thanks to a vain, the air sampled is always from the predominant wind direction. The air aspired is conducted to a 1.5 ml Eppendorf vial where it enters following an spiral movement that allows the allergens to remain attached to the vial walls. There is a revolver system containing 8 eppendorf vials and a rotation system that can be programmed. This permits each Eppendorf sampling the air between 0 and 24 hours each day. Following the manufacturers, this sampler offers an efficiency of 100% for particle sizes up to 1.06 µm and 93.28% for particle sizes 0.82-0.75 µm. The collector provides daily allergen samples and ensures comparability of the allergen and pollen data (Emberlin 1995). When collected, the samples were preserved at -80º C until the analysis time.

Figure 11. Burkard Multi-vial cyclone air sampler at the C Building of the Universitat Autònoma de Barcelona, in Bellaterra.

The airborne pollen allergens were studied through a temporal study considering the years from 2013 to 2015 (in a daily basis) in the aerobiological station of Bellaterra

(Barcelona) and a spatial study analyzing the year 2014 (in a daily basis) , in two cities: Bellaterra (Barcelona) and Vitoria-Gasteiz. In both cases, a cyclone collector was used. The collectors were installed at 23 m.a.g.l (meters above ground level) on the roof of the C Building at the UAB, in Bellaterra (Barcelona) and on the roof of the Pharmacy Faculty of the Universidad del País Vasco, in Vitoria-Gasteiz.

High volume TSP collector from MCV S.A. (Spain). The TSP (Total Suspended Particles) sampler by MCV (Figure 13) allows trapping the airborne particles thanks to a vacuum pump. The volume of air aspired by this trap was set of 40 m^3/h and it impacted onto a glass fiber filter of 15 cm in diameter.

This collector was used to sample allergens in the Bellaterra (Barcelona) aerobiological control station at the UAB, where it was located next to the multi-vial cyclone. Filters, corresponding to daily samples, from 0 to 24 hours, and for alternate days during the month of July 2015 were obtained, divided into two halves and stored at -80º C until the analysis time.

Figure 12. MCV high-volume air sampler at the C Building of the Universitat Autònoma de Barcelona, Bellaterra.

4. Pollen/spore analysis

The daily samples obtained were analyzed under the light microscope according to the Spanish Aerobiological Network (Red Española de Aerobiologia, REA) methodology (Galán et al.. 2007) and following the minimum requirements of the European Aerobiology Society, EAS (Galán et al.. 2014) and the results were expressed in pollen/spores grains per cubic meter of air (pollen/m^3, spore/m^3). The pollen and spores concentration data used in this study were provided by the corresponding aerobiological laboratories in the two study areas: Laboratory of Palynological Analyses, Xarxa Aerobiologia de Catalunya (XAC), of the UAB provided the data for Bellaterra (Barcelona), and the Laboratory of Public Health of Alava, in the Basque Country, provided the data for Vitoria-Gasteiz.

5. Allergen detection and quantification Hydration time determination

First of all, we needed to stablish the time we should submit the airborne allergen samples under hydration conditions. To do so, for each pollen type, 5, 50, 100 and 500 pollen grains approximate counts were prepared under the microscope using commercial pollen grains material. These prepared samples were introduced into 1.5 ml Eppendorf vials and hydrated using 1 ml of PBS-TW solution. The hydration times were set for 2, 4, 6 and 8 hours, with the aim to determine the optimum hydration time each pollen grain taxon requires to rupture and release its contents. 100 µl of each hydrated pollen grains solution were analyzed using INDOOR Biotechnology® ELISA kits for Phl p 5 and Bet v 1 protein isoforms (Annex 1 and 2). NUNC™ flat bottom micro plates were used as per recommendation by the kit suppliers. The absorbances were measured at 450 nm filter in a Plate reader model Halo MpR-96 from Dynamica®. The best result was found to be 6 hours at room temperature for both *Beula* and *Poaceae* pollens

5.1 Phl p 5 samples analyses

The daily samples from Multi/vial cyclone air sampler of the years 2013 and 2015 collected at Bellaterra (Barcelona) and stored were hydrated for 6 hours using 100 µl of PBS-TW solution before analysis to ensure allergen release of any present pollen grains. Hydrated samples were analyzed according to the INDOOR® Biotechnology provided protocol (Annex 1) NUNC™ microplates. Absorbance were measured using 450 filters in a Plate reader model Halo MpR-96 from Dynamica®.

5.2 Bet v 1 samples analyses

The daily samples from the Multi/vial cyclone air sampler of the year 2014 in both Bellaterra (Barcelona) and Vitoria-Gasteiz and of the year 2015 in Bellaterra (Barcelona) were collected and stored with the purpose to analyze Bet v1. The samples were hydrated for 6 hours before analysis using 100 µl PBS-TW solution. The samples were analyzed according to the INDOOR® Biotechnology provided protocol (Annex 2). in NUNC™ microplates. Absorbance were measured at 450 µm filter in a Plate reader model Halo MpR-96 from Dynamica®.

5.3 Alt a 1 samples analysis

The daily samples from the Multi/vial cyclone air sampler of the year 2015 in Bellaterra (Barcelona) were collected and stored with the purpose to analyze Alt a 1. The samples were hydrated for 6 hours before analysis using 100 µl PBS-TW solution. The samples were analyzed according to the INDOOR® Biotechnology provided protocol (Annex 3) in NUNC™ microplates. Absorbance were measured at 450 µm filter in a Plate reader model Halo MpR-96 from Dynamica®.

As will be shown in the Results section, Alt a 1 was not detected in the samples from the Multi/vial cyclone air sampler. For this reason, a new attempt of detection of the fungal allergen Alt a 1 was decided using the High volume TSP collector during July 2015. The samples obtained were processed following different methodologies looking for to obtain the maximum daily concentration.

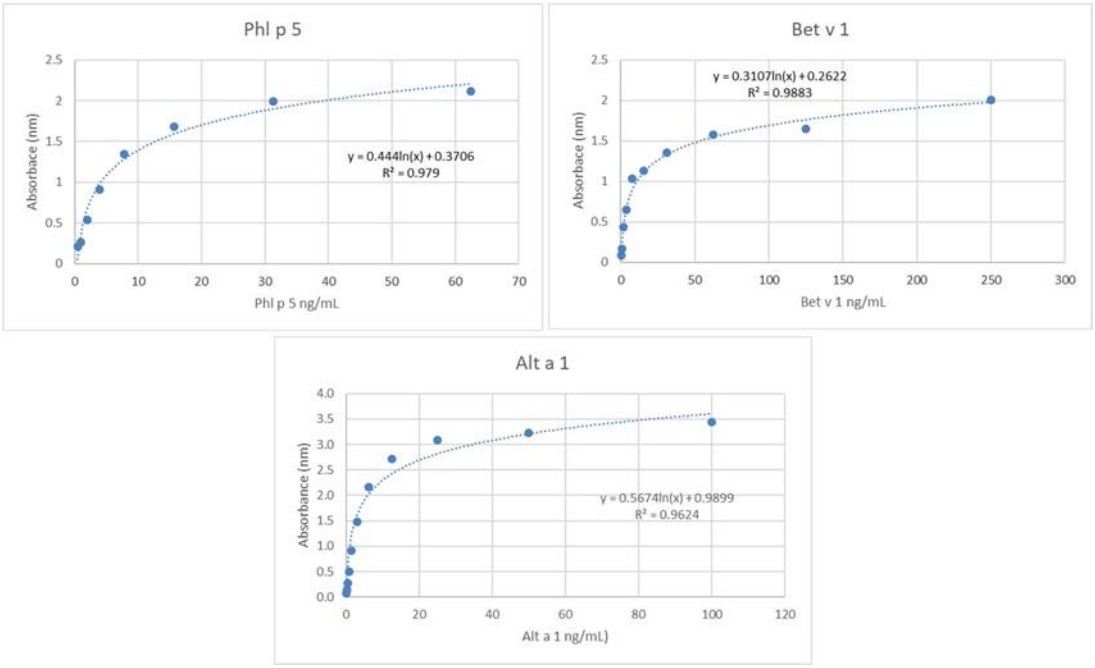


Figure A . ELISA assay response curve for Phl p 5, Bet v 1 and Alt a 1. Absorbance at 492nm.

5.3.1 Lyophilization

Half of the filter was washed using 30 ml of sterilized distilled water and the solution shacked for 1 hour at a speed of 100 rpm (round Per Minute) in a Reciprocating Shaker (MaxQ 2506). The resulting samples were filtered on a sterilized cotton mesh, then transferred to a 100 ml polypropylene screw-cap conical centrifuge tube, and finally stored frozen at -70 ºC. One day later, the samples were lyophilized using a Telstar-CRYODOS®.

5.3.2 Reconstitution of the dried samples

Lyophilized high volume air samples were reconstituted using 200µl of PBS buffer pH 7.4. A fraction of 50µl of the samples were digested using lysis buffer in order to solubilize the maximum amount of protein out of other airborne particles.

5.3.3 SDS-PAGE and immunoblotting for Alt a 1 detection

Samples were separated using 12% SDS-PAGE (Laemmli, 1970). Resulting bands for were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA), as described by Towbin et al.. (1992). Briefly, the membrane was cut into strips and incubated overnight with the diluted 1:10000 Rabbit sera containing Alt a 1 antibody at 4 °C. The reaction was developed using Clarity western ECL Blotting substrate from BIO-RAD (see Annex 4).

Bound IgE was detected via incubation with horseradish peroxidase (HRP) conjugate (Southern Biotech, Birmingham, AL, USA). The blots were developed by the addition of chemiluminescent reagents (ECL+, Amersham Biosciences, Bucks, UK).

5.3.4 Dot-blot detection of Alt a 1

Dot blot was carried out using usually 3-4 mm diam nitro-cellulose membrane strips, using narrow-mouth pipette tip, 1 µl of samples spotted onto the nitrocellulose membrane at the center. The membrane was left to dry then, the non-specific sites by was blocked by soaking the membrane in 5% BSA in TBS-T (1 hr, RT). A sterilized 10 cm Petri Dish was used as reaction chamber.

The sample loaded membrane was then incubated with (primary Anti-Alt a 1 rabbit serum and 1:1000, 1:5000 and 1:10000 dilutions dissolved in BSA/TBS-T) for 30 minutes.

Cross reaction test was done for different proteins such as, Asp f 1, Dust mite protein Der p 1, Phl p 1, *Curvularia lunata* extract. Where 3 pairs of nitro-cellulose test strips were prepared as above mentioned. Each test pair, the first strip was for Alt a 1 standard curve. Where 1 µl spotted of each of the following standard concentrations of Alt a 1 (Indoor® Biotechnology) (1000, 500, 250, 125, 62.5) ng/ml. The Other strip, is for sample and the cross-reaction antigens mentioned above.

The 1:10000 Anti-alt a 1 dilution did not show cross reaction with (Phl p 1, Der p 1, Asp f 1) but strong cross reaction with *Curvularia lunata* extract.

6. Meteorological data

The following daily meteorological data: mean precipitation, mean temperature, relative humidity, and wind speed were provided by The Catalan Meteorological service METEOCAT for the monitoring site in Sant Cugat del Vallès, the closest to the UAB and by Euskalmet-Agencia Vasca de Meteorología for Vitoria- Gasteiz.

7. Particulate Material data

Daily data of particles of size 10 µm (PM10) was obtained from the Generalitat de Catalunya website (http://dtes.gencat.cat/icqa/) from four stations in the vicinity of the UAB aerobiological sampling point. They were located in Sabadell, Sant Cugat del Vallès, Montcada and Barberà del Vallès (see Fig. 14 below).

Figure 13. Location map of A: Pollen and Allergen samplers at the UAB and B: Air quality monitoring stations for PM10.

8. Statistical Analysis

The non-parametric two-tailed Spearman rank correlation test between airborne allergen concentrations and airborne pollen concentrations and with the above mentioned meteorological parameters and PM10 data were conducted using software IBM® SPSS® (Version 22). The level of significance were assessed between $p< 0.01$ and $P < 0.05$.

IV. RESULTS

1. *Poaceae* pollen *vs* Phl p 5 2013 and 2015 in Bellaterra (Barcelona)

Table 4 and Figure 14 show the results of the comparative study of Phl p 5 and Poaceae pollen during 2013.

Phl p 5 was detected less days than pollen was (65 versus 180). Figure 14 shows that the first day with *Betula* pollen detection was 2 February with 1 pollen/m^3, almost two months before the first day with Phl p 5 detection, which was on April 4 with 87.8 ng/m^3. Similarly, the end of the pollen detection period was on November 21, 4 months after July 27, the last detection for Phl p 5. In general, both variables showed a similar behavior, increasing gradually in April, showing maximum values in May and decreasing in July. The maximum allergen and pollen concentrations were recorded in May-June. However, during the periods following the heaviest Poaceae pollination, the two curves showed differences in the sense of airborne pollen being present but no allergen detected.

May 19 was detected the Peak concentration of Phl p 5 at a concentration of 5101 ng/m3 (Table 4, and Figure14). It represented the 10.6% of the Annual Allergen Integral (AAIn, Table 4). The Peak concentration of Phl p 5 occurred on May 19 while the peak of Poaceae pollen took place on June 13 with 38 pollen/m^3 (Table4, Figure 14).

In 2015, the general results were lower than in 2013 in the case of Phl p 5 but higher for Poaceae pollen. AAIn was 4850 ng/m^3 (100 times lower than in 2013) while the Annual Pollen Integral (APIn) was 1477 pollen/m^3 (400 pollen/m^3 higher than in 2013). The number of days with presence of airborne allergen was 65 days in front of 193 days with pollen presence. The peak day for Phl p 5 was registered one day before the peak day for Poaceae pollen (April 20, with 2502 ng/m^3, vs. April 21, with 53 pollen/m^3 (Table 4).

In relation to the meteorological parameters (Table 4), 2013 registered more Precipitation and Relative Humidity than 2015 (493.7 mm - 66.4% and 336.3 mm -

71.2%, respectively). Temperature was practically the same with 1º C more in 2015 than in 2013.

Table 4. Phl p 5, *Poaceae* pollen and meteorological parameters in Bellaterra (Barcelona), annual summaries for years 2013 and 2015. AAIn (Annual Allergen Integral), APIn (Annual Pollen Integral), Tmax (mean annual maximum Temperature), Tmin (mean annual minimum Temperature), Tmean (mean annual mean Temperature), P (annual Precipitation), RH (mean annual Relative Humidity).

	Bellaterra (Barcelona)	
	2013	**2015**
Phl p 5		
AAIn (ng*day/m^3)	48286	4850
Peak (ng/m^3)	5101	510
Peak date	19-may	07-may
Days analyzed	346	308
Days with allergen presence	65	65
***Poaceae* Pollen**		
APIn (pollen*day/m^3)	1128	1477
Peak (pollen/m^3)	38	71
Peak date	13-jun	08-may
Days analyzed	358	359
Days with pollen presence	180	193
Meteorological data		
Tmax (ºC)	21,2	22,4
Tmin (ºC)	9,6	10,6
Tmean (ºC)	15,4	16,5
P (mm)	493,7	336,3
RH (%)	66,4	71,2

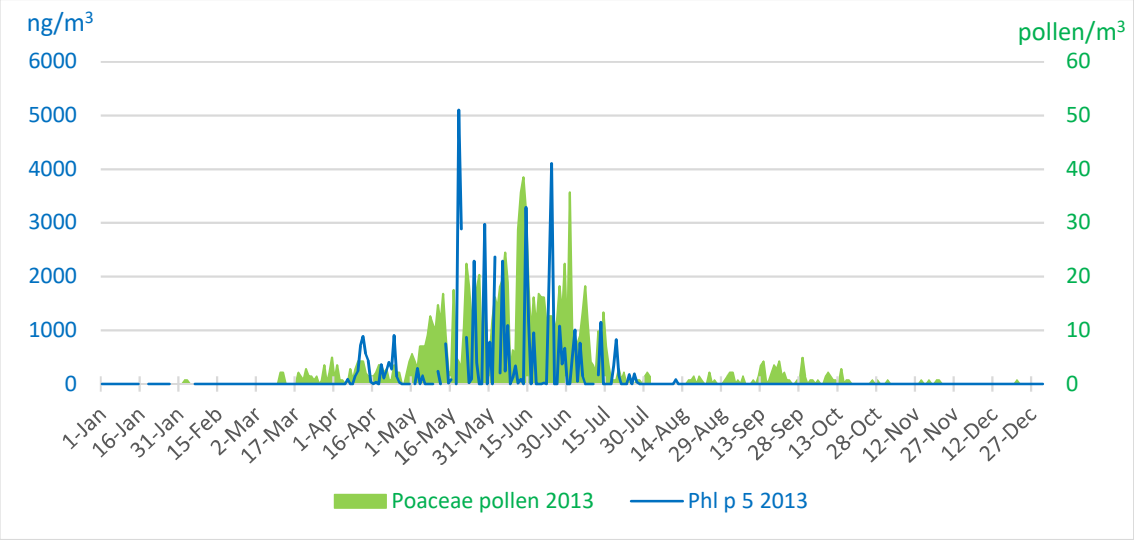


Figure 14. Annual dynamics of Phl p 5 (in ng/m^3) and *Poaceae* pollen (in pollen/m^3) in Bellaterra (Barcelona), daily concentrations during 2013.

Table 5 shows a monthly summary of Phl p 5, Poaceae pollen and the meteorological parameters for the detection period of 2013. The presence of Phl p 5 and pollen were maximum during May-June (in number of days, in the day and month of maximum concentration, and monthly integral). Poaceae pollen was present in the air in February-March and between September and December, while Phl p 5 was not detected in these months. The maximum Monthly Integral detected for Phl p 5 and Poaceae pollen coincided in the same month.

In relation to the meteorological parameters, April presented an increase of temperature (maximum, minimum and mean) of 3ºC with respect to March and March was the rainiest month, followed by November and April**.**

During 2015, the dynamics of Phl p 5 and Poaceae pollen (Figure 15 and Table 6) showed that allergen and pollen are present in the air since April to July. As in 2013, the maximum allergen and pollen concentrations were recorded in May and June. However, during the period succeeding the heaviest Poaceae pollination, the two curves showed differences registering airborne pollen but no allergens.

Monthly summaries for allergen and pollen detection in 2015 (Table 6) show that April and May registered the maximum number of days with Phl p 5 while July and May the more days with Poaceae pollen. May and June coincided presenting the maximum daily concentrations and the highest Monthly Integral values for pollen and allergen.

Table 5. Phl p 5, Poaceae pollen and meteorological parameters in Bellaterra (Barcelona), monthly summaries for the detection during 2013. Daily concentrations in ng/m^3 and pollen/m^3, Monthly Integrals in ng*day/m^3 and pollen*day/m^3, Tmax (mean annual maximum Temperature in ºC), Tmin (mean annual minimum Temperature in ºC), Tmean (mean annual mean Temperature in ºC), P (annual Precipitation in mm), RH (mean annual Relative Humidity in %).

		Number of days with detection		Max. daily concentration detected		Monthly Integral		Meteorological parameters				
		Phl p 5	*Poaceae* pollen	Phl p 5	*Poaceae* pollen	Phl p 5	*Poaceae* pollen	Tmax	Tmin	Tmean	P	RH
2013	January	0	0	0	0	0	0	14,4	2,6	8,5	22,4	67,6
	February	0	2	0	1 (2)	0	1	13,2	2,6	7,9	28,4	62,6
	March	0	14	0	5 (31)	0	27	16,6	6,0	11,3	110,7	68,2
	April	17	26	903 (24)	4 (10,11,12,30)	5622	60	19,3	7,5	13,4	80,6	65,0
	May	16	31	5101 (19)	22 (22)	16921	306	21,2	9,7	15,5	40,5	67,2
	June	18	30	4105 (24)	39 (13)	20344	494	26,1	13,5	19,8	18,9	62,2
	July	13	27	1150 (13)	36 (1)	5536	169	30,5	18,4	24,4	6,8	59,5
	August	1	9	80 (11)	2 (24)	80	11	29,7	17,7	23,7	6,6	63,2
	September	0	21	0	5 (29)	0	42	27,2	14,8	21,0	19,9	69,9
	October	0	13	0	3 (14)	0	14	24,4	13,4	18,9	39,8	74,0
	November	0	5	0	1 (1,14,17,20)	0	4	17,3	5,9	11,6	104,9	64,3
	December	0	1	0	1 (21)	0	1	14,2	2,4	8,3	14,2	72,4

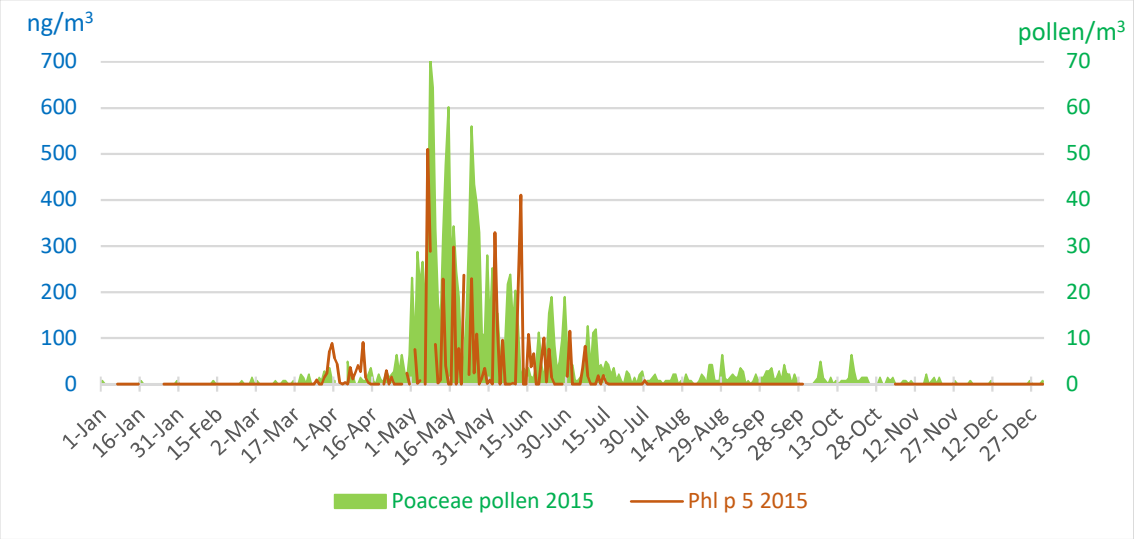


Figure 15. Annual dynamics of Phl p 5 (in ng/m^3) and *Poaceae* pollen (in pollen/m^3) in Bellaterra (Barcelona), daily concentrations during 2015.

Statistical analysis

Statistical analysis between the concentrations of Phl p 5 and the Poaceae pollen, meteorological parameters and PM10 for the years 2013 and 2015 is shown in Tables 7 and 8. A significant positive correlation between the allergen concentration and the pollen concentrations measured during the whole year was obtained (rs=0.542**, P < 0.01, N=339). However, this correlation could be due to the abundance of 0 values in both series. To eliminate this error, Table 7 shows the correlation results for other three periods of airborne allergen and of pollen presence. The first defined period, "March-July", compares the allergen and pollen concentrations of these five months. A second period, defined as period with "Poaceae pollen presence" contains only the values corresponding to the days with Poaceae pollen presence and the day before and after. and the third period, named period with "Phl p 5 presence" contains only the values corresponding to the days with Phl p 5 presence and the day before and after. Considering these three periods, the Spearman correlations showed positive correlations between both variables except in 2015 for the period "Phl p 5 presence" where not correlation was obtained (Tables 7 and 8).

In relation to the meteorological parameters, positive and significant correlations were observed between the allergen and the pollen concentrations with the mean, minimum and maximum Temperatures for the "January-December" and the "March-July" periods of 2013 (Table 7) and in all periods analyzed of 2015 (Table 8). On the other hand, negative and significant correlations were obtained with Precipitation and Relative Humidity in both years in the annual and in the "March-July" periods, but no correlation did not exist in the case of the "Poaceae pollen presence" period of 2013 (Table 7) and "Phl p 5 presence" period in 2013 and 2015 (Tables 7 abd 8).

The daily concentrations of Atmospheric PM10 monitored at the Montcada Air Quality station showed positive and significant correlations with Poaceae pollen and Phl p 5 in both years and all periods considered with the only exception of the "Phl p 5 presence" period (Tables 7 and 8).

Table 6: Phl p 5, *Poaceae* pollen and meteorological parameters in Bellaterra (Barcelona), monthly summaries for the detection during 2015. Daily concentrations in ng/m^3 and pollen/m^3, Monthly Integrals in ng*day/m^3 and pollen*day/m^3, Tmax (mean annual maximum Temperature in ºC), Tmin (mean annual minimum Temperature in ºC), Tmean (mean annual mean Temperature in ºC), P (annual Precipitation in mm), RH (mean annual Relative Humidity in %).

		Number of days with detection		Max. daily concentration detected		Monthly Integral		Meteorological parameters				
		Phl p 5	*Poaceae* pollen	Phl p 5	*Poaceae* pollen	Phl p 5	*Poaceae* pollen	Tmax	Tmin	Tmean	P	RH
2015	January	0	3	0	1 (1,6,30)	0	2	13,6	2,3	8,0	14,5	72,2
	February	0	3	0	2 (28)	0	3	13,8	1,9	7,9	8,8	66,8
	March	5	14	89 (31)	4 (30)	208	20	18,2	6,5	12,3	47,8	71,0
	April	16	23	90 (12)	6 (25)	422	55	20,8	8,3	14,6	12,5	70,4
	May	21	31	510 (7)	71 (8)	2298	880	26,2	12,7	19,5	10,9	62,7
	June	15	30	411 (12)	25 (16)	1626	291	29,6	16,9	23,2	19,2	61,7
	July	8	37	115 (1)	13 (8)	297	95	32,7	21,0	26,9	20,0	63,3
	August	0	25	0	6 (29)	0	39	30,2	18,6	24,4	30,9	72,9
	September	0	24	0	4 (5)	0	46	25,3	15,1	20,2	49,3	75,0
	October	0	19		6 (18)	0	31	21,9	11,6	16,8	11,1	78,2
	November	0	11	0	2 (3)	0	12	19,2	6,8	13,0	110,7	75,8
	December	0	4	0	1 (3,11,26,31)	0	3	16,2	5,2	10,7	0,6	83,7

Table 7. Spearman's Rho correlation coefficients for Bellaterra (Barcelona) 2013 between the Phl p 5 and *Poaceae* pollen concentrations; the meteorological parameters and the PM10 concentrations. Different periods of airborne allergen and or pollen presence considered. Tmax (mean annual maximum temperature in ºC), Tmin (mean annual minimum temperature in ºC), Tmean (mean annual mean temperature in ºC), P (annual Precipitation in mm), RH (mean annual Relative Humidity in %). ** p<0.001; * p<0.05.

				Sant Cugat					PM10			
		Phl p 5	*Poaceae*	Tmax	Tmin	Tmean	P	RH	Barberà	Montcada	Sabadell	Sant Cugat
January - December	Phl p 5	1,000	**,542****	**,194****	**,153****	**,186****	-,061	**-,160****	**-,141***	**,126***	-,084	,007
	N	346	339	346	346	346	346	346	202	344	168	184
	Poaceae	**,542****	1,000	**,462****	**,427****	**,462****	**-,151****	**-,209****	**-,253****	**,114***	**-,230****	-,098
	N	339	358	358	358	358	358	358	215	356	176	190
March - July	Phl p 5	1,000	**,387****	**,205***	,133	**,201***	**-,172***	-,047	,077	**,277****	,113	,197
	N	146	146	146	146	146	146	146	90	146	71	79
	Poaceae	**,387****	1,000	**,416****	**,360****	**,419****	**-,383****	**-,168***	,117	**,371****	,045	**,292****
	N	146	153	153	153	153	153	153	96	153	74	80
Poaceae pollen (1)	Phl p 5	1,000	**,481****	-,021	-,093	-,046	-,074	**-,160***	-,037	**,166***	,024	,102
	N	220	219	220	220	220	220	220	131	218	106	118
	Poaceae	**,481****	1,000	,113	,010	,075	**-,243****	**-,249****	-,039	**,207****	-,066	,154
	N	219	230	230	230	230	230	230	139	228	109	121
Phl p 5 presence (2)	Phl p 5	1,000	**,210***	-,059	-,105	-,078	-,032	,025	-,013	,134	-,001	,159
	N	98	98	98	98	98	98	98	57	98	47	48
	Poaceae	**,210***	1,000	,071	,069	,079	-,192	,023	-,023	,148	-,096	,088
	N	98	104	104	104	104	104	104	63	104	50	49

(1) The working file contains only the values corresponding to the days with *Poaceae* pollen presence and the day before and after.

(2) The working file contains only the values corresponding to the days with Phl p 5 presence and the day before and after.

Table 8. Spearman's Rho correlation coefficients for Bellaterra (Barcelona) 2015 between the Phl p 5 and *Poaceae* pollen concentrations; the meteorological parameters and the PM10 concentrations. Different periods of airborne allergen and or pollen presence considered. Tmax (mean annual maximum temperature in ºC), Tmin (mean annual minimum temperature in ºC), Tmean (mean annual mean temperature in ºC), P (annual Precipitation in mm), RH (mean annual Relative Humidity in %). ** p<0.001; * p<0.05.

				Sant Cugat					PM10			
		Phl p 5	*Poaceae*	Tmax	Tmin	Tmean	P	RH	Barberà	Montcada	Sabadell	Sant Cugat
January - December	Phl p 5	1,000	**,469****	**,249****	**,161****	**,208****	-,043	**-,332****	-,051	**,192****	-,159	,019
	N	308	302	308	308	308	308	308	160	308	149	185
	Poaceae	**,469****	1,000	**,645****	**,578****	**,626****	-,094	**-,429****	**-,312****	**,130***	**-,251****	-,084
	N	302	359	359	359	359	359	359	184	359	171	213
March - July	Phl p 5	1,000	**,317****	,085	-,013	,032	-,088	**-,186***	,066	**,172***	-,233	,001
	N	146	142	146	146	146	146	146	79	146	68	88
	Poaceae	**,317****	1,000	**,413****	**,351****	**,387****	**-,267****	**-,405****	-,099	**,224****	-,106	,062
	N	142	149	149	149	149	149	149	83	149	70	91
Poaceae pollen presence (1)	Phl p 5	1,000	**,417****	,106	-,003	,050	-,073	**-,341****	,123	**,248****	-,037	,139
	N	240	238	240	240	240	240	240	127	240	108	139
	Poaceae	**,417****	1,000	**,467****	**,384****	**,438****	**-,152***	**-,474****	-,109	**,205****	-,077	,069
	N	238	279	279	279	279	279	279	145	279	123	159
Phl p 5 presence (2)	Phl p 5	1,000	,031	,021	-,049	-,017	,058	-,022	,103	,149	-,171	,016
	N	96	92	96	96	96	96	96	55	96	47	59
	Poaceae	,031	1,000	**,311****	**,272****	**,291****	-,149	**-,275****	-,119	,185	,002	,086
	N	92	98	98	98	98	98	98	58	98	49	61

(1) The working file contains only the values corresponding to the days with *Poaceae* pollen presence and the day before and after.

(2) The working file contains only the values corresponding to the days with Phl p 5 presence and the day before and after.

2. *Betula* pollen vs Bet v 1 2014 and 2015 in Bellaterra, Barcelona

The comparative study of Bet v 1 and *Betula* pollen during 2014 is shown in Tables 9 and 10 and in Figure 16.

As shown in Table 9, Bet v 1 was detected in almost three times more daily samples than *Betula* pollen (98 versus 34) was. Figure 16 shows that the first day with *Betula* pollen detection was the January 26, with 0.7 pollen/m^3, almost two weeks before the first day with Bet v 1 detection, which started on February 9 with 2.8 pg/m^3. Similarly, the end of the pollen detection period was on July 18, 5 days after July 13, the last detection for Bet v 1. Although it seems that the pollen period was larger than the allergen period, it was not so because pollen concentrations happened in a very scattered way up to April 6 and since May 19. On the contrary, allergen concentrations, showed less interruption.

The Peak concentration of Bet v 1 was detected on April 11 at a concentration of 4797 ng/m^3 (Table 9, and Figure16). It represented the 32.3% of the Annual Allergen Integral. The Peak concentration of *Betula* pollen occurred one day after with 39.2 pollen/m^3 (Table 9, Figure 16). It represented the 20.1% of the Annual Pollen Integral.

In 2015, the results were lower than in 2014 (Table 9). The allergen and the pollen registered lower AAIn (2502 *vs.* 14865 ng/m^3) and APIn (53 *vs.* 194 pollen/m^3); the days with presence of airborne allergen was 63 days in front of the 31 days of pollen presence. The peak day for Bet v 1 was registered again, as in 2014, one day before the peak pollen day (April 20, 2502 ng/m^3 *vs*. April 21, 53 pollen/m^3 (see Table 9)).
In relation to the meteorological data (Table 9), 2014 registered more Precipitation and Relative Humidity than 2015 (714.5 mm - 73.8% and 336.3 mm - 72.2%, respectively). Temperature was practically the same except in Tmax there where was a difference of 0.8º (higher in 2015).
The dynamics of Bet v 1 and *Betula* pollen in 2014 reveals that both elements were present in the air (Figure 16). The maximum allergen and pollen concentrations were recorded in April. However, during the periods preceding and succeeding the heaviest *Betula* pollination period, the two curves showed differences, with airborne allergens being present while no pollen.

Table 10 shows a monthly summary of Bet v 1, *Betula* pollen and meteorological parameters for the detection period of 2014. The presence of Bet v 1 and pollen occurred between January until July and were maximum during April (in number of days, in the day and month of maximum concentration, and Monthly Integral). Taking into account the montly maximum values, in no case the maximum daily concentrations detected of Bet v 1 and *Betula* pollen coincided in the same day (see Figure 16) and in April a difference of one day was observed (Bet v 1 was detected one day before than *Betula* pollen). In relation to the meteorological parameters, April presented an increase of Temperature (maximum, minimum and mean) of 3ºC with respect to March.

Table 9. Bet v 1, *Betula* pollen and meteorological parameters in Bellaterra (Barcelona), annual summaries for years 2014 and 2015. AAIn (Annual Allergen Integral), APIn (Annual Pollen Integral), Tmax (mean annual maximum Temperature), Tmin (mean annual minimum Temperature), Tmean (mean annual mean Temperature), P (annual Precipitation), RH (mean annual Relative Humidity).

	Bellaterra (Barcelona)	
	2014	**2015**
Bet v 1		
AAIn (ng*day/m^3)	14865	2502
Peak (ng/m^3)	4797	766
Peak date	11-april	20-april
Days analyzed	325	365
Days with allergen presence	98	63
***Betula* pollen**		
APIn (pollen*day/m^3)	194	53
Peak (pollen/m^3)	39	8
Peak date	12-april	21-april
Days analyzed	362	359
Days with pollen presence	34	31
Meteorological data		
Tmax (ºC)	21,8	22,4
Tmin (ºC)	10,9	10,6
Tmean (ºC)	16,3	16,5
P (mm)	714,5	336,3
RH (%)	73,8	71,2

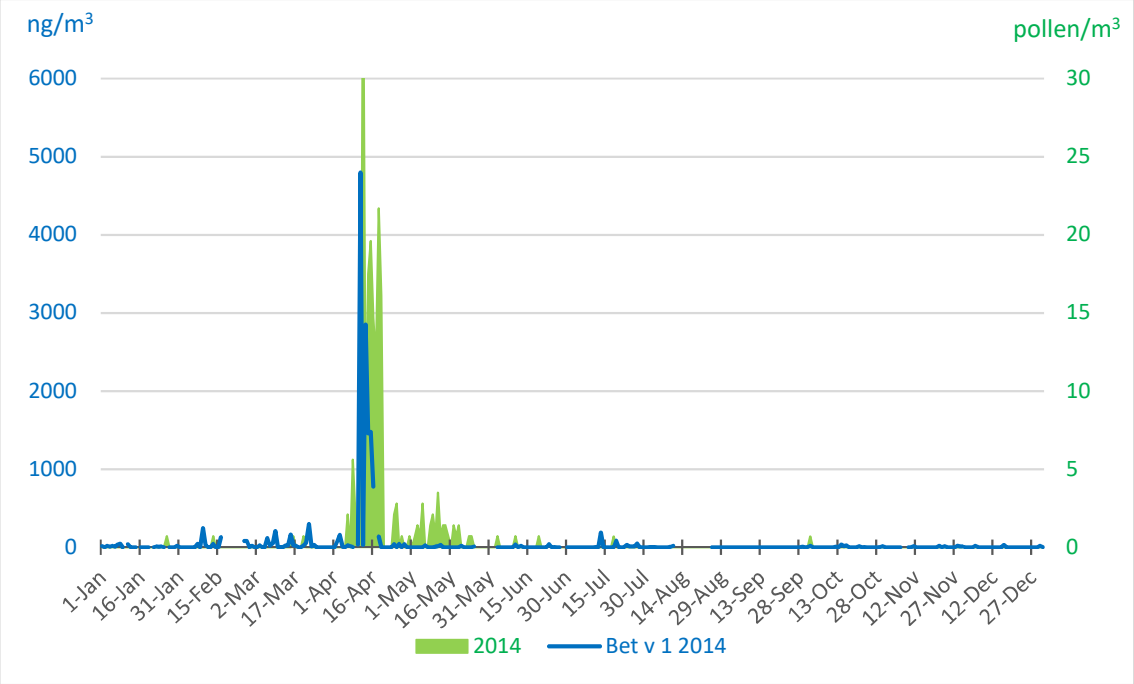


Figure 16. Annual dynamics of Bet v 1 (ng/m^3) and *Betula* pollen (pollen/m^3) in Bellaterra (Barcelona), daily concentrations during 2014.

Table 10. Bet v 1, *Betula* pollen and meteorological parameters in Bellaterra (Barcelona), monthly summaries for the detection period of 2014. Daily concentrations in ng/m^3 and pollen/m^3. Monthly Integral in ng*day/m^3 and pollen*day/m^3. Tmax (mean annual maximum Temperature in ºC), Tmin (mean annual minimum Temperature in ºC), Tmean (mean annual mean Temperature in ºC), P (annual Precipitation in mm), RH (mean annual Relative Humidity in %).

		Number of days with detection		Max. daily concentration detected (*day*)		Monthly Integral		Meteorological parameters				
		Bet v 1	*Betula* pollen	Bet v 1	*Betula* pollen	Bet v 1	*Betula* pollen	Tmax	Tmin	Tmean	P	RH
2014	Jan	12	1	49 (*08*)	1 (*26*)	233	1	14,0	4,7	9,4	46,9	78.1
	Feb	8	1	242 (*9*)	1 (*13*)	665	1	15,0	4,4	9,7	26,1	70.5
	March	17	2	298 (*22*)	1 (*16*)	1096	1	18,3	5,8	12,0	22,5	67.5
	April	17	17	4797 (*11*)	39 (*12*)	11967	165	21,5	9,7	15,6	64,2	73.6
	May	6	17	30 (*12*)	3 (*8*)	104	22	22,6	11,1	16,8	68,5	71.8
	June	4	3	37 (*23*)	1 (*3,10,19*)	90	2	28,1	15,6	21,8	10,1	64.7
	July	8	1	187 (*13*)	1 (*18*)	379	1	29,3	17,6	23,5	79,6	66.3

The dynamics of Bet v 1 and *Betula* pollen in 2015 showed that allergen and pollen were present in the air during the first seven month of the year (Figure 17). As in 2014, the maximum allergen and pollen concentrations were recorded in April followed by May and June. However, during the periods preceding and succeeding the heaviest *Betula* pollination, the two curves showed differences, with airborne allergens being present but no pollen detected.

With regard to the monthly summaries of detection (Table 11), similar results than in 2014 (Table 10) were observed. April registered the maximum number of days with Bet v 1 and Pollen, maximum daily concentration and maximum Monthly Integral. The differences of temperature between March to April was 2ºC.

The comparative annual dynamics of Bet v 1 in 2014 and 2015 is shown in Figure 18. In both years, this allergen appeared on January with low concentrations in the air being April the month with the higher concentrations. An outstanding fact was that in 2014 the concentrations registered were six times higher than in 2015 at the time that the period of presence in the air was shorter (Figure 18).

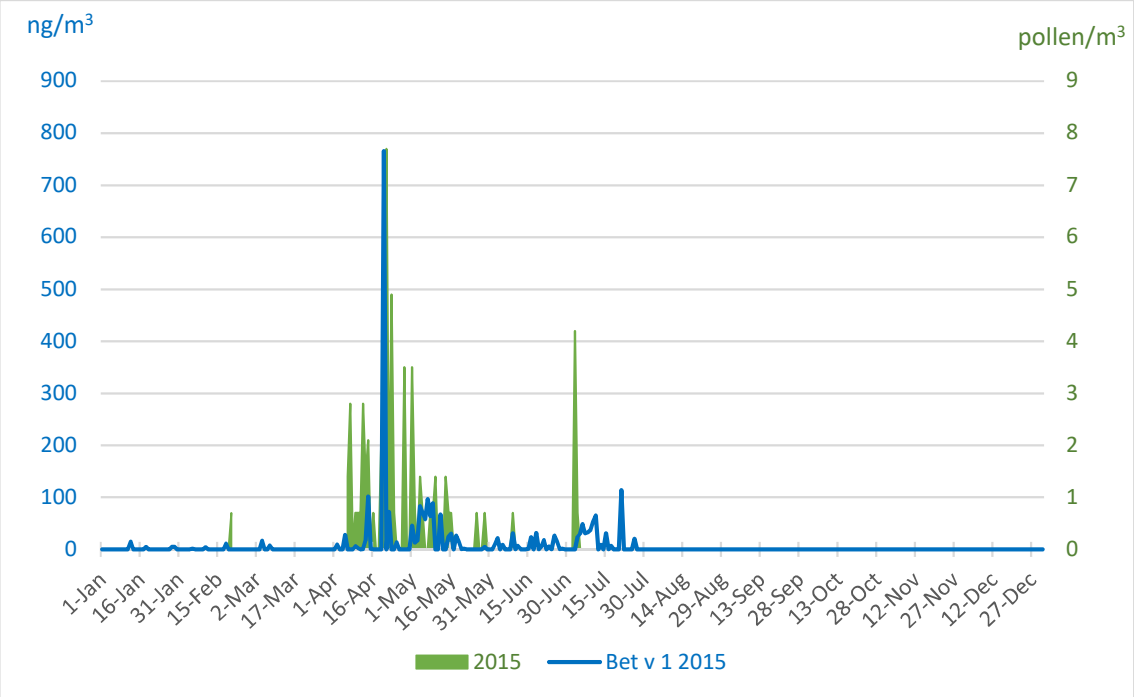


Figure 17. Annual dynamics of Bet v 1 (ng/m^3) and *Betula* pollen (pollen/m^3) in Bellaterra (Barcelona), daily concentrations during 2015.

Table 11. Bet v 1, *Betula* pollen and meteorological parameters in Bellaterra (Barcelona), monthly summaries for the detection period of 2014. Daily concentrations in ng/m^3 and pollen/m^3. Monthly Integral in ng*day/m^3 and pollen*day/m^3. Tmax (mean annual maximum Temperature in ºC), Tmin (mean annual minimum Temperature in ºC), Tmean (mean annual mean Temperature in ºC), P (annual Precipitation in mm), RH (mean annual Relative Humidity in %).

		Number of days with detection		Max. daily concentration detected (*day*)		Monthly Integral		Meteorological parameters				
		Bet v 1	*Betula* pollen	Bet v 1	*Betula* pollen	Bet v 1	*Betula* pollen	Tmax	Tmin	Tmean	P	RH
2015	Jan	4	0	15 (*12*)	0	29	0	13,6	2,3	8,0	14,5	78.1
	Feb	3	1	11 (*18*)	1 (*20*)	17	1	13,8	1,9	7,9	8,8	70.5
	March	2	0	16 (*4*)	0	24	0	18,2	6,5	12,3	47,8	67.5
	April	11	14	766 (*20*)	8 (*21*)	1017	33	20,8	8,3	14,6	12,5	73.6
	May	17	12	97 (*7*)	4 (*1*)	706	13	26,2	12,7	19,5	10,9	71.8
	June	16	2	31 (*9*)	1 (*29*)	206	1	29,6	16,9	23,2	19,2	64.7
	July	13	2	114 (*21*)	4 (*3*)	503	5	32,7	21,0	26,9	20,0	66.3

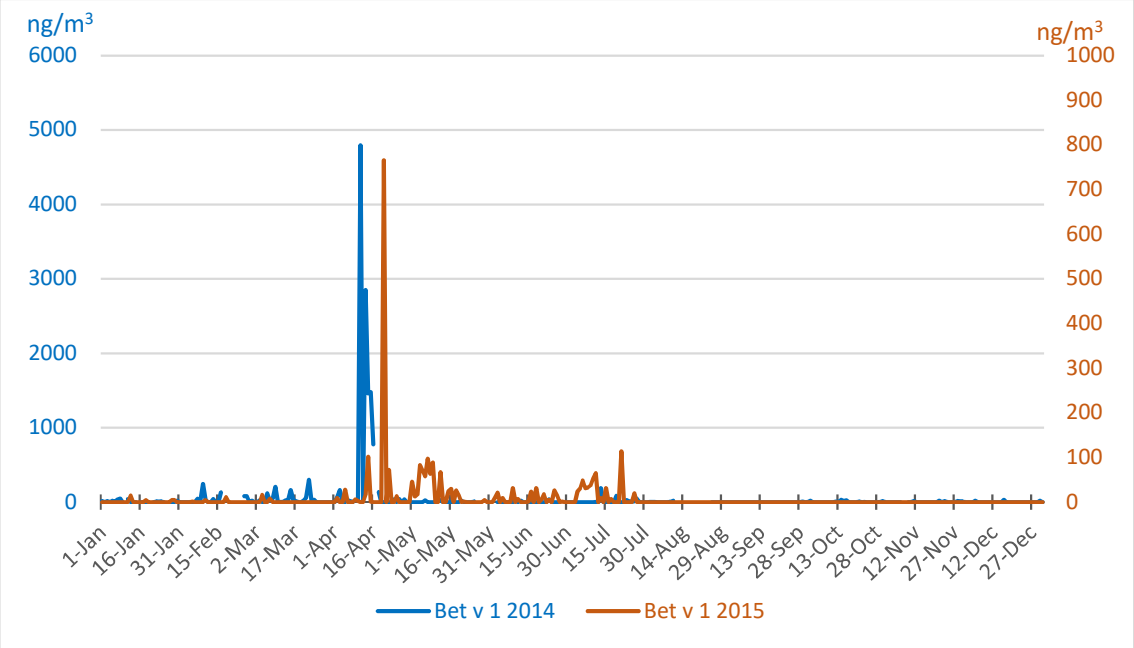


Figure 18. Annual dynamics of Bet v 1 in Bellaterra (Barcelona), daily concentrations during 2014 and 2015 expressed in ng/m^3.

Following Allergome information (www.allergome.org, accessed November 2018) Bet v 1 presents cross reactivity with allergens of other anemophilous plants like *Alnus*, *Castanea*, *Corylus* and *Quercus*. In the study area of Bellaterra (Barcelona) these taxa are present, and their pollination occur in different periods of the year and in some cases coincide with the *Betula* pollination. Thus, they could also have contributed to the Bet v 1 presence detected in the results. *Alnus* and *Corylus* pollen are present in the air early in the year (January-February), *Quercus* are spring pollinating species and *Castanea* is the latest pollen of this group (June-July). The main pollination parameters of these taxa during the study period of 2014 and 2015 are shown in Table 12.

Table 12. Summary of the main parameters of *Betula* and the airborne pollen taxa that present cross reactivity with Bet v 1 detected in Bellaterra (Barcelona), years 2014 and 2015. APIn (Annual Pollen Integral), SD daily conc. (Standard Deviation of the mean daily concentrations).

		Betula	*Alnus*	*Castanea*	*Corylus*	*Quercus*
2014	APIn (pollen*day/m^3)	194	96	63	155	11660
	Peak (pollen/m^3)	39	12	10	16	659
	(date)	(5-may)	(18-feb)	(5-july)	(11-march)	(10-may)
	SD daily conc.	3	1,1	0,8	1,7	86,7
	Days with presence	43	35	35	56	205
2015	APIn (pollen*day/m^3)	52	282	230	328	20626
	Peak (pollen/m^3)	8	37	37	50	1979
	(date)	(21-april)	(28-feb)	(24-june)	(9-march)	(15-may)
	SD daily conc.	0,7	3,4	3,3	4	201,2
	Days with presence	31	41	43	68	189

Statistical analysis

Spearman's Rho correlation coefficients of 2014 between the concentrations of Bet v 1 and the pollen concentrations of *Betula*, *Alnus*, *Castanea*, *Corylus* and *Quercus,* the meteorological parameters and the PM10 concentrations are shown in Table 13. A significant positive correlation between the allergen and the *Betula* pollen concentrations measured was obtained (r_s=0.184, P < 0.01, N=322), but this correlation could be due to the abundance of 0 values in both daily series. To eliminate this error, Table 13 shows also the correlation values for other three periods of airborne allergen and pollen presence. First, the correlation has been calculated for the concentrations of only the period "April-May". Then we stablished a period named "*Betula* pollen presence" containing only the values corresponding to the days with *Betula* pollen

presence and the day before and after. Finally, and the period "Bet v 1 presence" was prepared containing only the values corresponding to the days with Bet v 1 presence and the day before and after. In each of these three cases, the Spearman correlation showed positive correlation between Bet v 1 *Betula* pollen in "Bet v 1 presence" (r_s=0.197, P < 0.01, N=172).

The correlation in relation to the meteorological parameters did not show significance between pollen and allergen concentrations and the mean, minimum or maximum Temperatures (Table 13). Bet v 1 correlated only in the case of the "January-December" period, when a negative correlation with minimum Temperatures (r_s= -0.119, N= 325, P < 0.05) was observed. Regarding *Betula* pollen, it showed a negative significant correlation with Relative Humidity in the "January-December" period (r_s= -0.196, P < 0.01, N= 362) and for "April-May" period (r_s= -0.282, P < 0.01, N= 61) the "Bet v 1 presence"(r_s= -0.158, P < 0.05, N= 179). Finally, a negative correlation was observed between *Betula* pollen and Precipitation for the "April-May" period (r_s= -0.393, P < 0.01, N= 61). On the other hand, positive correlations with atmospheric PM10 monitored by neighboring Air Quality stations were obtained with Bet v 1 in the "Bet v 1 presence" period (Montcada: r_s= 0.429, N= 35, p< 0.05; Sabadell: r_s= 0.477, N= 35, p< 0.01; Sant Cugat: r_s= 0.339, N= 41, p< 0.05).

Finally, when considering the Spearman correlation tests between Bet v 1 and pollen types other than *Betula* with cross reactivity (Table 13) the only one showing a positive and significant correlation was *Alnus* for the "Bet v 1 presence" period (r_s= 0.198, N= 172, p< 0.01). There was also correlation between the concentrations for the whole year period but this one could be due to the existence of lots of 0 values in the two series and so it was not considered.

Two tailed Spearman correlation test between Bet v 1 and *Betula* pollen in 2015 (Table 14) for the annual results as well as for the days of presence results showed positive correlation (r_s=0.304, N=352 p< 0.01).

In all the cases in 2015, except for the "January-December" period, no significant correlations were obtained. Temperatures (maximum, minimum and mean), and Precipitation showed positive and significant correlation with Bet v 1 during the "Bet v 1 presence" period. In this same period, PM10 values from the Barberà station showed

negative significate correlation (r_s= -0.177, N= 124, P < 0.05) while those form the Sabadell showed positive significate (r_s= 0.257, N= 124, P < 0.01).

Table 13. Spearman's Rho correlation coefficients for Bellaterra (Barcelona) 2014 between the concentrations of Bet v 1 and the pollen concentrations of *Betula*, *Alnus*, *Castanea*, *Corylus* and *Quercus*; the meteorological parameters and the PM10 concentrations. Different periods of airborne allergen and or pollen presence considered. Tmax (mean annual maximum temperature in ºC), Tmin (mean annual minimum temperature in ºC), Tmean (mean annual mean temperature in ºC), P (annual Precipitation in mm), RH (mean annual Relative Humidity in %). ** p<0.001; * p<0.05.

								Sant Cugat					PM10			
		Bet v 1	*Betula*	*Alnus*	*Castanea*	*Corylus*	*Quercus*	Tmax	Tmin	Tmean	P	RH	Barberà	Montcada	Sabadell	Sant Cugat
January - December	Bet v 1	1,000	**,184****	**,262****	**-,127***	**,240****	-,023	-,067	**-,119***	-,092	-,007	-,094	,130	**,177***	**,153***	,076
	N	325	322	322	322	322	322	325	325	325	325	325	165	173	171	188
	Betula	**,184****	1,000	-,037	-,091	-,043	**,439****	,052	-,012	,022	-,126*	**-,196****	,022	**-,164***	-,075	-,115
	N	322	362	362	362	362	362	362	362	362	362	362	179	192	187	209
April - May	Bet v 1	1,000	,222	.	.	,133	-,247	,153	,088	,150	,075	-,018	,122	,372	**,622****	,336
	N	53	53	53	53	53	53	53	53	53	53	53	18	28	26	32
	Betula	,222	1,000	.	.	-,050	**,280***	,183	,046	,120	**-,393****	**-,282***	-,046	-,002	,181	,134
	N	53	61	61	61	61	61	61	61	61	61	61	20	33	29	36
Betula pollen presence (1)	Bet v 1	1,000	,219	,153	-,096	,160	-,158	,065	-,027	,032	,018	-,014	,258	**,429***	**,477****	**,339***
	N	72	72	72	72	72	72	72	72	72	72	72	27	35	35	41
	Betula	,219	1,000	-,113	-,202	**-,259***	**,306****	-,120	-,019	-,084	-,013	,089	-,091	-,173	,199	,013
	N	72	76	76	76	76	76	76	76	76	76	76	29	37	35	43
Bet v 1 presence (2)	Bet v 1	1,000	**,197****	**,198****	-,108	,146	,008	-,030	-,087	-,058	,023	-,060	,040	,105	,075	,043
	N	175	172	172	172	172	172	175	175	175	175	175	96	94	91	105
	Betula	**,197****	1,000	-,069	-,120	-,110	**,485****	,153*	,117	,144	-,113	**-,154***	-,036	**-,202***	,008	-,114
	N	172	179	179	179	179	179	179	179	179	179	179	99	98	94	109

(1) The working file contains only the values corresponding to the days with *Betula* pollen presence and the day before and after.
(2) The working file contains only the values corresponding to the days with Bet v 1 presence and the day before and after.

Table 14. Spearman's Rho correlation coefficients for Bellaterra 2015 between the concentrations of Bet v 1 and the pollen concentrations of *Betula*, *Alnus*, *Castanea*, *Corylus* and *Quercus*; the meteorological parameters and the PM10 concentrations. Different periods of airborne allergen and or pollen presence considered. Tmax (mean annual maximum temperature in ºC), Tmin (mean annual minimum temperature in ºC), Tmean (mean annual mean temperature in ºC), P (annual Precipitation in mm), RH (mean annual Relative Humidity in %). ** p<0.001; * p<0.05.

								Sant Cugat					PM10			
		Bet v1	*Betula*	*Alnus*	*Castanea*	*Corylus*	*Quercus*	Tmax	Tmin	Tmean	P	RH	Barberà	Montcada	Sabadell	Sant Cugat
January - December	Bet v1	1,000	**,304****	-,021	**,324****	-,049	**,470****	**,259****	**,184****	**,229****	-,066	**-,328****	,040	**,254****	,074	**,141***
	N	365	359	359	359	359	359	365	365	365	365	365	186	365	172	215
	Betula	**,304****	1,000	-,051	-,055	-,031	**,400****	,099	,007	,059	-,081	**-,195****	,010	**,217****	-,095	,071
	N	359	359	359	359	359	359	359	359	359	359	359	184	359	171	213
April - May	Bet v1	1,000	,117	-,164	.	-,139	**,316***	,202	,115	,186	,009	-,206	,294	**,326***	,144	,075
	N	61	57	57	57	57	57	61	61	61	61	61	33	61	26	37
	Betula	,117	1,000	,028	.	,123	-,130	-,002	-,150	-,072	-,060	-,048	,344	,161	-,106	,109
	N	57	57	57	57	57	57	57	57	57	57	57	32	57	26	36
Betula pollen presence (1)	Bet v1	1,000	,095	-,230	,026	-,214	**,283***	,201	,116	,181	-,160	-,230	,321	,247	,199	,101
	N	59	57	57	57	57	57	59	59	59	59	59	30	59	25	36
	Betula	,095	1,000	-,039	-,188	,038	-,110	-,066	-,143	-,107	,002	-,016	,206	,019	-,301	-,060
	N	57	57	57	57	57	57	57	57	57	57	57	30	57	25	35
Bet v 1 presence (2)	Bet v1	1	1	0,139	-0,123	**0,135**	**,181***	**,261****	**,229***	**,203***	**,229***	-0,101	**-,177***	0,026	**,257****	0,052
	N	.	124	120	120	120	120	120	124	124	124	124	124	60	124	65
	Betula	124	0,139	1	-0,143	**-,256****	-0,131	**,418****	-0,028	-0,114	-0,069	-0,09	-0,071	0,109	**,209***	**-,266***
	N	120	120	120	120	120	120	120	120	120	120	120	120	59	120	65

(1) The working file contains only the values corresponding to the days with *Betula* pollen presence and the day before and after.
(2) The working file contains only the values corresponding to the days with Bet v 1 presence and the day before and after

3. *Betula* pollen vs Bet v 1 2014 in Bellaterra and Vitoria-Gasteiz

The spatial study of Bet v 1 and *Betula* pollen in two localities of Northern Spain showed the following results. Bet v 1 showed different annual behavior in the two localities (Table 15). In Vitoria-Gasteiz Bet v 1 was detected in 23 days, whereas pollen was detected in 52 days. The Peak concentration of Bet v 1 was detected on April 8 at a concentration above the maximum concentration of our standard curve 6124.4 ng/m^3. The Peak concentration of Pollen was one week after, on April 15, with 29 pollen/m^3 (Table 15).

In comparison to Bellaterra (Barcelona), the Bet v 1 concentrations in Vitoria-Gasteiz were higher (6214 vs 4797 ng/m^3), the peak day appeared 3 days before (April 8 vs April 11) and they were present in the air less days (23 vs 98). Similarly, *Betula* pollen concentrations in Vitoria registered higher values (266 vs 194) and they stay longer in the air (52 vs 34 days) although the pollen concentration in the peak day was lower (29 vs 39).

The annual meteorological data of the two localities were different, registering a thermal amplitude (difference between maximum and minimum Temperature of the year) of 12º in Vitoria-Gasteiz against the 5º in Bellaterra (Barcelona).

Figure 19 shows the daily dynamics along the year of Bet v 1 and *Betula* pollen in both locations. While in Bellaterra (Barcelona) the dynamics of both particles showed their main behavior in April, in Vitoria-Gasteiz it is extended for one month more (April-May). In Bellaterra (Barcelona) some allergen peaks were observed in February and March while this was not the case in Vitoria-Gasteiz.

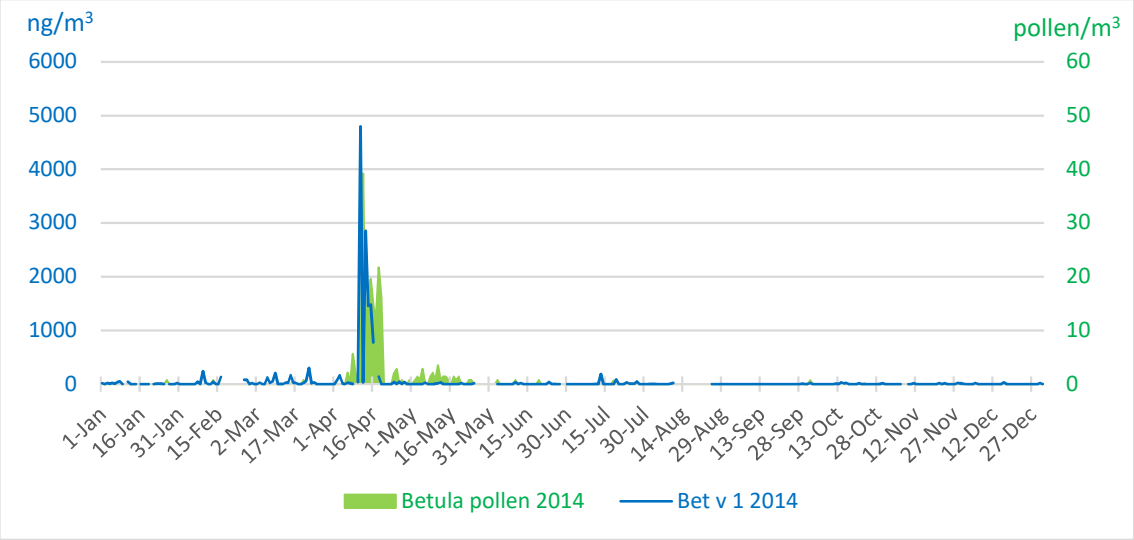
The monthly summary of Bet v 1, *Betula* pollen and meteorological parameters in Bellaterra (Barcelona) and Vitoria-Gasteiz areshown in Table 16.

In general, the higher concentrations of Bet v 1 and *Betula* pollen were detected during April. The Monthly Integral of pollen was outstanding in this month, with 165 pollen*day/m^3 in Bellaterra (Barcelona) registered in 17 days and 215 pollen*day/m^3 in Vitoria-Gasteiz in 30 days. In the other months the values did not exceed 22 pollen*day /m^3 distributed in 17 days (Bellaterra (Barcelona)) or 35 pollen*day /m^3 distributed in 16 (Vitoria-Gasteiz) in May.

Table 15. Bet v 1 allergen, *Betula* pollen and meteorological parameters in Bellaterra (Barcelona) and Vitoria-Gasteiz, annual summaries for 2014. AAIn (Annual Allergen Integral), APIn (Annual Pollen Integral), Tmax (mean annual maximum Temperature), Tmin (mean annual minimum Temperature), Tmean (mean annual mean Temperature), P (annual Precipitation), RH (mean annual Relative Humidity).0

	2014	
	Bellaterra (Barcelona)	**Vitoria-Gasteiz**
Bet v 1		
AAIn (ng*day/m^3)	14865	28608
Peak (ng/m^3)	4797	6124
Peak date	11-april	08-april
Days analyzed	325	356
Days with allergen presence	98	23
***Betula* Pollen**		
APIn (pollen*day/m^3)	194	266
Peak (pollen/m^3)	39	29
Peak date	12-april	15-april
Analyzed days	362	365
Days with pollen presence	34	52
Meteorological data		
Tmax (ºC)	21,8	19,2
Tmin (ºC)	10,9	13,2
Tmean (ºC)	16,3	7,2
P (mm)	714,5	807,2
RH (%)	73,8	78,3

a) Bellaterra



b) Vitoria-Gasteiz

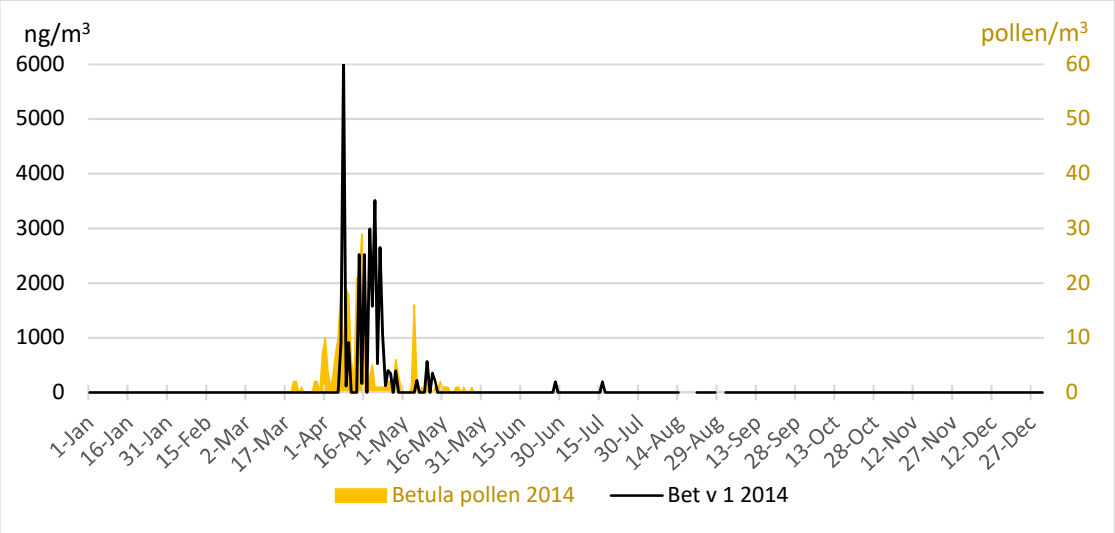


Figure 19. Annual dynamics of Bet v 1 (in ng/m^3) and *Betula* pollen (in pollen/m^3) daily concentrations during 2014 in a) Bellaterra (Barcelona) and b) Vitoria-Gasteiz.

Table 16: Bet v 1, *Betula* pollen and meteorological parameters in Bellaterra (Barcelona) and Vitoria-Gasteiz, monthly summaries for the detection period of year 2014. Daily concentrations in ng/m^3 and pollen/m^3. Monthly Integrals in ng*day/m^3 and pollen*day/m^3. Tmax (mean annual maximum Temperature in ºC), Tmin (mean annual minimum Temperature in ºC), Tmean (mean annual mean Temperature in ºC), P (annual Precipitation in mm), RH (mean annual Relative Humidity in %).

		Number of days with detection		Max. daily concentration detected		Monthly Integral		Meteorological parameters				
		Bet v 1	*Betula* Pollen	Bet v 1	*Betula* pollen	Bet v 1	*Betula* pollen	Tmax	Tmin	Tmean	P	RH
Bellaterra (Barcelona)	Jan	12	1	49 (08)	1 (26)	233	1	14,0	4,7	9,4	46,9	78.1
	Feb	8	1	242 (9)	1 (13)	665	1	15,0	4,4	9,7	26,1	70.5
	March	17	2	298 (22)	1 (16)	1096	1	18,3	5,8	12,0	22,5	67.5
	April	17	17	4797 (11)	39 (12)	11967	165	21,5	9,7	15,6	64,2	73.6
	May	6	17	30 (12)	3 (8)	104	22	22,6	11,1	16,8	68,5	71.8
	June	4	3	37 (23)	1 (3,10,19)	90	2	28,1	15,6	21,8	10,1	64.7
	July	8	1	187 (13)	1 (18)	379	1	29,3	17,6	23,5	79,6	66.3
Vitoria-Gasteiz	Jan	0	0	0	0	0	0	11,2	3,8	7,5	91,6	81,1
	Feb	0	0	0	0	0	0	12,0	1,4	6,7	80,5	76,0
	March	0	6	0	7 (31)	0	16	15,2	2,2	8,7	138,4	77,2
	April	17	30	6124 (8)	29 (15)	26863	215	20,3	6,0	13,1	36,8	75,3
	May	4	16	564 (10)	16 (5)	1352	35	19,6	6,4	13,0	35,0	73,7
	June	1	0	196 (28)	0	196	0	26,0	10,7	18,3	35,0	74,0
	July	1	0	196 (16)	0	196	0	26,3	12,5	19,4	37,6	76,5

Statistical analysis

The Spearman correlation analyses between Bet v 1 and *Betula* pollen showed positive correlation in both cities (Bellaterra (Barcelona) rs=0.184, P < 0.001, N=322 and Vitoria-Gasteiz rs=0.556, P < 0.001, N=356), as shown in Table 17 a and b. However, as already said in the previous chapters, this could be due to the abundance of 0 values in both series. Thus, Table 17 shows also other three periods of airborne allergen and of pollen presence: the "April-May" period used to compare the concentrations during these two months; the "*Betula* pollen presence" period which contains only the values corresponding to the days with *Betula* pollen presence and the day before and after; and the "Bet v 1 presence" period which contains only the values corresponding to the days with Bet v 1 presence and the day before and after. Taking into account these new periods, it is observed that in Vitoria-Gasteiz, Bet v 1 presented , in all the cases and again, positive and significant correlation with *Betula* pollen (Table 17b).

The meteorological correlation showed negative signification for Precipitation and Relative Humidity during April-May in both localities and positive and significance with *Betula* pollen in Vitoria-Gasteiz during the months of maximum concentration ("April-May") and the period "*Betula* pollen presence" for maximum and mean Temperature, and negative in case of Relative Humidity (Table 17b).

According to the information from Allergome, (www.allergome.org, accessed November 2018), Bet v 1 presents cross reactivity with allergens of other anemophilous plants

like *Alnus*, *Castanea*, *Corylus* and *Quercus*. In the study areas of Bellaterra (Barcelona) and Vitoria-Gasteiz these taxa are present, and their pollination occur in different periods of the year and in some cases coincide with the *Betula* pollination. Thus, they could also have contributed to the Bet v 1 presence detected in the results. Tables 17 a and b show the results of the Spearman correlations. As said in previous chapters, in this case we will not consider the results of the period January-December, because the abundance of 0 values give a false correlation coefficient. Takning this into account, the only correlation obtained between Bet v1 and the cross reactive pollen types is with *Alnus* pollen in the case of Bellaterra (Table 17 a).

Finally, Spearman correlation tests between atmospheric PM 10 concentrations and Bet v 1 and *Betula* pollen concentrations (Tables 17 a and b) showed that in the Montcada station (which is in the vicinity of Bellaterra, Barcelona) there was a negative significant correlation with Bet v 1 (r_s= -0.202, N= 98, P < 0.05), while in Vitoria-Gasteiz a positive significant correlation was observed between PM10 and Bet v 1 concentrations of the "April-May" period (r_s= 0.287, N= 54, P < 0.05).

Table 17. Spearman's Rho correlation coefficients for a) Bellaterra (Barcelona) and b)Vitoria-Gasteiz 2014 between the concentrations of Bet v 1 and the pollen concentrations of *Betula*, *Alnus*, *Castanea*, *Corylus* and *Quercus*; the meteorological parameters and the PM10 concentrations. Different periods of airborne allergen and or pollen presence considered. Tmax (mean annual maximum temperature in ºC), Tmin (mean annual minimum temperature in ºC), Tmean (mean annual mean temperature in ºC), P (annual Precipitation in mm), RH (mean annual Relative Humidity in %). ** p<0.001; * p<0.05.

a) Bellaterra (Barcelona)

								Sant Cugat					PM10			
		Bet v1	*Betula*	*Alnus*	*Castanea*	*Corylus*	*Quercus*	Tmax	Tmin	Tmean	P	RH	Barberà	Montcada	Sabadell	Sant Cugat
January - December	Bet v1	1,000	**,184****	**,262****	**-,127***	**,240****	-,023	-,067	**-,119***	-,092	-,007	-,094	,130	**,177***	**,153***	,076
	N	325	322	322	322	322	322	325	325	325	325	325	165	173	171	188
	Betula	**,184****	1,000	-,037	-,091	-,043	**,439****	,052	-,012	,022	-,126*	**-,196****	,022	**-,164***	-,075	-,115
	N	322	362	362	362	362	362	362	362	362	362	362	179	192	187	209
April - May	Bet v1	1,000	,222	.	.	,133	-,247	,153	,088	,150	,075	-,018	,122	,372	**,622****	,336
	N	53	53	53	53	53	53	53	53	53	53	53	18	28	26	32
	Betula	,222	1,000	.	.	-,050	**,280***	,183	,046	,120	**-,393****	**-,282***	-,046	-,002	,181	,134
	N	53	61	61	61	61	61	61	61	61	61	61	20	33	29	36
Betula pollen presence (1)	Bet v1	1,000	,219	,153	-,096	,160	-,158	,065	-,027	,032	,018	-,014	,258	**,429***	**,477****	**,339***
	N	72	72	72	72	72	72	72	72	72	72	72	27	35	35	41
	Betula	,219	1,000	-,113	-,202	**-,259***	**,306****	-,120	-,019	-,084	-,013	,089	-,091	-,173	,199	,013
	N	72	76	76	76	76	76	76	76	76	76	76	29	37	35	43
Bet v 1 presence (2)	Bet v1	1,000	**,197****	**,198****	-,108	,146	,008	-,030	-,087	-,058	,023	-,060	,040	,105	,075	,043
	N	175	172	172	172	172	172	175	175	175	175	175	96	94	91	105
	Betula	**,197****	1,000	-,069	-,120	-,110	**,485****	,153*	,117	,144	-,113	**-,154***	-,036	**-,202***	,008	-,114
	N	172	179	179	179	179	179	179	179	179	179	179	99	98	94	109

(1) The working file contains only the values corresponding to the days with *Betula* pollen presence and the day before and after.
(2) The working file contains only the values corresponding to the days with Bet v 1 presence and the day before and after.

b) Vitoria-Gasteiz

		Bet v1	*Betula*	*Alnus*	*Castanea*	*Corylus*	*Quercus*	Tmax	Tmin	Tmean	P	RH	PM10
January - December	Bet v1	1,000	**,556****	-,091	-,047	**-,126***	**,395****	,097	-,062	,040	-,007	-,084	,034
	N	356	356	356	356	356	356	356	356	356	356	356	307
	Betula	**,556****	1,000	-,089	**-,170****	**-,137****	**,538****	,063	**-,149****	-,020	-,100	**-,225****	-,018
	N	356	365	365	365	365	365	365	365	365	365	365	316
April - May	Bet v1	1,000	**,413****	.	.	.	,028	,155	-,258*	,017	,049	,048	,190
	N	61	61	61	61	61	61	61	61	61	61	61	54
	Betula	**,413****	1,000	.	.	.	,074	**,454****	-,123	**,397****	-,248	**-,279***	**,287***
	N	61	61	61	61	61	61	61	61	61	61	61	54
Betula polen presence (1)	Bet v1	1,000	**,383****	-,138	.	-,161	,168	,210	-,094	,122	,064	,084	,159
	N	69	69	69	69	69	69	69	69	69	69	69	62
	Betula	**,383****	1,000	-,029	.	-,044	,101	**,445****	,032	**,413****	**-,276***	**-,272***	,237
	N	69	69	69	69	69	69	69	69	69	69	69	62
Bet v 1 presence (2)	Bet v1	1,000	**0,300***	.	-,313	.	,112	-,152	-,275	-,233	,371*	,104	-,077
	N	38	38	38	38	38	38	38	38	38	38	38	38
	Betula	**0,300***	1,000	.	**-,580****	.	**,451****	,092	-,293	-,001	,082	-,208	,078
	N	38	38	38	38	38	38	38	38	38	38	38	38

(1) The working file contains only the values corresponding to the days with *Betula* pollen presence and the day before and after.
(2) The working file contains only the values corresponding to the days with Bet v 1 presence and the day before and after.

4. *Alternaria* spore vs Alt a 1 2015 in Bellaterra

The last objective of the present thesis was to detect the airborne fungal allergen (Alt a 1) in the atmosphere of Bellaterra (Barcelona) and to quantify its allergenic load. To do this, daily airborne samples obtained during 2015 with a multi-vial air cyclone sampler were analyzed with the ELISA technique. In no case Alt a 1 was detected while *Alternaria* spores were present in the air during the whole year, mainly from April on (Figure 20).

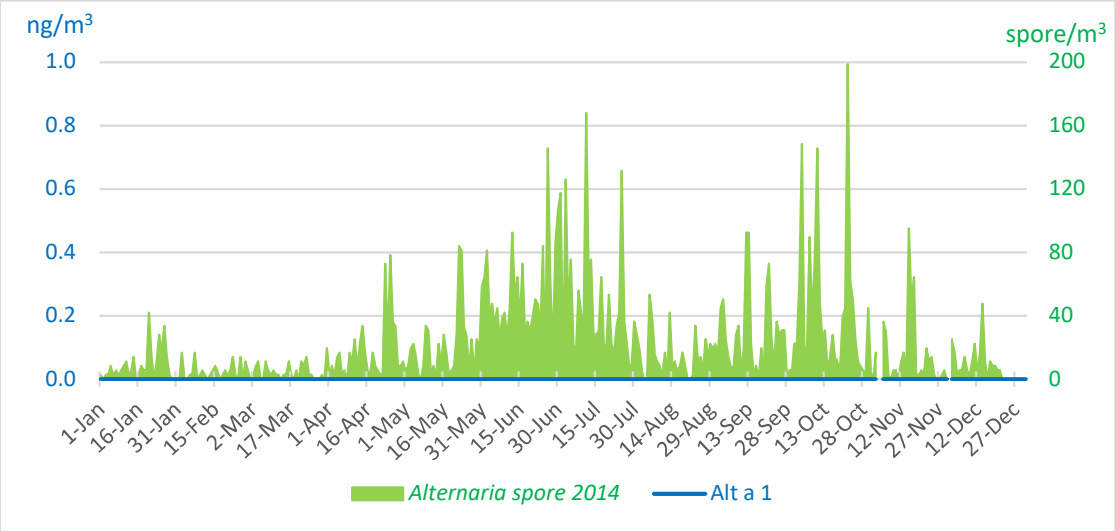


Figure 20. Comparison between daily airborne *Alternaria* spores (spore/m^3) and airborne Alt a 1 allergen (ng/m^3) concentrations during 2014 analyzed using a Hirst sampler and a Cyclone sampler, respectively (Note: the measurements of Alt a 1 in whole the year resulted in 0 ng/m^3).

With the hypothesis that if there are *Alternaria* spores in the air there should be also airborne the allergen Alt a 1, and having seen the results obtained with the multi-vial cyclone, we decided to use another sampler-collector to try to detect Alt a 1. In this case, we used a High volume TSP (Total Suspended Particles) collector (MCV sampler) with an aspiration rate of 40 m^3/h during a period of 15 days (July 16-30, year 2015). The samples were processed as shown in the "Methods" section to realize the qualitative and quantitative studies.

A qualitative study was done by a dot-blotting immunoassay analysis, using serum from a Rabbit. As a result, all the days analyzed (12 days in total) exhibited reaction while with

different intensity (see Figure 21). According to these results, we realized a quantitative analysis by means of the ELISA technics.

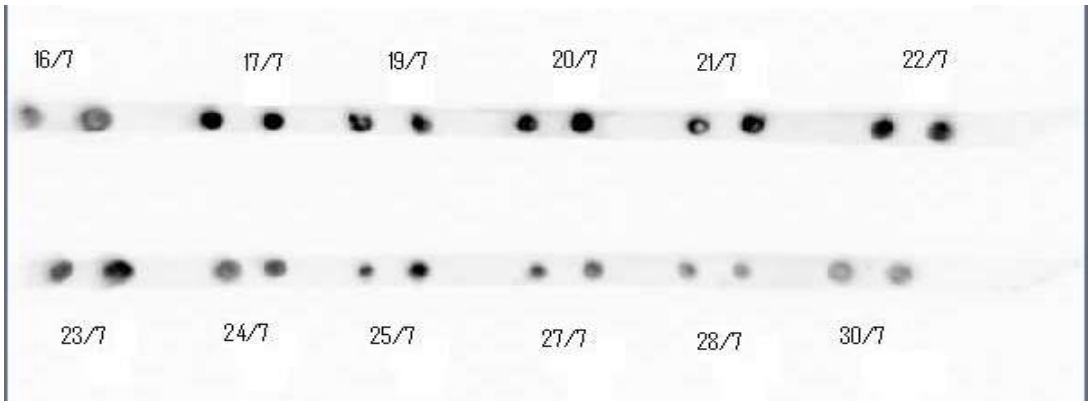


Figure 21. Qualitative study by Dot-blot analysis of Alt a 1 of MCV samplers (16th to 25th-July).

Purified Alt a 1 was used as reference material in a standard curve with concentrations between 1000 and 0.09 ng/ml. The working range of the assay was defined as the lineal portion of the curve. (Figure A, Material and methods section).

As shown in Table 18, airborne *Alternaria* spores and airborne Alt a 1 were detected during July 16-30, year 2015. The samples obtained with the MCV sampler registered Alt a 1 in 10 out of the 15 days of sampling. The highest *Alternaria* spore concentration was recorded in July 28 with 87 spore/m^3 and at this day also 0.63 pg/m^3 of Alt a 1 were registered. The maximum allergen concentration was obtained in July 17 (0.89 pg/m^3) when the airborne *Alternaria* concentration was 14 spore/m^3 (Table 18 and Figure 22). Another interesting result is that on the samples of the days19 and 20 of July 2015 Dot-blot could detect Alt a 1, whereas, ELISA could not detect it. This means that Dot-Bot is more sensible than ELISA in the detection of the allergen, although the method do not permit the quantification of the allergen.

Table 18. Quantitative study of *Alternaria* spores (spore/m^3) and Alt a 1 (pg/m^3) obtained from different samplers during 16th to 25th-July 2015. ND. No detected.

		16-jul	17-jul	18-jul	19-jul	20-jul	21-jul	22-jul	23-jul	24-jul	25-jul	26-jul	27-jul	28-jul	29-jul	30-jul
Hirst sampler	**spores**	14	14	28	39	22	6	3	11	34	25	39	42	87	17	22
Cyclone sampler	**Alt a 1**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
MCV sampler	**Alt a 1**	0,42	0,89	ND	ND	ND	0,78	0,51	0,67	0,48	0,77	ND	0,58	0,63	ND	0,73

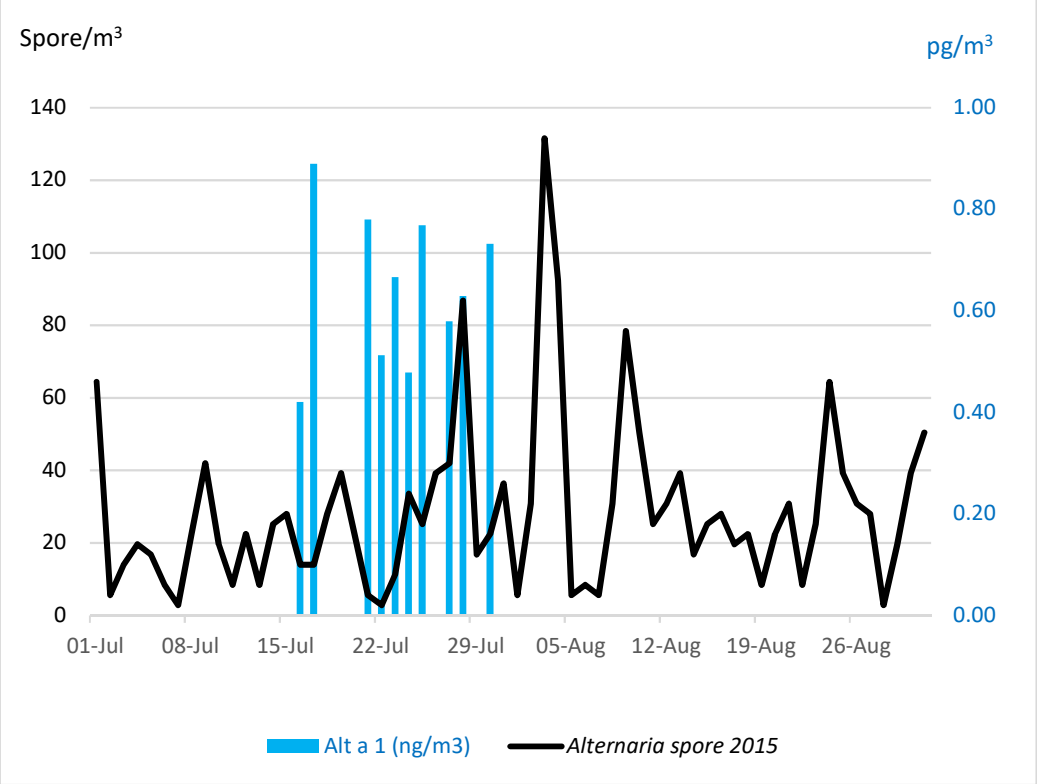


Figure 22 Figure 22. Dynamics of daily concentrations of Alternaria spores (spore/m^3) and Alt a 1 concentrations (pg/m^3) during the period 16th to 25th-July (Note: there are two measurements which resulted in 0 ng/m3, on 19 April and on 20 July)

V. DISCUSSION

The analysis of the allergenic load carried out in the environments of Bellaterra (Barcelona) and Vitoria-Gasteiz is presented as a complementary study to aerobiological analyzes carried out on airborne pollen and spores.

As in other researches, this thesis has shown that, together with pollen grains and fungal spores in the air, there are also other biological particles with the same or greater degree of allergenicity (Moreno-Grau et al. 2006, De Linares et al., 2007, Rodríguez-Rajo et al. 2011; Buters et al. 2012). This thesis has shown the existence of these particles (allergens), their annual dynamics and the relationship with airborne pollen and spores as well as with the meteorological parameters and the PM10.

The use of sampling collectors such as Multi-vial Cyclone samplers (with an aspiration rate of 16 L / min) has allowed to collect enough sample of pollen allergens for its detection and quantification, with the only limitation of the possibility to measure only one allergen type per sample. However, the detection of fungal allergens such as Alt a 1 has not been possible. That is why it was carried out a test for a few weeks with a different sampler, a high volume sampler (MCV sampler with a suction capacity of 40m^3/h) which was able to collect enough material for the detection and quantification of this fungal allergen.

The immunological techniques used (ELISA and Dot- Blotting) in this thesis have also been shown to be good detection methods, amplifying the signal sufficiently despite obtaining data with values in picograms per milliliter (pg / mL) as the case of Alt a 1. ELISA was the method that permitted the quantification.

Phl p 5 and *Poaceae* Bellaterra Barcelona 2013 - 2015

This study investigated the airborne Phl p 5 dynamics and its correlation with *Poaceae* pollen in the years 2013 and 2015. The results obtained showed a parallel dynamics between the pollen and the allergen concentrations.

The peak month of Poaceae pollen was in June in 2013 but in 2015 was in May. This variation (May and June) was similarly reported in a study done in Catalonia where it was shown that the maximum Poaceae daily pollen concentration can be reached from mid-May

to mid-June (Latorre and Belmonte 2004). The cause of this variation was attributed to several factors such as the flowering grass taxon, some regional characteristics, the meteorological parameters of the year, etc.

In our study, and in the case of Phl p 5 these variations were also detected and even more markedly. Perhaps, as was reported in Plaza et al. (2017), the use of different sampler types e.g. multi-vial Cyclone sampler (Burkard®) and High volume cascade impactor (ChemVol®) can be a potential cause of the detection dates variations.

Despite the timing mismatch between Phl p 5 and Poaceae pollen, the Spearman correlation tests showed a positivecorrelation between Poaceae pollen and allergen daily concentrations for the both years under study. This result agrees with the results of other studies (De Linares et al. 2010, Plaza et al 2016, Buters et al. 2012). On the contrary, several studies concerned about Poaceae concluded that aeroallergen and pollen concentrations did not show significant correlations (Gonzalez-Parrado et al. 2014; Rodríguez-Rajo et al. 2011; Fernández-González et al. 2010).

In the present study, in the locality of Bellaterra (Barcelona) the peak detection of Phl p 5 in both years (2013 and 2015) was on 19 and 7th of May respectively. Similarly, the peaks of Phl p 5 were also reported in May for the years (2012, 2013 and 2014) in Cordoba- Spain (Plaza et al., 2016).

Many days were observed when pollen did not release allergens at the beginning and the end of the year. This could be justified due to the potency variation of each pollen grain to release allergens (Buters et al. 2015). Moreover, several days in both years were observed to have Phl p 5 and no Poaceae pollen. Such observation could be justified due to the difference in particle sizes between the particles bearing the allergen and the pollen grains, therefore, they might show different aerodynamics in terms of flying durations and transport (Jochner et al., 2015; Plaza et al., 2016; Schäppi et al., 1999).

Poaceae pollen existed at the end of both years in September, October and November. But no pollen allergen could be detected. One explanation could be that the flowering season of some *Poaceae* subfamilies e.g. Panicoideae as *Paspalum sp.* was reported in Catalunya region (Verloove, 2005). The above mentioned subfamily was notably reported to contain group 1 and but not 5 allergens, the allergen group of Phl p 5 (Marth et al., 2004; Scala et al., 2010)

The results of the correlation tests of this study between Phl p 5 and *Poaceae* with meteorological parameters (Precipitation, Temperature and Relative Humidity) was found to be similar to those of other simultaneous studies done in Cordoba, Spain (Plaza et al. 2017; Jochner et al. 2018).

The start of Phl p 5 detection in 2015 was in March and in 2013 it was in April, one month later. According to Behrendt et al. (1999), the release of allergens is pH and temperature dependent and maximum values of 37°C and pH 9.0 can produce the release of Phl p 5. About 10% of the total proteins released from grass pollen has shown to be Phl p 5 (Behrendt et al. 1999). In an early study (Behrendt et al. 1991), the mechanism of protein release from *Dactylis glomerata* grass pollen after a two-hour incubation period of pollen with ammonium carbonate buffer—a method to obtain extracts of high allergen content—was investigated by conventional transmission and scanning electron microscopy. Transmission electron microscope analysis revealed a reduction of electron density and lamellation of the intine, fusion of cytoplasmic p-particles and exposure of granular matrices to the intine. In addition, granular material appeared outside the exine, just on top of microchannels—indicating liberation of probably allergenic material. This was also shown ten years later—by means of immunogold field emission scanning and transmission electron microscopy—for hydrated rye-grass pollen (Grote et al. 2000).

Phl p 5 and pollen results from the present study were positively correlated with temperature. The correlation increased during 2015, the drier year and the one with the higher average temperature. A similar comparison between 2012 and 2014 in Cordoba showed also positive correlation between Phl p 5 and pollen with temperature. Also, there were higher allergen and pollen concentrations in the warmer year (Plaza et al., 2017).

In 2015, Poaceae pollen and Phl p 5 were negatively correlated with Precipitation. In the case of airborne pollen, this can be explained by the rainfall washing out the big particles from the air. (Schäppi et al. 1999, Taylor et al. 2002). In the case of the allergen, in rare situations—for example during a thunderstorm— pollen may break by osmotic shock, releasing allergen-containing starch particles. This finding might explain the higher frequency of asthma attacks during thunderstorms as reported in Australia (D'Amato, Liccardi, and Frenguelli 2007). In the case of Bellaterra (Barcelona) this phenomenon was not detected. It may have occurred, as with pollen, that the airborne allergen could have been washed out by the precipitation.

In the both study years, the humidity was negatively correlated with both Phl p 5 and *Poaceae* pollen. The recorded average value of the RH during the peak months (May and June) of pollen and allergen integral was 62% to 67%. Similar to the in vitro observations that showed that relatively dry air with relative humidity oh 65% increased particle counts in the test chamber and also increased the allergen release. However, in the same experiment when relative humidity increased to 88% and above, it caused the anther to close and encapsulate the pollens. (Taylor et al., 2002).

In both study years, the humidity was negatively correlated with both Phl p 5 and Poaceae pollen. This meteorological variable is related with the precipitation. If there is rainfall, the RH increases. As occurred with precipitation, this meteorological variable can provoke the decrease of airborne pollen and allergen.

Phl p 5 and Poaceae pollen in both years were positively correlated with PM10 concentrations monitored in the Montcada station. This coincides with the findings that Poaceae pollen and 80% allergen bearing particles were found in particles bigger than 7.2 µm (Rantio–Lehtimäki, Viander, and Koivikko 1994) and in particles bigger than 10 µm in Melborne Australia (Schäppi et al. 1997) and similarly in Germany (Jochner et al. 2015).

Bet v 1 and *Betula* pollen in Bellaterra – Barcelona Spain.

The present study reveals the annual dynamics of Bet v 1 and its correlation with *Betula* pollen, meteorological factors and pollution.

The main pollen and allergen season coincided during weeks 2 and 3 of April. In 2014 the detection start was 9 days before than in 2015 for both pollen and allergen. These small variations were also reported during 2009 for *Betula* and Bet v 1 in other four European cities: Munich (Germany), Turku (Finland), Lyon (France) and Worcester (UK) (Buters et al. 2012a).

In both, 2014 and 2015, allergenic load were detected in the periods preceding and succeeding peak pollen grains. This phenomenon was also reported in *Betula* (Schäppi et al. 1997) and in other taxa as *Olea* (De Linares et al. 2007; Galan et al. 2013).

Results showed a variation for Bet v 1 and *Betula* pollen between the years 2014 and 2015, in terms of concentration, number of detection days and also in timing. 2014 showed higher

values, both in pollen as in Bet v 1 concentrations. These variations could be explained by the differences in meteorological factors between the years. The precipitation during 2015 was significantly lower in amount and in number of days with rain especially during the peak month of detection. Also lower minimum and average temperatures were recorded in 2015 during the peak month. This could have make impossible an adequate flowering. The effect of year to year variation in meteorological factors on *Betula* pollen and Bet v 1 concentrations was similarly reported by Buters et al (2008).

In both 2014 and 2015, Bet v 1 peak was one day before the pollen peak . Also, in both years the day before peak Bet v 1 coincided with light rainfall (2014, 1.4 mL/24h) and (2015, 3.4mL/24h). Some researches have postulated that the airborne allergen particles increase after rainfall (Rantio–Lehtimäki, Viander, and Koivikko 1994). Also, that light rainfall (1mL/24h) can be responsible for the germination of 80 % of the leaves in which sticking birch pollen is adhered and then release as aerosols combined with starch granules and with other particles in the atmosphere (Schäppi et al. 1997).

Several studies have shown the presence of Bet v 1 in airborne particles of different sizes and in these cases it has been stated, that 80% of Bet v 1 was detected in particles bigger than 7.2 μm (Schäppi et al. 1997); in particles bigger than 10 μm (Buters et al. 2012); and in particles smaller than PM2.5 (Miguel et al. 1999; Schäppi et al. 1997; Knox et al. 1997). According to them, the statistical results of this thesis where Bet v 1 was positively correlated in 2014 with *Betula* pollen and PM10 during the "Bet v 1 presence" period could be related to the distributions of this allergen in particles smaller than 10 μm.

In 2015 results showed a positive correlation between *Betula* pollen and temperature and in 2014 there was also a positive correlation between Bet v 1 with temperature. The explanation of this positive correlation was reported by Hjelmroos (1991) when it was proved that temperature has a direct influence on the date of *Betula* flowering start. In addition, another study of 18 to 30 years period for 4 different cities in Europe has proven that temperature has also decisive positive influence on *Betula* pollen counts (Spieksma et al., 1995)

In the present study, for the two years relative humidity was negatively correlated with *Betula* pollen. In a three years study *Betula* pollen showed also negative correlation with relative humidity (Sousa et al., 2008). Buters et al (2008) showed that the year and the area

with lower average relative humidity showed less flying *Betula* pollen than other year and location.

The immunochemical and molecular characterization of Bet v 1 has therefore led to the definition, purification, and partial amino acid sequence characterization of major allergens of Bet v 1 in different plants (Viander et al. 1979; Ipsen and Løwenstein 1983). Bet v 1, a 17-kd protein, has been identified as the major birch pollen allergen that shares epitopes with the major pollen allergens of trees belonging to the pollen of *Fagales* order and its plant-derived food (Ipsen and Hansen, 1990; Ipsen and Løwenstein, 1983; Vik and Elsayed, 1986). By means of qualitative IgE inhibition experiments, it was demonstrated that recombinant Bet v 1 and Bet v 2 share IgE epitopes with allergens from various pollens and plant-derived foods and thus can be responsible for clinically relevant IgE-mediated cross-reactions (Ebner et al., 1995; Valenta et al., 1992; Vallier et al., 1992). In the present study and for the two years, Bet v 1 could be correlated with other cross reaction pollen species such as in 2014 *Alnus, Corylus* and *Quercus*, and in 2015 Bet v 1 with *Castanea,* and *Quercus*. The cause of such cross-reaction is due to the presence of homologous allergens in pollen grains and other parts of other plant families such as fruits or leaves. These homologues are mainly members of the Bet v 1 (the major birch pollen allergen) and profilin families, as well as cross-reactive carbohydrate determinants, seems to be reported in a number of studies such as *Corylus* (Ortolani et al., 2000), *Castanea* (Salcedo et al., 2004), and *Alnus* (Gajhede et al., 1996).

Bet v 1 and *Betula* pollen in Bellaterra and Vitoria-Gasteiz.

This study investigated Bet v 1 and *Betula* pollen dynamics in 2 different locations simultaneously. Additional investigation about the correlations between the pollen and the allergens and the meteorological factors in both locations was undertaken.

The Annual Pollen Integral (APIn), Annual Allergen Integral (AAIn) and the value of maximum daily concentration in Vitoria-Gasteiz were higher than in Bellaterra (Barcelona). This could be explained by the geographical location of Vitoria-Gasteiz that is characterized for being surrounded by Atlantic vegetation where the birch is a more common plant than in the Mediterranean vegetation surrounding Bellaterra (Barcelona).

The peak dates of both pollen and allergen coincided in April. The peak dates were similarly and frequently occurring in April in many other European localities: Basel, Vienna, London and Leiden (Spieksma et al. 1995a), Munich (Buters et al., 2008) or Córdoba (Plaza et al., 2017).

In Vitoria-Gasteiz, Bet v 1 showed positive and significant correlations with *Betula* pollen in all the periods studied, while Bellaterra (Barcelona) did not. The explanation could be that the comparably high pollen concentration in Vitoria-Gasteiz facilitates the coincidence of pollen and allergens in the air while in Bellaterra (Barcelona), they do not coincide because of the lower concentrations and in this case other factors play a role.

Betula pollen was positively correlated with temperature during the main pollen period in both locations. Also, the start of pollen detection in Barcelona (warmer) was in January, two months earlier than in Vitoria-Gasteiz. Such observations could be explained by the earlier flowering start in warmer areas. This is in concordance with the observation that temperature proved to be a decisive factor on the start of flowering time (Hjelmroos, 1991).

Alt a 1 and *Alternaria* Bellaterra 2015

The objective of this study was to establish a reliable sampling technique for the fungal allergen quantification and to study and compare the efficiency of different analytical methodologies to quantify Alt a 1.

Results have shown that the volume of air sampled in addition to the nature of the sample play an important role in the detection and quantification of fungal allergens. The analyses of the samples collected with the multi-vial cyclone air sampler were scarce and the results obtained were outside the range of detection of the ELISA technique.

Considering that other studies using high volume samplers obtained good results (Agarwal et al. 1983; Feo-Brito et al. 2012; Buters et al 2008, Caravalho 2008), in the present study we decided to use a MCV high volume air sampler looking for to obtain the daily maximum concentration of particles. Due to the higher air volume sampled, higher quantity of particles and consequently, higher amount of the fungal material could be obtained.

As in the present study, Cage et al (1996) wanted to compare four different bioaerosol samplers in the outdoor environment to detect Alt a 1. Test samplers used included a Rotorod, a Kramer-Collins suction trap, an all-glass impinger (AGI-30), and a high-volume cyclonic liquid impinger (Spin-Con). The High volume SpinCon® sampler collected a larger number of spores than the other devices. The allergen Alt a I and GP70 were collected by both collectors; however, the SpinCon collected more Alt a I and the AGI-30 collected more GP70. Similar results were shown in the present thesis. The comparative study between Multi-vial cyclone sampler and MCV high volume sampler showed that the fungal allergenic load in the air is so low that is necessary the use of a high volume sampler.

There are no enough reports on if allergen could be present while no spore are in the atmosphere. Twaroch et al. (2015) reported that Alt a 1 was found exclusively in the cell wall of the spores and not in the cytoplasm. In contrast to data obtained in 2 earlier studies describing a diffuse labeling of the cytoplasm and cell wall (Paris et al. 1991; Ibarrola et al. 2004). Other study reports that liquid atmosphere similar to rain may have caused elution and dislocation of Alt a 1 in liquid cultures, a phenomenon that has been found to cause diffusion of allergens also from pollen grains upon hydration (Grote 1999). Results showed that airborne *Alternaria* spores were present in all days with Alt a 1 detection. Due to the low number of samples no statistical analysis was done in the present study. Similar studies performed by Feo-Brito et al. (2012) and Agarwal et al. (1983) obtained weak statistical significance. .

In this study it is shown two possible techniques to detect and quantify Alt a 1. As the result of Dot blot is 100 times more sensitive than ELISA (J. S. Hu. 1995; Ekebom, Vesterberg, and Hjelmroos 1996) in this studied is decided detect Alt a 1 with this technique and then to use the samples to quantify Alt a 1 with ELISA analysis.

In comparison to ELISA, Dot-blot has shown higher detection capacity for Alt a 1. During 2 days (July 19 and 20) Alt a 1 could be detected by dot-blot and not ELISA. However with this techique is obtained a qualitative study and is not possible to know if the Alt a 1 increase or decrease throughout time. However, ELISA technique, despite being less sensitive can quantify the airborne Alt a 1 and show it behavior.

VI. CONCLUSIONS

With the results obtained in this Doctoral Thesis and considering the objectives that were intended to be achieved, the following conclusions have been drawn:

General results:

1. The allergenic load of Phl p 5 and Bet v 1 using a Multi-vial Cyclone has been detected for the first time in Bellaterra (Barcelona) and Vitoria-Gasteiz.
2. The daily samples of the Multi-vial Cyclone permitted only the detection of an allergen at the time (not of several of them as initially pretended).
3. The allergenic load of Phl p 5 and Bet v 1 has been quantified by means of ELISA analyses.
4. To detect the allergenic load of Alt a 1 in Bellaterra (Barcelona) was not possible with the Multi-vial Cyclone sampler and has required to set up a different sampling and quantifying methodologies.
5. A High Volume Sampler for Total Suspended Particles has proven to be useful for the collection, and posterior detection and quantification, of the fungal allergen Alt a 1 for the first time in Bellaterra (Barcelona).
6. The allergenic load of Alt a 1 has been detected using Dot-Blotting Immunological analyses.
7. The allergenic load of Alt a 1 has been quantified by means of ELISA analyses.
8. Dot-Blotting Immunological analyses showed to be more sensible to Alt a 1 presence than ELISA analyses.
9. The total allergen released per year varied from one year to another as well as from one location to another.
10. Phl p 5 and Bet v 1 Annual Allergen Integral in year 2015 were 10 times lower than in 2013 and 2014, respectively.
11. Poaceae pollen was detected in days where Phl p 5 was not detected.
12. Bet v 1 was detected in days where *Betula* pollen was not found.
13. There was a positive significant correlation between daily concentrations of Phl p 5 and Poaceae pollen in Bellaterra (Barcelona), years 2013 and 2015.
14. There was a positive significant correlation between daily concentrations of Bet v 1 and *Betula* pollen in Vitoria-Gasteiz, year 2015.

15. There was no significant correlation between daily concentrations of Bet v 1 and *Betula* pollen in Bellaterra (Barcelona), years 2014 and 2015.
16. Bet v 1 was present in the atmosphere outside the main pollination season of *Betula* and this could be related to the release of Bet v 1 from other cross-reactive species.
17. Bet v 1 daily concentrations showed a positive correlation with daily *Quercus* pollen concentrations in Bellaterra (Barcelona), year 2014, but not in year 2015 neither in Vitoria-Gasteiz 2015.
18. No correlation was found between the daily concentrations of Bet v 1 and the pollen concentrations of *Alnus*, *Castanea* and *Corylus*, neither in Bellaterra (Barcelona) nor in Vitoria-Gasteiz.
19. The relationship between daily concentrations of Alt a 1 and airborne *Alternaria* spores could not be reported due to the short period of sampling with the trap that permitted to detect it.
20. Phl p 5 and Bet v 1 daily concentrations did not show correlation with the meteorological parameters (Maximum, minimum and mean Temperature, Precipitation, Relative Humidity).
21. Phl p 5 and Bet v 1 daily concentrations in Bellaterra (Barcelona) showed positive correlation with the PM10 values from Montcada while no correlation was found in Vitoria-Gasteiz.
22. The abundance of plants in the vicinity of the aerobiological sampling station helped the correlation between allergen and pollen concentrations.

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VIII. Annexes

1. Annex 1: ELISA protocol for Phl p 5 of Indoor Biotechnology^{INC}

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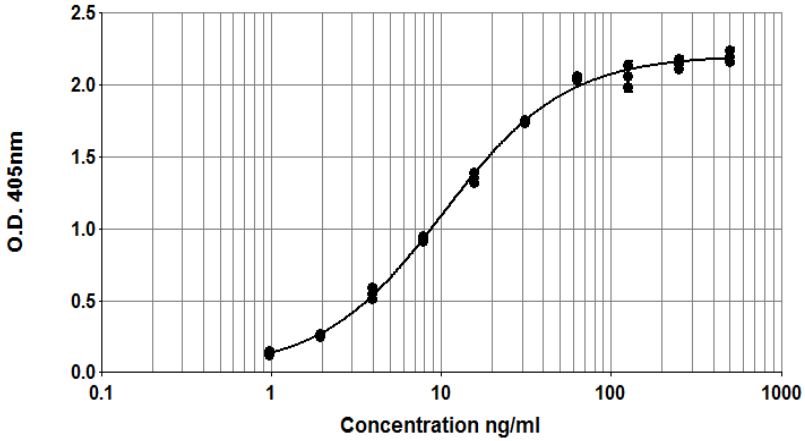
Phl p 5 ELISA kit (1D11/Bo1)

Product Code: EL-PP5

Lot Number: xxxxx

Sample Curve:



Content:

Vial 1 (red top) 100 µL
Monoclonal antibody 1D11
Concentration: 1.5mg/ml in PBS

Vial 2 (white top) 400 µL
Phl p 5 Standard
Concentration: 5000ng/ml rPhl p 5a

Vial 3 (brown) 100 µL
Biotinylated monoclonal antibody Bo 1
Dilute: 1:1000 for use

Storage: All reagents should be stored at 4°C

For research and commercial use in vitro: not for human in vivo or therapeutic use.

Certificate of Analysis

Monoclonal Antibody:	1D11 (clone 1D11 C8)
Immunogen:	Timothy pollen extract
Isotype:	Mouse IgG1
Specificity:	Binds to species specific epitope present on Timothy Grass Pollen Allergen, Phl p 5a & b.
Purification:	Produced in tissue culture and purified by chromatography using Protein A. Single heavy and light chain bands on SDS-PAGE.
Concentration:	1.5 mg/ml in phosphate buffered saline, pH 7.4. Based on A_{280} for IgG (1.42=1mg/ml) 0.22µm filtered, preservative free.
Lot Number:	**xxxxx**

Monoclonal Antibody:	Bo 1
Immunogen:	Crude timothy pollen extract
Isotype:	Mouse IgG1
Specificity:	Binds to species specific epitope present on Timothy Grass Pollen Allergen, Phl p 5a & b.
Purification:	From tissue culture by ammonium sulphate precipitation and purified by affinity chromatography using Protein A. Single heavy and light chain bands on SDS-PAGE.
Biotinylation:	Biotinylated and titrated for use in ELISA at 1/1000 dilution. Prepared in 1% BSA/50% glycerol/PBS, pH 7.4, 0.22µm filtered, preservative free.
Lot Number:	**xxxxx**

Allergen Standard:	rPhl p 5a
Composition:	Recombinant Phl p 5a prepared in 1% BSA/50% glycerol/PBS. 0.22µm filtered, preservative free, pH 7.4.
Concentration:	5000ng/ml
Calibration:	The rPhl p 5a was from E. coli and purified by conventional biochemical methods. Validity of the rPhl p 5 standard was confirmed by comparison with the European Pharmacopoeia reference CRS (Y0001566), recombinant major allergen rPhl p 5 containing 8.56 µg of rPhl p 5 per vial.
Lot Number:	**xxxxx**

ELISA Protocol for Phl p 5

1. Coat polystyrene microtiter plates (NUNC Maxisorp Cert. NUNC catalog # 439454) with 100µl mAb 1D11 at 10µl/10ml, i.e. 1/1000 dilution of stock, in 50mM carbonate-bicarbonate buffer, pH 9.6, incubate overnight at 4°C.

2. Wash wells 3x with PBS-0.05% Tween 20, pH 7.4 (PBS-T). Incubate for 30 min. at room temperature with 100µl/well of 1% BSA, PBS-T. Wash 3x with PBS-T.

3. Use doubling dilutions of the rPhl p 5a standard to make a control curve ranging from 500 - 1ng/ml Phl p 5: Pipette 20µl Phl p 5 standard into 180µl 1% BSA PBS-T into wells A1 and B1 on the ELISA plate. Mix well and transfer 100µl across the plate into 100µl 1% BSA, PBS-T diluent to make 10 serial doubling dilutions. Wells A11, B11 and A12, B12 should contain only 1% BSA, PBS-T as blanks.

4. Add 100µl of diluted allergen samples and incubate for 1 hour at room temperature. House dust extracts for Phl p 5 analysis are routinely diluted two-fold from1/10-1/80. Other sample types, like air filter extracts and allergen extracts, may require different dilutions.

5. Wash wells 3x with PBS-T and add 100µl diluted biotinylated anti Phl p 5 mAb Bo1. The antibody solution contains 50% glycerol and should be diluted 1/1000 in 1%BSA, PBS-T. Incubate for 1 hour at room temperature.

6. Wash wells 3x and add 100µl diluted Streptavidin - Peroxidase (Sigma S5512, 0.25mg reconstituted in 1ml distilled water). The Streptavidin Peroxidase should be diluted 1/1000 in 1% BSA, PBS-T. Incubate for 30 minutes at room temperature.

7. Wash wells 3x and develop the assays by adding 100µl 1mM ABTS in 70mM citrate phosphate buffer, pH 4.2 and 1/1000 dilution of H_2O_2. Read the plate when the absorbance at 405nm reaches 2.0-2.4.

Notes:

The Phl p 5 standard is recommended for immunoassay calibration purposes only. Not recommended for in-vitro antibody measurements, T cell studies, immunization purposes, or other uses.

Buffer recipes, storage conditions and a list of frequently asked questions can be found under "Protocols" on our web site: www.inbio.com.

For research and commercial use in vitro: not for human in vivo or therapeutic use.

2. Annex 2: ELISA protocol for Bet v 1 of Indoor Biotechnology^{INC}

References:

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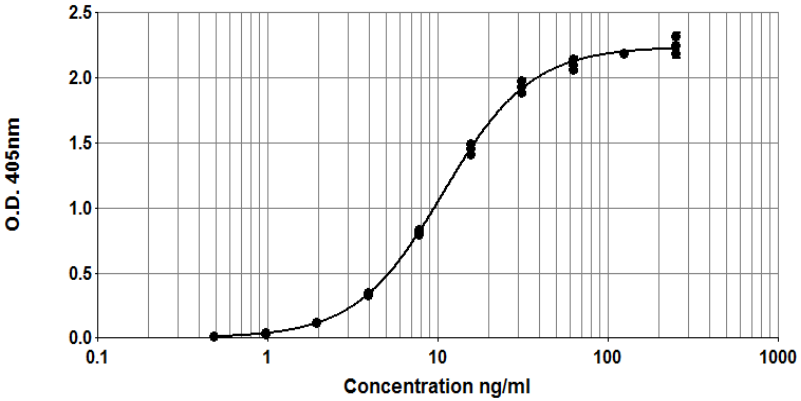
Bet v 1 ELISA kit (4B10/2E10)

Product Code: EL-BV1

Lot xxxxx

Sample Curve:



Content:

Vial 1 (red top) 100 µl
Monoclonal antibody 4B10
Concentration: 1mg/ml in PBS

Vial 2 (white top) 400 µl
rBet v 1 Standard
Concentration: 2500ng/ml

Vial 3 (brown) 100 µl
Biotinylated monoclonal antibody 2E10
Dilute: 1:1000 for use

Storage: All reagents should be stored at 4°C

For research and commercial use in vitro: not for human in vivo or therapeutic use.

Certificate of Analysis

Monoclonal Antibody:	4B10 (clone 4B10 D10 F8)
Immunogen:	Birch pollen extract
Isotype:	Mouse IgG1
Specificity:	Binds to multiple isoforms of *Betula verrucosa* allergen, Bet v 1.
Purification:	Produced in tissue culture and purified by affinity chromatography using Protein A. Single heavy and light chain bands on SDS-PAGE.
Concentration:	1mg/ml in phosphate buffered saline, pH 7.4. Based on A280 for IgG (1.42=1mg/ml) 0.22µm filtered, preservative free.
Lot Number:	**xxxxx**

Monoclonal Antibody:	2E10 (clone 2E10 G6 G7)
Immunogen:	Birch pollen extract
Isotype:	Mouse IgG1
Specificity:	Binds to a non-overlapping, conserved epitope on Bet v 1.
Purification:	From tissue culture by ammonium sulphate precipitation and purified by affinity chromatography using Protein A. Single heavy and light chain bands on SDS-PAGE.
Biotinylation:	Biotinylated and titrated for use in ELISA at 1/1000 dilution. Prepared in 1% BSA/50% glycerol/PBS, pH 7.4, 0.22µm filtered, preservative free.
Lot Number:	**xxxxx**

Allergen Standard:	rBet v 1a
Composition:	Recombinant Bet v 1a prepared in 1% BSA/50% glycerol/PBS, 0.22µm filtered, preservative free, pH 7.4
Concentration:	2500ng/ml Bet v 1
Calibration:	The rBet v 1a was produced from E. coli and purified by a multi-step chromatography procedure. Validity of the rBet v 1 standard was confirmed by comparison with the European Pharmacopoeia reference CRS (Y0001565), recombinant major allergen rBet v 1 containing 10.25µg of rBet v 1 per vial.
Lot Number	**xxxxx**

ELISA protocol for Bet v 1

1. Coat polystyrene microtiter plates (NUNC Maxisorp Cert. NUNC catalog # 439454) with 100µl mAb 4B10 at 10µl/10ml, i.e. 1/1000 dilution of stock, in 50mM carbonate-bicarbonate buffer, pH 9.6, incubate overnight at 4°C.

2. Wash wells 3x with PBS-0.05% Tween 20, pH 7.4 (PBS-T). Incubate for 30 min. at room temperature with 100µl/well of 1% BSA, PBS-T. Wash 3x with PBS-T.

3. Use doubling dilutions of the rBet v 1 standard to make a control curve ranging from 250 - 0.5ng/ml Bet v 1: Pipette 20µl Bet v 1 standard into 180µl 1% BSA, PBS-T into wells A1 and B1 on the ELISA plate. Mix well and transfer 100µl across the plate into 100µl 1% BSA, PBS-T diluent to make 10 serial doubling dilutions. Wells A11, B11 and A12, B12 should contain only 1% BSA, PBS-T as blanks.

4. Add 100µl of diluted allergen samples and incubate for 1 hour at room temperature. House dust extracts for Bet v 1 analysis are routinely diluted two-fold from 1/10-1/80. Other sample types, like air filter extracts and allergen extracts, may require different dilutions.

5. Wash wells 3x with PBS-T and add 100µl diluted biotinylated anti-Bet v 1 mAb 2E10. The antibody solution contains 50% glycerol and should be diluted 1/1000 in 1%BSA, PBS-T. Incubate for 1 hour at room temperature.

6. Wash wells 3x with PBS-T and add 100µl diluted Streptavidin-Peroxidase (Sigma S5512, 0.25mg reconstituted in 1ml distilled water). The reconstituted Streptavidin should be diluted 1/1000 in 1% BSA, PBS-T. Incubate for 30 minutes at room temperature.

7. Wash wells 3x and develop the assays by adding 100µl 1mM ABTS in 70mM citrate phosphate buffer, pH 4.2 and 1/1000 dilution of H_2O_2. Read the plate when the absorbance at 405nm reaches 2.0-2.4.

Notes:

The Bet v 1 standard is recommended for immunoassay calibration purposes only. Not recommended for in-vitro antibody measurements, T cell studies, immunization purposes, or other uses.

Buffer recipes, storage conditions and a list of frequently asked questions can be found under "Protocols" on our web site: www.inbio.com.

For research and commercial use in vitro: not for human in vivo or therapeutic use.

3. Annex 3: ELISA protocol for Alt a 1 of Indoor Biotechnology[INC]

Sabina Wünschmann, PhD September, 2012

Focus on…Alt a 1

Exposure to high levels of *Alternaria* spores in the US Midwest during the spring and summer months is a risk factor for asthma attacks and has been associated with respiratory arrest among children and young adults[1]. Sensitization and exposure to *Alternaria* species was also associated with asthma in the Inner City Asthma Studies and the most recent National Health and Nutrition Examination Survey[2-6]. Most research has focused on *A. alternata*.

Thirteen allergens of *Alternaria alternata* have been identified, however only Alt a 1 is considered a major allergen. Alt a 1 is a dimer of 29 kDa that dissociates into 14.5 and 16 kDa subunits under reducing conditions and is recognized by approximately 80% of *Alternaria*-allergic patients[7]. Alt a 1 has been cloned and the recombinant allergen has been used to measure IgE and IgG antibody responses in patients with *Alternaria* allergy. Recombinant Alt a 1 induces skin prick reactivity comparable with natural Alt a 1 or *A. alternata* extract and is sufficient for a reliable diagnosis of *A. alternata* sensitization [7-9]. Homologs of Alt a 1 have been identified as allergens primarily in other *Alternaria* species[10].

The three-dimensional structure of the *E. coli* expressed recombinant Alt a 1 has just recently been solved through collaborative NIH supported research studies between scientists at INDOOR Biotechnologies and crystallographers and structural biologists at the University of Virginia. Chruszcz et al published the crystal structure of Alt a 1, determined by means of x-ray crystallography in the JACI[11]. The study reveals that Alt a 1 has a unique β-barrel structure, comprised of 11 β-strands, which form a “butterfly-like” dimer that is linked by a single disulfide bond (Figure 1). Dimerization of Alt a 1 provides an explanation for the ability to use the same monoclonal antibody for capture and detection of the allergen in a sandwich ELISA.

INDOOR Biotechnologies recently expressed rAlt a 1 in *Pichia pastoris* (rAlt a 1-P). rAlt a 1-P was purified and compared to the *E. coli* expressed rAlt a 1 (rAlt a 1-E) by SDS-PAGE, mAb ELISA and IgE Ab ELISA. Under non-reducing conditions SDS-PAGE shows the rAlt a 1-P dimer at ~29kD (Fig. 2A, Lane 2) and the rAlt a 1-E dimer and monomer at ~29kD and ~15kD, respectively (Lane 3). Under reducing conditions only the rAlt a 1 monomer is visible (inset). Immunoreactivity of rAlt a 1 was measured using a mAb ELISA and by chimeric ELISA assay for IgE. No difference in mAb binding was seen between rAlt a 1 constructs (Fig.2B), and IgE binding of rAlt a 1-P showed an excellent correlation to that of rAlt a 1-E (Fig. 3).

Applications for recombinant Alt a 1 produced in *Pichia pastoris* (Product Code: RP-AA1) include T-cell studies, histamine release assays and mouse models of asthma and it will also be useful for allergen standardization and the development of improved allergy diagnostics.

Sabina Wünschmann, PhD

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Figures:

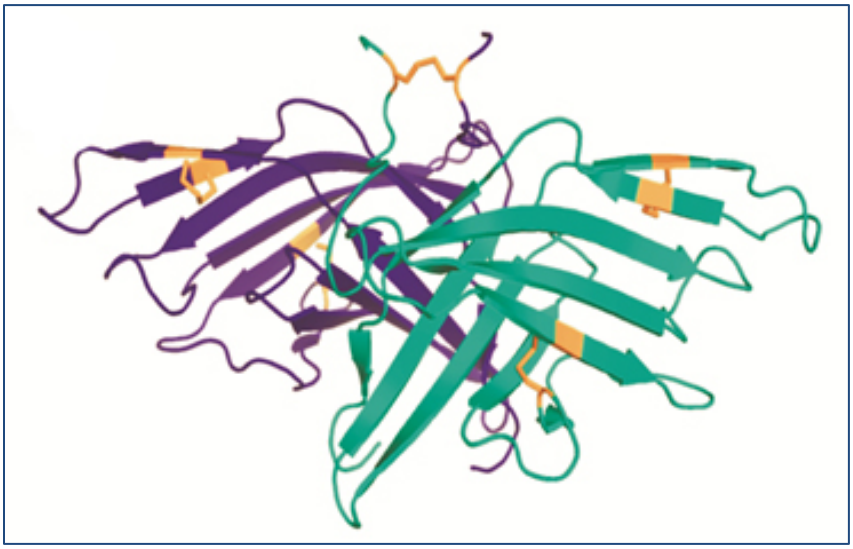

Fig.1: Alt a 1 dimer shown as a cartoon representation (Chruszcz et al, 2012)

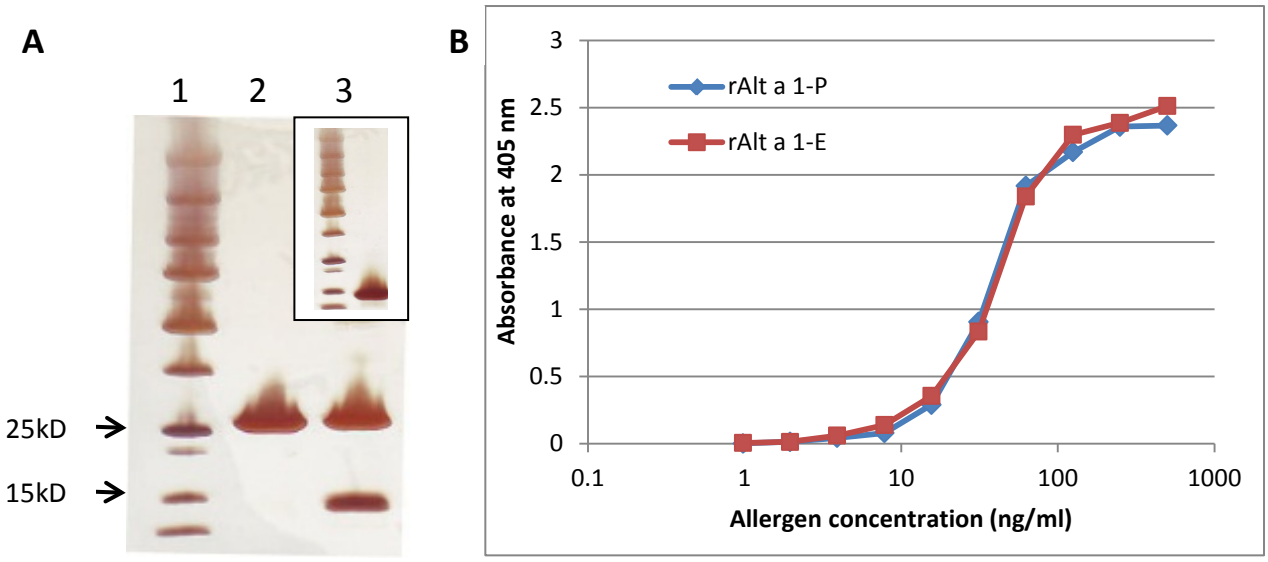

Fig.2. A: Non-reducing SDS PAGE of rAlt a 1 produced in *Pichia pastoris* (Lane 2) and in *E.coli* (Lane 3). SDS-PAGE of rAlt a 1 produced in *Pichia pastoris* under reducing conditions (inset).B: Alt a 1 ELISA.

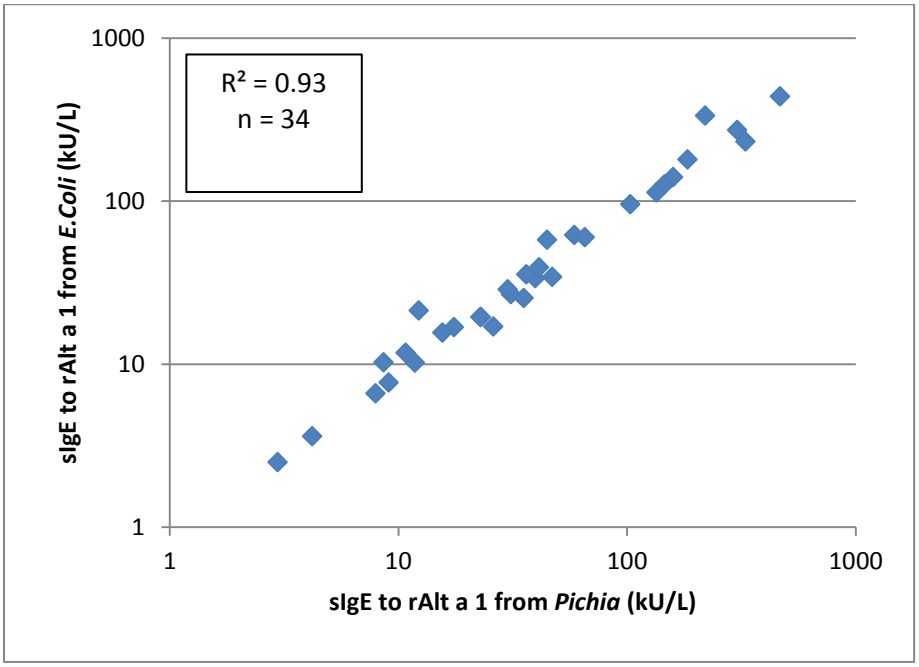

Fig.3: Correlation of IgE binding to rAlt a 1from *Pichia pastoris* and *E.coli.* IgE antibody binding was quantified using a chimeric IgE ELISA assay and sera from 34 Alternaria alternate allergic patients.

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(1) O'Hollaren MT, Yunginger JW, Offord KP, Somers MJ, O'Connell EJ, Ballard DJ et al. Exposure to an aeroallergen as a possible precipitating factor in respiratory arrest in young patients with asthma. N Engl J Med 1991; 324(6):359-63.

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4. Annex 4: Protocol of buffer recipes of Indoor Biotechnology^{INC}

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Solutions and reagents for ELISA

1. 50mM carbonate/bicarbonate buffer, pH 9.6:

- Na_2CO_3 1.59g
- $NaHCO_3$ 2.93g
- Dissolve in 1 liter deionized water

2. Phosphate buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-T):

- NaCl 8.00g
- KH_2PO_4 0.20g
- Na_2HPO_4 1.15g
- KCl 0.20g
- Tween-20 0.5ml

Dissolve in deionized water to a final volume of 1 liter.
To make 1% BSA PBS-T, add 1g bovine serum albumin (BSA, Sigma A-7030*) to 100ml PBS-T.

3. Streptavidin-Peroxidase:

Reconstitute 0.25mg Streptavidin-Peroxidase (Sigma S5512) in 1ml distilled water and store at -20°C in 50µl aliquots. Dilute 1/1,000 in 1% BSA PBS-T for use in the assay.

4. Substrate solution, 1mM ABTS in 70mM citrate-phosphate buffer, pH4.2:

- 70mM citrate-phosphate buffer, pH 4.2
- Solution A = 0.1M anhydrous citric acid, 19.21g/L
- Solution B = 0.2M Dibasic Na Phosphate.$7H_2O$, 53.65g/L

For 500ml buffer, mix 147ml A + 103ml B and make up to 500ml with deionized H_2O. Add 274mg ABTS to 500ml buffer to make the substrate solution (contains 1mM ABTS).

ABTS = 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), Sigma A1888. The substrate solution is stable at 4°C in the dark. Immediately prior to adding to assay plates, add 1µl 30% H_2O_2 solution/ml ABTS. The assay will not work if you do not add the H_2O_2.

*To prevent buffers from becoming turbid, BSA from Roche Diagnostics (catalog number 0311696400) is recommended for ELISA and MARIA buffer preparations. Buffers that become cloudy should be sterile-filtered or discarded.