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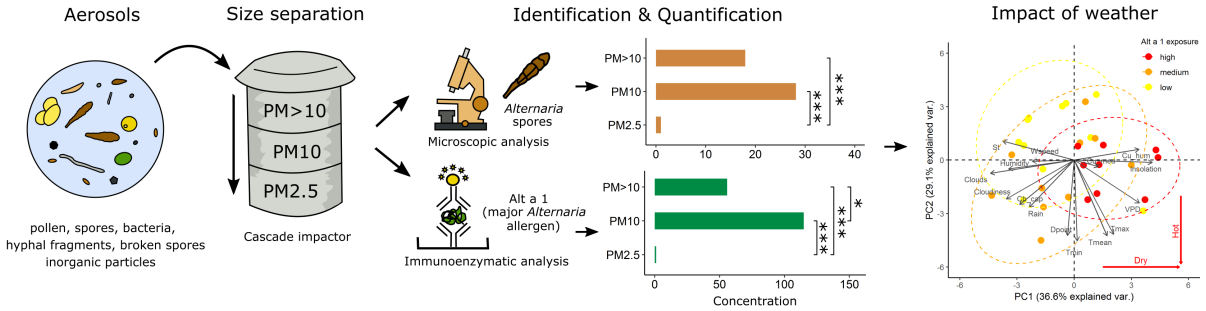

MANUSCRIPT

Particle size distribution of the major *Alternaria alternata* allergen, Alt a 1, derived from airborne spores and subspore fragments.

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Abstract

Fungal fragments are abundant immunoreactive bioaerosols that may outnumber the concentrations of intact spores in the air. To investigate the importance of *Alternaria* fragments as sources of allergens compared to *Alternaria* spores, we determined the levels of *Alternaria* spores and Alt a 1 (the major allergen in *Alternaria alternata* spores) collected on filters within three fractions of particulate matter (PM) of different aerodynamic diameter: (1) $PM_{>10}$, (diameter>10µm); (2) $PM_{2.5-10}$ (2.5-10µm); (3) $PM_{2.5}$ (0.12-2.5µm). The airborne particles were collected using a three stage high-volume ChemVol cascade impactor during the *Alternaria* sporulation season in Poznań, Poland (30 days between 6 July and 22 September 2016). The quantification of Alt a 1 was performed using the enzyme-linked immunosorbent assay. High concentrations of Alt a 1 were recorded during warm and dry days characterized by high sunshine duration, lack of clouds and high dew point values. Atmospheric concentrations of *Alternaria* spores correlated significantly ($r=0.930$, $p<0.001$) with Alt a 1 levels. The highest Alt a 1 was recorded in $PM_{2.5-10}$ (66.8% of total Alt a 1), while the lowest in $PM_{2.5}$ (<1.0%). Significantly more Alt a 1 per spore (>30%) was observed in $PM_{2.5-10}$ than in $PM_{>10}$. This Alt a 1 excess may be derived from sources other than spores, e.g. hyphal fragments. Overall, in outdoor air the major source of Alt a 1 are intact *Alternaria* spores, but the impact of other fungal fragments (hyphal parts, broken spores, conidiophores) cannot be neglected, as they may increase the total atmospheric Alt a 1 concentration.

Keywords: fungal allergy; bioaerosols; hyphal fragments; ELISA; cascade impactor.

Highlights

1. Alt a 1 (major allergen of *Alternaria*) was quantified in different air fractions
2. *Alternaria* spores and Alt a 1 levels correlated significantly ($r=0.930$, $p<0.001$)
3. The highest Alt a 1 level was detected in $PM_{2.5-10}$, while the lowest in $PM_{2.5}$
4. Significantly more Alt a 1 per spore (31.3%) was observed in $PM_{2.5-10}$ than in $PM_{>10}$
5. Spores are the main source of Alt a 1, but the impact of hyphae cannot be neglected

Introduction

Fungal aerosols include generative and vegetative particles of different size and shapes, fragmented, whole or aggregated, that can be passively or actively released into the air (Afanou et al. 2014; Despres et al. 2012; Green et al. 2006). Airborne fungal particles are very common both in indoor and outdoor environment comprising a large proportion of total aerosol particle mass (Elbert et al. 2007; Frohlich-Nowoisky et al. 2009; Womiloju et al. 2003). The size of reproductive fungal propagules (spores, conidia) varies from approximately 1µm to over 100µm (Lacey and West 2006). Airborne fragments of vegetative mycelium may be even smaller, reaching submicron dimensions (Green et al. 2006). Spores, conidiophores and hyphal fragments could be released simultaneously, but the releasing mechanism is different and depends on factors such as fungal species, weather conditions, mechanical disturbance (e.g. action of animals) as well as the texture, moisture and the vibration of the substrate (Afanou et al. 2014; Afanou et al. 2015; Frankel et al. 2014; Górny et al. 2002; Green et al. 2005a; Green et al. 2005b; Green et al. 2006; Madsen et al. 2016). Furthermore, morphological differences between intact spores and fungal fragments suggest that the atmospheric behaviour of these particles, e.g. deposition velocity, may vary substantially. For instance, measurement of fungal aerosols during aerolisation experiments have shown that counts of fungal fragments do not always correlate well with spore concentrations (especially in low air velocity) (Górny et al. 2002).

Fungal particles contain potentially harmful substances (mycotoxins and allergens) that may cause serious health problems (Rick et al. 2016). Development, persistence, and severity of allergic rhinitis and asthma have been associated with mold sensitivity, and it is estimated that around 6.5 million people worldwide have severe asthma with fungal sensitizations (Andersson et al. 2003; Denning et al. 2006; Knutsen et al. 2012). Allergenic proteins have been detected both in fungal spores (that are traditionally linked with allergic

reactions) (Twaroch et al. 2015) and in hyphal fungal fragments (Green et al. 2005c; Levetin et al. 2009). The degree that these fragments function as sources of allergens and contribute to adverse health effects has not, however, been determined (Green et al. 2006). On the one hand, subspore fragments (fragments of hyphae and conidiophores, and broken spores) are abundant bioaerosols and due to their small size may stay airborne longer and penetrate into the lower regions of the respiratory tract more easily than larger intact spores (Cho et al. 2005; Lacey and West, 2006; Pady and Gregory 1963; Pulimood et al. 2007). Also, as fragments have large surface area relative to their mass (in comparison to larger particles) they may show higher biological activity (Frankel et al. 2014). For instance, a positive association has been observed between asthma admission and the level of broken spores (but not hyphae) during thunderstorms (Pulimood et al. 2007). On the other hand, fungal allergens have predominantly been localized in the cell wall of mature spores, as shown for *Alternaria* sp. - one of the most clinically important fungal taxa (Burbach et al. 2009; Twaroch et al. 2012). Also, significant positive correlations have been found between atmospheric levels of the major *Alternaria alternata* allergen (Alt a 1) and both *Alternaria* spore concentrations (Agarwal et al. 1983; da Silva et al. 2019) and allergy symptoms (Feo Brito et al. 2012) suggesting close dependencies between intact spores, allergens, and symptoms.

Intact spores, due to the action of unfavourable weather conditions or mechanical disturbances, may be additionally fragmented to enrich the subspore fraction of fungal aerosols (China et al. 2016; Pulimood et al. 2007). Also, many fungal spores are hygroscopic and can absorb water from the surrounding atmosphere. Eventually, due to osmotic shock, spores may burst in humid conditions releasing submicronic size fragments (China et al. 2016; Pasanen et al. 1991). Whether these fragments contain allergens remains unclear, but pollen-orientated studies revealed high amounts of pollen allergens in cytoplasmic content derived from fragmented pollen grains (Buters et al. 2015; Hoidn et al. 2005; Schäppi et al.

1997b; Schäppi et al. 1999; Taylor et al. 2002). The bursting of hyphal tips in water has also been documented (Bartnicki-Garcia and Lippman 1972). This process resembles the abortive pollen germination in rainwater, when allergens are expelled from the tip of the pollen tube (Grote et al. 2003; Schäppi et al. 1997b). A recent study showed a positive correlation between Alt a 1 and *Alternaria* spores was only observed in buildings that had high relative humidity (da Silva et al. 2019). In general, moisture is essential in fungal spore germination (Dagno et al. 2011; Hatzipapis et al. 2002; Vloutoglou et al. 1996), and it has been shown that *Alternaria* spores in extraction liquid release allergens in just 15 minutes (Sweeney et al. 1985). Germinating spores were also observed in the warm and moist environment of the nasal cavity, and this mechanism was postulated as an additional source of allergens (Green et al. 2003; Sercombe et al. 2006).

This study aims to determine whether subspore fragments of *Alternaria* can be a significant source of Alt a 1 in ambient air (in comparison to intact *Alternaria* spores) and to investigate the relationship between allergen content and environmental conditions, especially those related to moisture. This was achieved by examining the levels of Alt a 1 allergen released from airborne particles collected on filters within three fractions of particulate matter (PM) and relating these to various weather parameters.

Materials and Methods

Fungal aerosol collection and quantification

Airborne *Alternaria* particles were collected using a high-volume (400 l/min) ChemVol cascade impactor (Butraco Inc., Son, Netherlands) (Buters et al. 2012; Demokritou et al. 2002). The ChemVol contains three impaction stages for collecting particles of different aerodynamic cut-off diameters i.e. (1) >10µm ($PM_{>10}$); (2) 2.5-10 µm ($PM_{2.5-10}$); (3) 0.12-2.5

µm ($PM_{2.5}$). *Alternaria* spores are large with the aerodynamic diameters ranging from about 10 to 30 µm (McCartney et al. 1993). The ChemVol sampler therefore seemed a suitable collecting device for separation intact *Alternaria* spores from smaller fungal fragments (hyphal parts and dissected spores). *Alternaria* particles were collected in Poznań, during the main *Alternaria* sporulation season (30 days between 6 July and 22 September 2016, Tab. 1S). The highest daily *Alternaria* spore concentrations in Poznań are observed from the end of June to the middle of September, with the seasonal peak recorded usually in the beginning of August (Grewling et al. 2019; Kasprzyk et al. 2013). A previous study showed that the mean seasonal *Alternaria* spore concentration in Poznań was the highest among other cities in Central and Eastern Europe (Kasprzyk et al. 2015), which is related to the fact that the city is located in the agricultural region of Western Poland. Field crops are considered the main source (host plants) of various *Alternaria* species. In the studied area the predominant species are *Alternaria alternata, A. brassicicola*, and *A. brassicae* as they infect oilseed rape fields that are abundant in Western Poland (Jajor et al. 2012; Baranowski et al. 2015). In addition, other crop pathogens are locally common, such as *A. solani* (infecting potatoes), *A. porri* (pathogen of onion), and *A. dauci* that infects carrots (Gawińska-Urbanowicz and Kapsa 2013, Ogórek et al. 2011). However, it should be stressed that spores of different *Alternaria* species are morphologically similar and so, when airborne samples are investigated, *Alternaria* spores cannot be identified to species level and are therefore grouped to genus level. The ChemVol was located at roof level (18 m a.g.l.) in the northern part of Poznań (52.46'N, 16.92'E) (Fig. S1). The sampling time was 24 hours, from 12:00 to 12:00 of the next day, but is described as a "daily average" throughout. The collection substrates were polyurethane foam filters. Each filter (three filters per day) was cut into three equal pieces and extracted in the dark (4 hours in 0.1 M ammonium bicarbonate buffer). After filter extraction the content was centrifuged (10 min at 1699g). The supernatant was used for quantification of

the major *Alternaria alternata* allergen (Alt a 1), while sediment was used for *Alternaria* spore calculation. The quantification of Alt a 1 in each air fraction was performed using the enzyme-linked immunosorbent assay (ELISA) following the protocol described in Grewling et al. (2019). Due to a high degree of structural similarity between Alt a 1 homologues proteins (Saenz-de-Santamaria et al., 2006; Hong et al. 2005, Amado et al. 2016), fungal species closely related to A. alternata could also be detected by ELISA. Therefore, when describing the Alt a 1 concentration in the air the results are not limited only to *A. alternata*, but may also refer to other *Alternaria* species. Other fungal genera, e.g. *Urocladium* and *Stemphylium* that show close phylogenetic relationship to *Alternaria* (Gutierrez-Rodriguez et al., 2011), should not markedly affect the obtained results as concentrations of their spores in the air are often very low (Bednarz and Pawłowska 2016; Ščevkova and Kovac 2019). The daily mean Alt a 1 concentration was expressed as pg/m^3. The number of spores extracted from filters was calculated using a method adopted from the estimation of pollen production (Bogawski et al. 2016). After centrifugation, the sediment with spores was diluted in 200µl of distilled water. This was vortexed to obtain a homogenous solution and 25ul was transferred to a microscope slide and gently spread within 1.5 x 1.5 cm area. The spores were counted under a light microscope (magnification 200x) in three horizontal lines. Taking into account the microscope field of view and number of lines the total examined area was 49.5 mm^2, i.e. 22% of total slide area (225 mm^2). This procedure was repeated three times and the number of spores was averaged. The obtained value was used to calculate the mean spore concentration in 1µl of solution. We decided to not express the spore concentration in 1 m^3 of air due to the uncertainty in estimating the total number of spores collected on filters (this value cannot be precisely calculated using proposed method of spore extraction).

To validate the correctness of spore enumeration from filters, the results were compared with the mean “daily” level of spores (from 12:00 to 12:00 of the next day) obtained by routine

methods used in aerobiology, i.e. volumetric Hirst spore trap (Hirst, 1952), located next to the ChemVol impactor. The Hirst (1952) spore trap is a impaction type sampler where air is sucked at a rate of 10 l/min through a 2 mm ×14 mm orifice. Behind the orifice the air flows over a rotating drum that moves past the inlet at 2 mm/h and is covered with an adhesive coated, transparent plastic tape. Airborne spores impact on the tape to give a time related sample. Following its removal from the trap, the tape is divided into segments corresponding to 24-h periods (48 mm in length). Each segment is mounted between a glass slide and cover slip, and the samples are examined by light microscopy (×400 magnification). Spores were counted along two longitudinal transects following the method described in literature (Maya-Manzano et al. 2016). The correlation between both datasets was positive and statistically significant (Pearson correlation coefficient, r =0.930, $p<0.05$) ensuring that selected methods give comparable results. The usefulness of the ChemVol impactor for bioaerosols collection has also been validated during the EU funded HIALINE project (Buters et al. 2015).

Weather data collection

Weather data were retrieved from the official weather station of the National Institute of Meteorology and Water Management located at the Poznań Ławica airport (app. 5 km southwest from aerobiological station) (Fig. S1). The temporal resolution of weather data was adjusted to the Alt a 1 collection time (12:00-12:00). The following meteorological parameters were analysed: daily mean, minimum and maximum air temperature (°C), dew point (°C), vapour pressure deficit - VPD (kPa), relative humidity (%), rainfall (mm), sunshine duration (h), wind speed (m/s), cloudiness (unit 1/8), daily fraction of specific cloud types (%): *Cumulus humilis* (*Cu hum*)/*Cumulus fractus* (*Cu fra*), *Cumulus mediocris* (*Cu med*)/*Cumulus congestus* (*Cu con*), *Stratocumulus* (*Sc*), *Cumulonimbus capillatus* (*Cu cap*), and daily fraction of all types of clouds (%).

Statistical analysis

The concentrations of Alt a 1 and *Alternaria* spores collected in three air fractions were compared by the Kruskal-Wallis H test and Dunn's procedure for multiple pairwise comparison. *P*-values have been adjusted using Benjamini-Hochberg correction. Relationships between Alt a 1 levels and *Alternaria* spore concentrations in selected air fractions were checked by simple linear regression analysis. Daily mean concentration of Alt a 1 in every stage of ChemVol sampler has been correlated (by Pearson correlation coefficient) with meteorological parameters. Also, the ratio in the level of Alt a 1 (or spores) between three investigated air fractions has been correlated with meteorological parameters. Data that were right (positively) skewed have been transformed (log+1). The multivariate principal component analysis (PCA) was performed to select the major weather conditions affecting the daily Alt a 1 concentration. Days with Alt a 1 concentration were divided into three groups based on two cut points estimated via probability quantiles (33.3% and 66.6%), i.e.: "low" (daily Alt a 1 levels< 1.98 pg Alt a 1/m^3, n=10), "medium" (1.98-6.71 pg Alt a 1/m^3, n=10) and "high" concentration (>6.71 pg Alt a 1/m^3, n=10). Before PCA analysis, the data were Box-Cox transformed, scaled and centred. All statistical analysis has been performed using computing environment R (R Core Team 2018) and packages: FactoMineR (Lê et al. 2008), corrplot (Wei and Simko 2017), caret (Kuhn 2008), and factoextra (Kassambara and Mundt 2017).

Results

Distribution of Alt a 1 in different fraction of particulate matter

The highest amount of Alt a 1 was detected in the 2.5-10 µm ($PM_{2.5-10}$) air fraction (Fig. 1) and was significantly higher ($p<0.05$) than in two other air fractions (Fig. 2). The Alt a 1 concentration was extremely low (<1% of total Alt a 1) in the $PM_{2.5}$ air fraction that contained

the smallest particles (i.e. <2.5 µm). A similar pattern was observed in relation to *Alternaria* spores, as the highest spore level was observed in $PM_{2.5-10}$, while the lowest in $PM_{2.5}$. Airborne concentrations of Alt a 1 were significantly ($p<0.001$) related to *Alternaria* spore levels collected in $PM_{>10}$, $PM_{2.5-10}$ and $PM_{2.5}$ ($R^2=0.801$, $R^2=0.819$, and $R^2=0.454$, respectively) (Fig. 3). The correlation between total Alt a 1 and *Alternaria* spores was positive and significant ($R^2=0.865$, $p<0.001$). Significantly more Alt a 1 per spore (31.3%) was observed in $PM_{2.5-10}$ than in $PM_{>10}$ ($p=0.015$).

Impact of weather on Alt a 1 distribution

There were significant positive correlations ($p<0.05$) between daily *Alternaria* spore levels collected in the Hirst type trap and daily mean, maximum and minimum temperature, VPD, dew point, and sunshine duration. Similar relationships were recorded between daily levels of Alt a 1 (collected by ChemVol sampler) and weather conditions (Fig. 4). For instance, the Alt a 1 in every air fraction correlated positively with daily maximum ($r>0.387$, $p>0.05$) and mean temperature ($r>0.384$, $p>0.05$). Furthermore, statistically significant positive correlations were recorded between Alt a 1 in the $PM_{2.5}$ fraction and fair weather *Cumulus humilis* clouds ($r= 0.584$, $p<0.001$), sunshine duration ($r= 0.567$, $p=0.001$), and VPD ($r= 0.505$, $p=0.004$).

On the other hand, humidity ($r= -0.447$, $p=0.013$), the occurrence of all types of clouds ($r= -0.552$, $p=0.001$) and cloudiness ($r= -0.495$, $p=0.005$) all had significant negative associations with levels of Alt a 1 in the $PM_{2.5}$ fraction. In addition, there was a significant negative correlation between Alt a 1 and wind speed ($r= -0.372$) in the larger fractions. There were no significant relationships between Alt a 1 concentrations and rainfall or *Cumulonimbus capillaris* clouds. Finally, no significant relationships have been observed between *Alternaria* spores and meteorological parameters related to “humid conditions”, such

as cloudiness, the occurrence of *Stratocumulus* and all types of clouds, rainfall and increased humidity.

Considering the impact of meteorological conditions on the ratio of Alt a 1 (or spores) recorded in different air fractions, only two significant correlations have been observed: (1) Alt a 1 in PM2.5 to Alt a 1 in >PM2.5 was negatively correlated with humidity ($r = -0.411$, $p=0.024$); (2) Spores in PM2.5 to spores collected in >PM2.5 correlated significantly with sunshine duration ($r=-0.413$, $p=0.023$) (Fig. 5).

PCA supports results of correlation analysis, i.e. higher Alt a 1 concentrations were generally recorded during warm and dry days characterized by high sunshine duration (PC1), dew point and daily mean, maximum and minimum temperatures (PC2) (Fig. 6, Fig. S2 & S3). In addition, the occurrence of *Stratocumulus* clouds and all types of clouds (PC1) showed strong negative relationship with Alt a 1 concentrations. The first two principal components explained 65.7% of variability in the dataset (Fig. S2).

Discussion

Sources of Alt a 1

Our study demonstrates that the daily levels of *Alternaria* spores correlated significantly with Alt a 1 ($r=0.930$, $p<0.001$), and we can therefore assume that the majority of atmospheric Alt a 1 was derived from intact *Alternaria* spores. Spores take part in the infection of plants and Alt a 1 is a protein involved in plant pathogenesis, i.e. interacts with plant defence proteins such as PR5 (Garrido-Arandia et al. 2016). In other words, spores need Alt a 1 to block plant defences and to favour fungal entry into the plant. In view of these findings, it is not surprising that Alt a 1 was located in the highest concentrations in the cell walls of old and

germinating spores (Mitakakis et al. 2001; Twaroch et al. 2012) because the allergen is located exactly where it is most needed. The length of *Alternaria* spores vary from approximately 20 µm to as much as 200 µm (Simmons 2007) and are many times larger than micronic sized fungal fragments. In addition, only part of the hyphal fragments are immunoreactive, as around 25% of all hyphae expressed detectable allergens (Green et al. 2005c). Hundreds of fragments are therefore needed to exceed the allergen load of a single spore, and comparative studies showed that the differences between the levels of airborne hyphal fragments and spores are not in fact as high. According to Green et al. (2005c) fungal hyphae concentrations surpassed spores only by around 2-3 times. Higher differences (exceeding even 300-fold) have been observed in aerosolization experiments (Górny et al. 2002), but the mean difference between the number of hyphal fragments and spores was much lower (varying from 10 to 60-fold depending on air velocity). In addition, in a study conducted in two US cities, fungal fragments were present on 99% of all days, although spores rather than hyphae predominated in the air (Levetin et al. 2009). What is more, it should be stressed that in environmental samples, it is extremely difficult to morphologically distinguish the hyphae of different fungal species (immunostaining and DNA extraction techniques may be a solution) (Green et al. 2005b; Rittenour et al. 2012). In some of the studies mentioned previously (Green et al. 2005c; Levetin et al. 2009), only total hyphae fragments were counted (without species recognition). This approach, although valuable, does not allow for the direct comparison between spores and hyphae of particular fungal species, so the contribution of mycelial fragments could be overestimated.

The highest level of *Alternaria* spores (and Alt a 1) was observed in the $PM_{2.5-10}$ air fraction. This is surprising as, according to previous studies, the aerodynamic diameter of *Alternaria* spores exceeds 10 µm (McCartney et al. 1993; Yamamoto et al. 2014). Most of the spores should therefore be deposited in the $PM_{>10}$ air fraction, as it was presented in pollen-

oriented studies (based on the same experimental setup) where around 90% of pollen allergens (and therefore also pollen grains) were detected in $PM_{>10}$ fraction (Buters et al. 2012; Buters et al. 2015; Galan et al. 2013; Grewling et al. 2016). It is worth noting, however, that *Alternaria* colonies were also isolated from air samplers with particle diameter lower than 10µm (Kim et al. 2010; Sayer et al. 1969). Furthermore, DNA barcoding analysis (Yamamoto et al. 2012) showed that the concentration of *Alternaria* DNA was also very high in the $PM_{2.5-10}$ fraction (although DNA might originate from both fungal spores and fragments). Presumably, the high number of *Alternaria* spores in the $PM_{2.5-10}$ fraction derived from their characteristic elongated club shape (in contrast to spherical pollen grains). McCartney et al. (1993) stressed that because of the shape of *Alternaria* spores, the mass is not uniformly distributed along their length, and thus it is difficult to predict their aerodynamic characteristics. In addition, the aerodynamic diameter of fungal spores cannot be accurately estimated solely based on the physical diameter but needs additional information, e.g. on the density of the spores and ambient air humidity (Reponen et al. 2001). For instance, it has been shown that high humidity may increase the diameter of *Cladosporium* spores by as much as 180% (Pasanen et al. 1991).

Our study revealed that the proportion of Alt a 1 to spores in the $PM_{2.5-10}$ fraction was around 30% higher than in $PM_{>10}$. Presumably, the 30% excess of Alt a 1 in $PM_{2.5-10}$ derived from subspore hyphal fragments. However, it should be noted, that we did not quantify the level of *Alternaria* fungal fragments (based on their morphology) in air samples in this study (only Alt a 1 derived from fragments). When interpreting the peculiarities in allergen concentrations in the air, one should also remember about high variation in the allergenicity of fungal spores (Grewling et al. 2019; Mitakakis et al. 2001). Grewling et al. (2019) revealed differences of up to eightfold in day-to-day variations in *Alternaria* spore allergenicity that could be linked to varying species composition during the sporulation season. Spores of

different *Alternaria* species vary in their aerodynamic properties (McCartney et al. 1993), and so species-specific variations in the aerodynamic behaviour of spores (with different Alt a 1 content) may also affect the amount of Alt a 1 recorded in different fractions of particulate matter.

Impact of weather on Alt a 1 concentration

The results of PCA and correlation analysis showed that the highest Alt a 1 levels were recorded during sunny, warm and dry days, when weather conditions favoured the upward movement of air currents (high temperature, sunshine duration, dew point and VPD, and presence of *Cumulus* clouds). These conditions are known to positively affect the daily concentrations of *Alternaria* spores (Grinn-Gofroń and Bosiacka 2015; Hjelmroos 1993; Stennett and Beggs 2004; Troutt and Levetin 2001). It is striking to note that the strength and direction of correlations between meteorological factors and both Alt a 1 (especially in $PM_{>10}$ and $PM_{2.5\text{-}10}$ fractions) and *Alternaria* spore concentrations were generally very similar (see Fig. 4), which supports the idea that the main sources of Alt a 1 are *Alternaria* spores.

In contrast to findings from pollen-oriented studies where humid conditions increased the fraction of allergens related to small fragments (Buters et al. 2015; Schäppi et al. 1997a), our experiment showed an opposite situation. The ratio of Alt a 1 in $PM_{2.5}$ to Alt a 1 in larger fractions increased with decreasing relative humidity. This result concurs with previous findings (Madsen 2012) showing that the fraction of the fungal particles being of respirable size was the highest for particles aerosolized at low relative humidity. We suspect that the different behavior of pollen and fungal spores arises from differences in the wall structure and the role of water in pollen/fungal spore germination. The release of subpollen allergens through pollen wall bursting (due to high humidity) was mainly described for pollen grains

characterized by a delicate and thin pollen wall like grasses (Poaceae) (Schäppi et al. 1999, Taylor et al. 2002; Buters et al. 2015). It has not, however, been documented in pollen with thicker walls like mugwort (*Artemisia* sp.). In this species the concentration of subspore particle does not show positive relationship with air humidity (Grewling et al. 2020). Water has an adverse effect on pollen longevity and viability and so plants developed certain protective mechanisms (e.g. specific floral morphology, production of germination inhibitors) to prevent pollen from water damaging or undesirable germination (outside the stigma) (Eisikowitch and Woodell, 1974; Mao and Huang 2009). In contrast, water is essential for fungal spore germination, and spores of some *Alternaria* species only germinate at 100% relative humidity (Dickinson and Bottomley, 1980; Hatzipapas et al., 2002). Fungal spores are therefore adapted to moisture conditions and likely more resistant to rupturing by osmotic shock than thin-wall pollen grains. The recent study by Lawler et al. (2020) documented the occurrence of fungal nanoparticles in the air, which peaked around 1.5 days after the rainfall. Similar behavior was also observed in the Amazon, where daily increase in fungal particles was related to high nighttime relative humidity (China et al. 2016). This suggests that post-rain processes related to fungal spore germination may play a role in the release of nanoparticles (Lawler et al. 2020). Considering the fragmentation of *Alternaria* spores, the mechanical damage to spores (e.g. observed during grass moving or harvesting (Pulimood et al. 2007)) seems to be more important than osmotic rupture.

In addition, our study showed that fine fungal fragments were more likely, than larger particles, to become airborne when *Cumulus humilis* clouds were observed. This type of cloud indicates unstable atmospheric conditions below the clouds, especially during their formation. Such turbulent conditions could occur during intense sunshine duration preceding *Cumulus humilis* formation and/or in the presence of *Cu hum* clouds (Stull 1985). Indeed, *Cu hum* and sunshine duration are highly positively correlated with the amount of Alt a 1 in smallest air

fraction (see Figure 4). Consequently, higher numbers of small, immunoreactive fungal particles may occur during weak or moderate convection (e.g. warm air ascending with velocity 2-5 m s^{-1}). Larger fungal fragments (>2.5µm) therefore seem to be more loosely connected with weak convection, probably because they require stronger air movements to overcome gravity and drag.

Deposition velocity of airborne particles is the lowest (<0.03 cm s^{-1}) for particles of aerodynamic diameter between 0.1-1.0µm. For larger particles (>5.0µm) deposition velocity strongly increases (>1.0 cm s^{-1}) and sedimentation becomes a predominant atmospheric process of particle removal (Nicholson 1995). According to Woo et al. (2018) *Alternaria* spores with aerodynamic diameter of 10µm had a deposition velocity of 0.63 cm s^{-1}. Fine hyphal fragments (0.12-2.5µm) might hypothetically gain in importance in indoor environments (Górny et al. 2002) or during specific weather conditions, e.g. thunderstorm events when many particles are uplifted, mixed and damaged (D'Amato et al. 2017; Pulimood et al. 2007). In our study, we investigated the effect of "storm-like" conditions, e.g. presence of *Cumulonimbus* clouds on Alt a 1 concentrations, but no significant relationships have been observed. Also, episodes of rain were uncommon during the study period and so we could not test the hypothesis linking occurrence of light rainfall (<1 mm) with increased level of allergens (Schäppi et al. 1997a; Schäppi et al. 1997b).

Alt a 1 in finest air fraction (PM 0.12-2.5 µm)

Previous studies have shown that the concentration of the smallest fungal fragments (~1µm), in comparison to spores, can be relatively high in the air (Reponen et al. 2007, Adhikari et al. 2009, Lee and Liao 2014). In these studies, the detection and enumeration of fungal fragments was mainly based on the concentration of (1→3)-ß-D-glucan, i.e. polysaccharide abundant in

fungal cell walls (Rylander 1999). Such methods do not, however, allow fragments belonging to specific fungal taxa to be identified, so all fungal fragments were grouped and counted together. When a molecular technique was applied, no sign of *Alternaria* DNA was found in the $PM_{2.5}$ fraction (Yamamoto et al. 2012). In the current study, we used a family-specific allergen that occurs in *A. alternata* and other members of Pleosporaceae family (as Alt a 1 homologs) (Hong et al. 2005; Sáenz-de-Santamaría et al. 2006). Based on this detection method, the total level of Alt a 1 in 0.12-2.5 µm air fraction was extremely low (1% of total Alt a 1). This result concurs with pollen allergen studies where no allergens was found in the $PM_{2.5}$ fraction (Buters et al. 2010; Buters et al. 2012). Green et al. (2005b) postulated that the amount of allergens released from a hyphal fragment might be a function of the critical fragment size, which is the minimum size at which a fungal fragment remains viable. In the study conducted by Górny et al. (2002) it was shown that immunoreactive fungal fragments of *Aspergillus*, *Penicillium* and *Cladosporium* might be as small as 0.3µm. The critical sizes for *Alternaria* species have not, however, been established. In addition, Buters et al. (2010) showed that airborne pollen allergens of micronic size are easily absorbed by diesel soot particles. It was postulated that this phenomenon could be responsible for the lack of pollen allergens in micron-sized air fraction (de Weger et al. 2013). These studies and our results suggest that the vast majority of immunoreactive airborne *Alternaria* particles belongs to spores and larger fungal fragments.

Conclusions

Our study showed that the main source of airborne Alt a 1 in the outdoor environment are intact *Alternaria* spores (app. 80%), which are deposited in both the $PM_{>10}$ and $PM_{2.5\text{-}10}$ air fractions. The possible contribution of other fungal particles is the most visible in $PM_{2.5\text{-}10}$, where fungal fragments may be responsible for more than 30% of total Alt a 1. The amount of

allergen related to the finest fungal fragments ($PM_{2.5}$) is very low, almost negligible from clinical and epidemiological point of view. This is important news, as the quantification of *Alternaria* spores in the air (without mycelial fragments) is currently a routine practice in many aerobiological laboratories. Our results suggest that such information could be used as a relevant approximation of exposure to airborne Alt a 1 (based on very strong correlation between spores and Alt a 1). Nevertheless, high variations in allergen content between individual spores should also be considered to fully evaluate the exposure level to *Alternaria* allergens.

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Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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712 Figures

713 Fig. 1. Distribution of Alt a 1 in three investigated air fractions. *Alternaria* spores
714 concentration (line curve) collected using Hirst type volumetric trap (samples description in
715 Table S1).

716 Figure 2. Ratio (%) between the concentrations of Alt a 1 (pg/m^3) and *Alternaria* spores
717 (spore/m^3) in selected air fractions to the total levels of Alt a 1 and total *Alternaria* spores.
718 The statistically significant differences between spores and Alt a 1 levels in three air fractions
719 are marked by asterisks, i.e. *p<0.05, **p<0.01, ***p<0.001.

720 Figure 3. Correlations between Alt a 1 concentrations and *Alternaria* spore levels in selected
721 air fractions.

722 Figure 4. Correlations matrix showing relationships between daily weather parameters and
723 both *Alternaria* spores (collected by Hirst trap) and Alt a 1 in three investigated fractions of
724 particulate matter (statistically significant correlations with p<0.05 are in bold).

725 Figure 5. Correlations matrix showing relationships between daily weather parameters and
726 ratio in the level of Alt a 1 (or spores) between investigated air fraction of particulate matter
727 (statistically significant correlations with p<0.05 are in bold).

728 Figure 6. Principal component analysis, using weather data collected during sampling period,
729 for Alt a 1 concentration levels (ellipses represent the 90% confidence interval of selected
730 groups). Additional information of PCA analysis in Suppl. Materials, Fig. S2 & S3.

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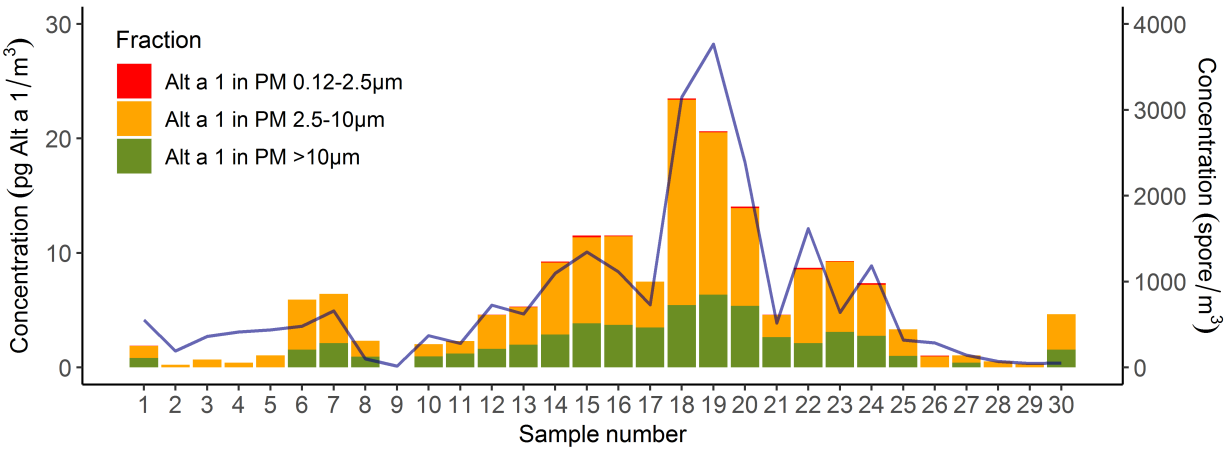

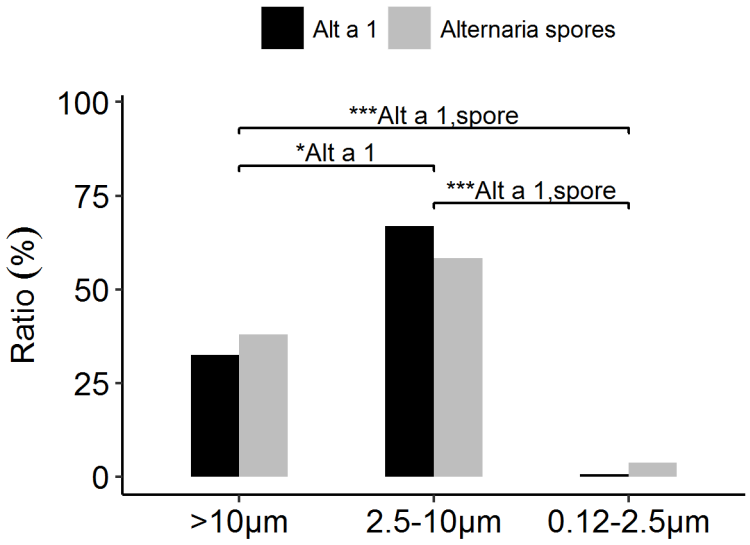

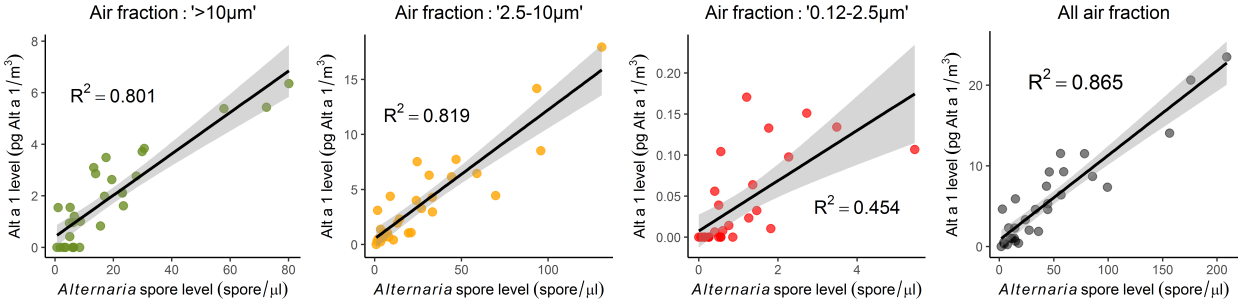

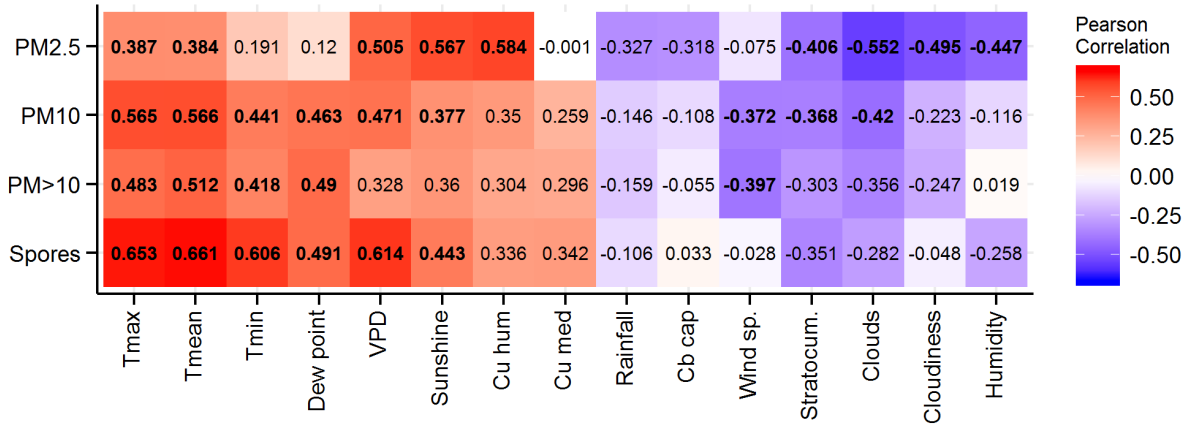

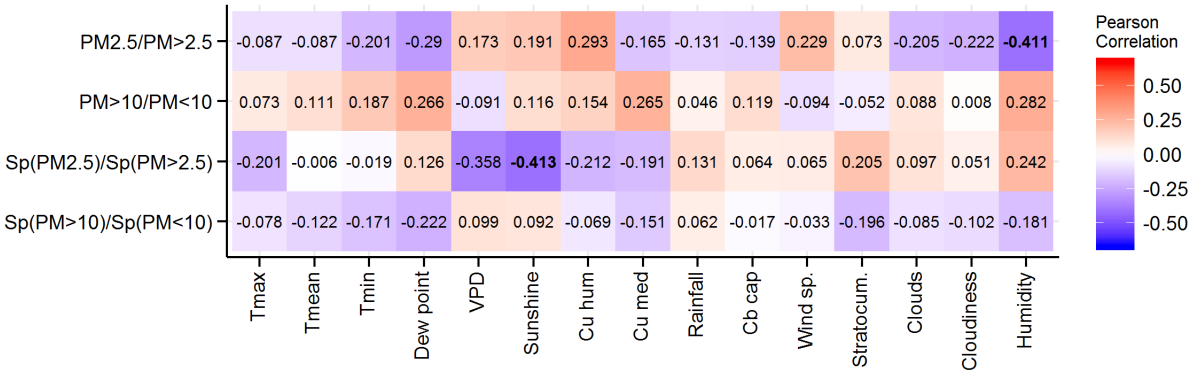

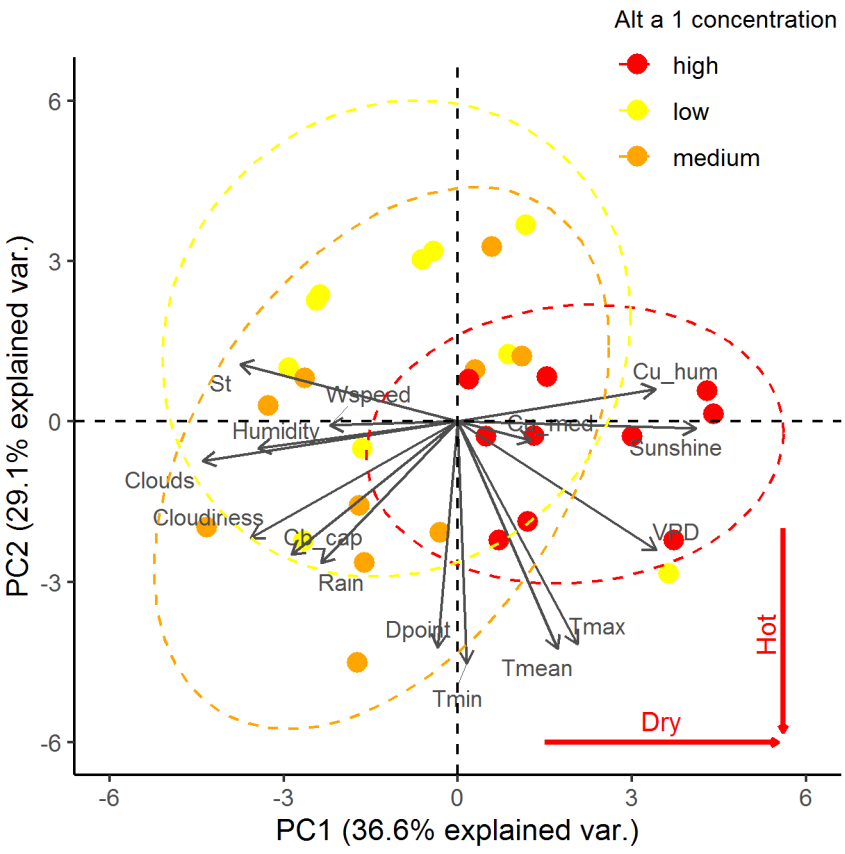

Highlights

1. Alt a 1 (major allergen of *Alternaria*) was quantified in different air fractions
2. *Alternaria* spores and Alt a 1 levels correlated significantly ($r=0.930$, $p<0.001$)
3. The highest Alt a 1 level was detected in $PM_{2.5-10}$, while the lowest in $PM_{2.5}$
4. Significantly more Alt a 1 per spore (31.3%) was observed in $PM_{2.5-10}$ than in $PM_{>10}$
5. Spores are the main source of Alt a 1, but the impact of hyphae cannot be neglected