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Species of Fungi and Pollen in the PM_1 and the Inhalable Fraction of Indoor Air in Homes

Rui Lu [1,2], Ann Dorte Pørneki [3], Jonas N. Lindgreen [3], Yanpeng Li [2] and Anne Mette Madsen [1,*]

[1] The National Research Centre for the Working Environment, Lersø Parkallé 105, DK-2100 Copenhagen, Denmark; Rui.Lu.work@outlook.com
[2] School of Water and Environment, Chang'an University, Xi'an 710054, China; liyanp01@chd.edu.cn
[3] House Test ApS, Petersmindevej 1A, DK-5000 Odense C, Denmark; adp@housetest.dk (A.D.P.); jnl@housetest.dk (J.N.L.)
* Correspondence: amm@nrcwe.dk; Fax: +45-39165201

Abstract: Airborne microbial fragments in the PM_1 fraction (particles with aerodynamic diameters less than 1 µm) are a cause for concern as they may potentially deposit in the alveoli of the human airways. This study aimed to use qPCR to identify and quantify 24 different species or groups of genera in the PM_1 and the inhalation fraction (particles that may enter the mouth or nose during breathing) of indoor air and to relate this to what has previously been found for each species. Results showed that eight fungal species, and *Aspergillus/Penicillium/Paecilomyces variotii*, as well as *Alnus/Corylus* and actinobacteria belonging to the *Streptomyces* genus were detected both in the PM_1 and the inhalable fraction. Five fungal species were only detected in the inhalable fraction. A significant effect of season was found on the fungal composition in the PM_1 ($p = 0.001$) and the inhalable ($p = 0.017$) fraction. This study demonstrated that it is possible to use qPCR to identify and quantify different microbes in the PM_1 fraction, and it has improved our understanding of the qualitative and quantitative relationship between the PM_1 and the inhalable microbial particles in indoor air. Combined with the literature review it also shows a large variation within and between species in the share of fungi which is present as fragments.

Keywords: fungal fragments; pollen; PM_1; inhalable; indoor air; qPCR

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1. Introduction

Airborne fungi and pollen are always present as inhalable particles in indoor environments and they are important risk factors for asthma symptoms [1,2]. According to a review study, fungal fragments are derived from broken or fractured conidia and hyphae that have been aerosolized following disturbances to fungi colonies [3]. Of the airborne fungi, fragments smaller than spore size have received attention since 1999 when they were first found to be released from gypsum boards colonized by fungi [4]. Studies of fungal fragments using scanning electron microscopy (SEM) show that they do not have common characteristics regarding shape and size [5–9], and cannot be identified to genus or species level by morphology.

The fungal fragments have been found in air samples from different settings, such as biofuel plants [10], open horticultural fields [11], agricultural farms [12], indoor air [13–15], and outdoor air [16,17]. Due to their small size, these fragments are expected to stay airborne for a longer time than larger particles, and thus the period of exposure may be extended. Under laboratory conditions, some fungal species, like *Stachybotrys chartarum*, can release more fragments than spores, and these fragments showed higher respiratory deposition than their spores [18]. Other laboratory studies have found that a larger number of spores than fragments are released from e.g., mixed fungal cultures [19], *Aspergillus fumigatus* [7], and *Aspergillus versicolor* under certain air velocity condition [20]. Fungal fragments can be

allergenic [3] and inflammogenic [19], and have been found in higher concentrations in homes with asthmatic children than in homes without asthmatic children [21].

Grass pollen has been found to be associated with the development of dermatitis [22], asthma [23], and other allergic respiratory diseases [24]. In a controlled-environment chamber study, flowering grasses exposed to a cycle of wetting and drying followed by wind disturbance released pollen fragments smaller than <1 μm; the fragments were detected using high-resolution microscopy and immunolabeling with antibodies [25]. Furthermore, grass-pollen allergens have been detected in the atmospheric aerosol fraction of particles smaller than 0.6 μm in outdoor air [26]. A recent study showed that the concentration of submicron pollen fragments (0.25–1.0 μm) as detected by microscopy can increase to peak value during rain events, and can persist in outdoor air for several hours [27].

Apart from microscopy, there is no method to discriminate between fragments of pollen and fungi and intact pollen and fungi in environmental samples, and therefore the size-selective sampling is important to differentiate them from each other. Fungal spores and pollen have an aerodynamic diameter larger than 1 μm, and to exclude non-fragmented spores and pollen from the sample, fragments of fungi can be sampled as part of the PM_1 fraction. The fungal fragments were first detected in pure fungal aerosols generated at laboratory level using an aerodynamic particle sizer e.g., [4,28–30], and later also as different fungal components, such as β-d-glucan [14,31], chitinase [10], and immunostaining combined with a field emission SEM [32].

Streptomyces is the most commonly found genus of actinobacteria in indoor environments [33], some *Streptomyces* species have aerial hyphae that consist of long, straight filaments, which bear spores [34]. The health effects of exposure to the spores of *Streptomyces* were described in a previous study [35]. The presence of airborne *Streptomyces* spp. as single spores is of relevance as a species within this genus has caused hypersensitivity pneumonitis [36].

In this study, we wanted to investigate whether it is possible to identify and quantify fragments of fungi, pollen, and actinomycetes in the PM_1 fraction from indoor air using qPCR. We have used specific primers for targeting 16 fungal species, three groups of fungal genera, in addition to pollen from one plant species and two groups of plant species, and one actinomycetes. Pairs of PM_1 and inhalable particle samples were taken at the same time in the same houses during different seasons have been analyzed. By combining the results obtained in this study and a review of the literature, we also want to see whether the degree of fragmentation is related to species.

2. Materials and Methods

2.1. Selection of Homes and Sampling of the Inhalable and PM_1 Fraction in Five Homes

Five homes in Greater Copenhagen were selected, and out of the five homes, there were three detached houses, one townhouse, and one apartment. One of the detached houses has previously had moisture problems while the other did not have any water-damage [37].

Four samples were taken simultaneously each time, two samples of the PM_1 fraction and another two of the inhalable fraction. In total 4 × 15 air samples were taken in five homes with an average 6-h sampling period [37]. All samples were taken in the living room of the homes. For a sampling of the inhalable fraction, Gesamtstaubprobenahme (GSP) conical inhalable samplers (CIS by BGI, INC Waltham, Watertown, MA, USA) were mounted with polycarbonate filters (37 mm, pore size 1.0 μm, GE Water Technologies & Solutions, Trevose, PA, USA), more details of the GSP sampler can be found in a previous study [38]. For a sampling of the PM_1 fractions, Triplex cyclones (BGI, MA) were also mounted with polycarbonate filters. The Triplex cyclone has a well-defined, sharp penetration curve, with a D_{50} = 1 μm at a flow rate of 3.5 L per minute, but still, about 1% of particles with a D_{50} between 1.7 and 2.0 μm penetrate the cyclone [39]. Some spores will be present as clusters or associated with other particles. We assume that 30% of the

fungal spores and none of the pollen grains are between 1.7 and 2.0 µm [40]. Based on this, 0.3% of the spores will enter the triplex cyclone. The two samplers were suspended 1.5 m above floor level in the living room of the homes and they sampled simultaneously during the day time with an average sampling period of 345 min (average volume = 1208 l air). Most of the time the occupants were not at home during sampling, and all windows of the homes were kept closed during sampling. One hour after sampling, the filters were frozen at −80 °C and later extracted and used for this study. The inhalable particles collected by the GSP sampler include respirable particles (aerodynamic diameter < 10 µm) but also large particles (aerodynamic diameter up to 100 µm) [38] and they are called GSP samples in the results and discussion.

2.2. Extraction of Filters and Plating

One of the PM_1 samples and one of the GSP samples from each sampling position were extracted in 5.0 mL sterile solution (0.05% Tween 80 and 0.85% NaCl) by orbital shaking (500 rpm) for 15 min at room temperature. Following, 100 µl solution of extracts were plated on dichloran glycerol agar (DG-18 agar, Oxoid, Basingstoke, UK) plates for quantification of cultivable fungi. The plates were incubated at 25 °C and the number of colony-forming units (CFUs) were counted after 3, 5, and 7 days of incubation. The data are presented as time-weighted average exposures (TWA) in CFU/m^3 air. The CFU data have been part of a previous study [37].

2.3. qPCR of the Inhalable (GSP) and PM_1 Fraction

Particles were extracted from the polycarbonate filter samples in 5.0 mL buffer by centrifugation (12,300× *g*) for 5 min. For total-DNA isolation, the GeneJET DNA Purification Kit (Thermo Scientific, Roskilde, Denmark) was applied with minor modifications. The particles in the GSP samples and PM_1 fraction were dissolved in 180 µl of digestion solution and 20 µL of proteinase K (supplied by the manufacturer) and disrupted by bead-beating (Disruptor Genie, Scientific Industries, Bohemia, NY, USA) using acid-washed glass beads (Sigma-Aldrich, Copenhagen, Denmark) for twenty minutes at 2840 rpm. The homogenized supernatant was transferred to a silica-based spin-column for the purification of the DNA and removal of PCR inhibitory compounds. The final elutants were used directly for qPCR analysis. Quantitative polymerase chain reaction (qPCR) assays were prepared using SYBR Green Master Mix (Bio-Rad Laboratories Inc., Hercules, CA, USA), individually optimized concentrations of target-specific primers for the 24 assays, and DNA template for a total volume of 7 µL. qPCR-assays were performed in a Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Estimation of spore equivalents was based on the use of standard curves specific for each target (fungal species/fungal group). Standard curves were made from spores harvested from pure cultures. Post spore harvesting and spore counting using a hemocytometer and microscopy, dilution series were made in the range of 1 up to 3×10^7 cells and each dilution was used for separate DNA extractions and qPCR to produce the standard curves. DNA from the following microorganisms was detected using primers for (1) one bacterial genus (*Streptomyces* spp.), (2) 16 fungal species (*Acremonium strictum*, *Alternaria alternata*, *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aspergillus niger*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Cladosporium herbarum*, *Cladosporium sphaerospermum*, *Penicillium chrysogenum*, *Pencillium expansum*, *Rhizopus stolonifer*, *Stachybotrys chartarum*, *Trichoderma viride*, *Ulocladium chartarum*, *Wallemia sebi*), (3) three fungal groups (*Mucor/Rhizopus* spp., *Aspergillus glaucus* spp., *Penicillium/Aspergillus/Paecilomyces variotii* spp.), and (4) one universal measure for the total fungal load. All primer sequences used are designed to target the internal transcribed spacer (ITS) region of the nuclear ribosomal repeat unit. The design is made in-house at House Test and are proprietary. Sequence requests can be made to House Test.. DNA from at least one of these microorganisms was detected in all samples. qPCR results are presented as spore equivalents (SE) per m^3 air (SE/m^3). In addition,

DNA from the following pollen was included in the study, *Alnus*/*Corylus*, *Betula*, and Grasses/*Artemisia*. The lowest values considered as positive are shown in Table A1.

2.4. Data Treatment

The proportion of fragments (PM_1) to inhalable particles (GSP) were calculated as a percentage, and are in the following called PM_1/GSP; these values were used to evaluate which species were present as fragments. According to the limitation of the sampler as mentioned in Section 2.1, 0.3% of the particles found in the GSP sampler may by chance have entered the PM_1 fraction. Consequently, it could be relevant to have a quantitative approach. As the Triplex cyclone (PM_1 sampler) and the GSP samplers sampled the same air volume, the samples can be compared directly, and the DNA copies in the PM_1 sample should constitute at least 0.3% of the amount of DNA sampled by the GSP, to be sure that the measured DNA in the PM_1 fraction is actually from particles smaller than spores. Hence, when the PM_1/GSP was more than 0.3%, it was considered that fungal fragments were detected in PM_1 samples.

The data analyses were performed in IBM SPSS Statistics 25 for Windows and SAS 9.4. The concentration data were log-transformed before analysis. Paired samples *t*-test analysis was used to compare the concentrations in PM_1 and GSP samples. An independent sample *t*-test was used to compare the difference in the concentration between qPCR and CFU. The Pearson correlation between the different measures were calculated. A mixed-effects linear regression model was used to test whether there was an effect of season on the concentration of fragments with the home as a random effect. A positive β-coefficient means there is an elevated concentration in the season. A *p*-value below 0.05 was considered statistically significant. Statistical analyses for microbial community composition and structure for the airborne fragments were performed and visualized using RStudio with the following R CRAN packages: ggplot2, ampvis2 and vegan. Canonical correspondence analysis (CCA) plots based on weighted Bray–Curtis distance measurements were used to explore the β-diversity. Analysis of similarity (ANOSIM) using Bray–Curtis index values was used to estimate significant differences in microbial diversity between the different sampling seasons and between different homes.

3. Results

3.1. Detection of Different Fungi in PM_1 and GSP Samples

The qPCR results showed the universal fungi and the *Asp/Pen/Pae* group had the most copies of all the fungal species and groups in PM_1 (811 and 196 SE/m^3) and GSP samples (2.8×10^4 and 4.0×10^3 SE/m^3). The universal primers for fungi were positive in all PM_1 and GSP samples, and the *Asp/Pen/Pae* group was positive in 80% (12/15) and 100% (15/15) of PM_1 and GSP samples, respectively. The species *Cl. cladosporioides*, *Cl. herbarum*, and *Cl. sphaerospermum* were the most commonly detected species of all the fungi in both PM_1 and GSP samples.

Eight fungal species, *Asp. fumigatus*, *Asp. versicolor*, *Cl. cladosporioides*, *Cl. herbarum*, *Cl. sphaerospermum*, *Pen. expansum*, *U. chartarum,* and *W. sebi* were detected in the PM_1 samples from the indoor environments, and these fungi were detected in the GSP samples as well (Table 1). In addition to the species found in both the GSP and PM_1 samples, five other fungal species: *Ac. strictum*, *Asp. niger*, *Ch. globosum*, *R. stolonifer*, and *T. viride*, and two fungal groups: the *Aspergillus glaucus* group and *Mucor/Rhizopus* group were detected in the GSP samples.

Table 1. The number of positive PM_1 and Gesamtstaubprobenahme (GSP) samples as measured using qPCR; PM_1/GSP (%); and references to other papers where fragments have also been found using other methods.

	This Study			Fragments Found in Previous Studies			
	Number of Positive Samples out of 15 Samples		PM_1/GSP (GM, %)				
	PM_1	GSP	PM_1/GSP	Fragment/ Spore	Place of Sampling	Method of Quantification	Reference
Fungal species							
Acremonium strictum	Bd	8	Bd	-	-	-	-
Alternaria alternata	Bd	Bd	Bd	NM	Laboratory [c]	No. by microscopy	[41]
Aspergillus fumigatus [b]	1	14	0.37%	~1%	Laboratory	No. by microscopy	[7]
Aspergillus niger	Bd	2	Bd	0–19.2%	Laboratory	No. particles	[42]
Aspergillus versicolor [b]	6	15	0.59%	100%	Laboratory	No. by microscopy	[18]
				<30%	Laboratory	No. of particles	[30]
				~2%	Laboratory	No. by microscopy	[7]
				10%	Laboratory	No. of particles	[28]
				~50%	Laboratory	No. of particles	[43]
Chaetomium globosum	Bd	2	Bd	-	-	-	-
Cladosporium cladosporioides [b]	5	13	0.71%	0–22.4%	Laboratory	No. of particles	[42]
				2%	Laboratory	No. of particles	[28]
				~50%	Laboratory	No. of particles	[43]
Cladosporium herbarum [a, b]	9	15	0.63%	-			-
Cladosporium sphaerospermum [a, b]	2	15	1.08%	-			-
Penicillium chrysogenum	Bd	Bd	Bd	~0.01%	Laboratory	No. of particles	[44]
				~2%	Laboratory	No. by microscopy	[7]
				~40%	Laboratory	No. of particles	[43]
Penicillium expansum [b]	1	8	16.47%	-	-	-	-
Rhizopus stolonifer	Bd	1	Bd	-	-	-	-
Stachybotrys chartarum	Bd	Bd	Bd	51,400%	Laboratory	No. by microscopy	[18]
Tricoderma viride	Bd	4	Bd	-		-	-
Ulocladium chartarum [b]	1	2	39.64%	~15%	Laboratory	No. of particles	[30]
Wallemia sebi [b]	2	11	1.16%	-	-	-	-
Fungal Groups							
Aspergillus glaucus group	Bd	6	Bd	-	-	-	-
Mucor/Rhizopus group	Bd	14	Bd	-	-	-	-
Asp/Pen/Pae group [a, b]	12	15	4.68%	-			-
Universal fungi [a, b]	15	15	2.95%	~35%	Open field	No. by microscopy	[11]
				<1%	Laboratory gypsum	No. particles	[19]
				~5%	Laboratory gypsum	(1→3)-β-D-glucan	[19]
				0.3–1.3%	Laboratory gypsum	No. particles	[6]
				170%	Basement	No. by microscopy	[45]
				1–216%	Homes	(1→3)-β-D-glucan	[15]
				6.3%	Home	No. by microscopy	[46]
				0.08–19.5%	Farms	(1→3)-β-D-glucan	[12]
Pollen							
Betula	Bd	4	Bd	-	-	-	-
Alnus/Corylus [a, b]	4	10	16.70%	-	-	-	-
Grasses/*Artemisia*	Bd	6	Bd	<62%	Outdoor	No. by microscopy	[26]
Bacterial genus							
Streptomyces spp. [b]	9	15	3.66%	-	Field	Cultivable	[47]

[a] Significant correlation between PM_1 and GSP concentration. [b] significant difference between PM_1 and GSP concentration. Bd, below detection limit; GM, geometric mean; NM, not mentioned.

The concentration of cultivable fungi in GSP samples was between 20 and 1.78×10^3 CFU/m^3, with a geometric mean value (GM) of 148 CFU/m^3, and it was significantly higher ($p < 0.05$) than the fungal concentration in PM_1 samples. Of the PM_1 samples, 6 out of 15 contained cultivable fungi ($n = 6$, GM of positive samples = 15 CFU/m^3). The concentration of universal fungi in GSP samples measured using qPCR was significantly higher than the concentration of cultivable fungi in GSP samples ($p < 0.05$).

3.2. PM_1/GSP and Correlation between PM_1 and GSP Samples—Fungi

The total concentration of fungi as measured using universal fungal primers in GSP samples was significantly higher than that in PM_1 samples ($p < 0.05$) (Table 1).

The PM_1/GSP was studied to evaluate whether the fungal DNA found in the PM_1 fraction is from fragments or intact spores; a percentage above 0.3 is considered as positive

for fragments. The PM_1/GSP for the universal fungal primer were in all the samples above 0.3% (n = 15, GM = 2.95%). This shows that fungal fragments smaller than spore size were present and can be detected. For each fungal species with at least 1 positive PM_1 sample, the geometric mean of the PM_1/GSP was above 0.3%. Of the fungi, the following species had the largest PM_1/GSP: *U. chartarum* (39.64%), *Pen. expansum* (16.47%), *W. sebi* (1.16%), and *Cl. sphaerospermum* (1.08%). The PM_1/GSP was 4.68% for the *Asp/Pen/Pae* group (Table 1).

Correlations between the fungal taxa found in most samples and between PM_1 and GSP samples were studied, and only positive correlations were significant (Table A2). For the PM_1 samples, the universal fungi correlated significantly with all three *Cladosporium* species, *Asp. versicolor*, *Asp. fumigatus*, and the *Asp/Pen/Pae* group (all $r > 0.50$, $p < 0.05$). For the GSP samples, the universal fungi correlated significantly with the concentration of all three *Cladosporium* species and with the *Asp/Pen/Pae* group (all $r > 0.50$, $p < 0.05$).

The concentrations of the three *Cladosporium* species in the PM_1 samples correlated significantly with each other and the same was found for the GSP samples (all $r > 0.50$, $p < 0.05$). The concentration of *Cl. herbarum*, *Cl. sphaerospermum*, the *Asp/Pen/Pae* group, and universal fungi in the PM_1 samples correlated significantly with their concentration in the GSP samples (all $r > 0.50$, $p < 0.05$, Table A2).

The concentration of universal fungi in GSP samples correlated significantly with the cultivable fungi in GSP samples ($r = 0.84$, $p < 0.001$).

3.3. Differences in Microbial Composition between Seasons—Fungi

A significant effect of season was found in regards to the fungal β-diversity in the PM_1 ($p = 0.001$; Figure 1a) and GSP samples ($p = 0.017$; Figure 1b), but no significant difference in β-diversity was found between the 5 homes ($p > 0.05$, no further data shown). For some of the targeted fungal species, differences in concentrations of DNA copies between seasons were found in the PM_1 (Table 2) and GSP (Table 3) samples.

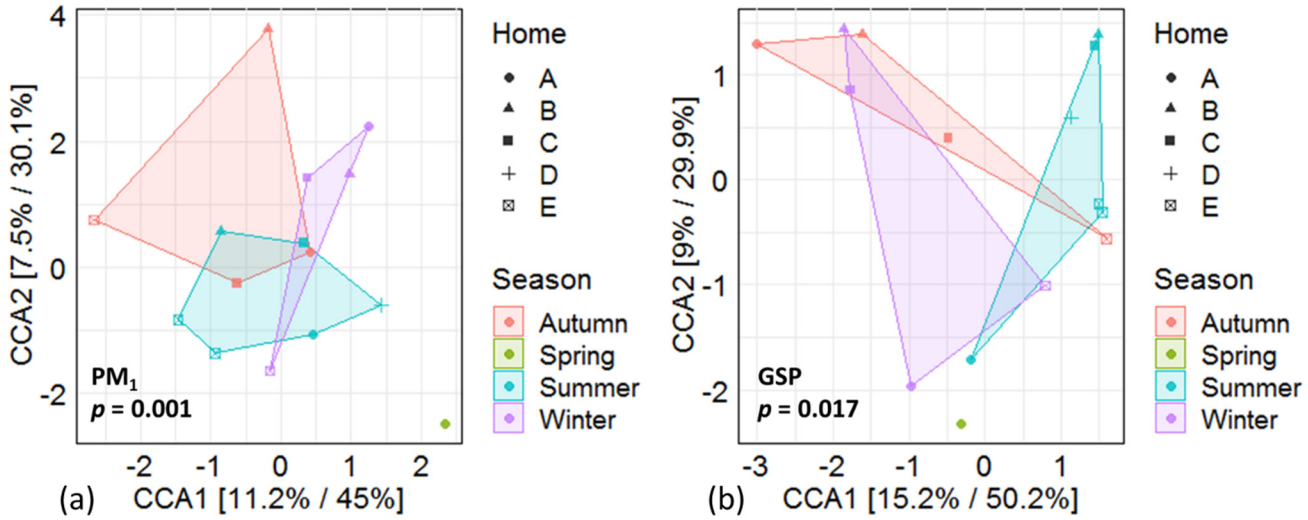

Figure 1. Canonical correspondence analysis (CCA) of the fungal β-diversity of the different fungi in PM_1 (**a**) and GSP (**b**) samples both constrained by season.

Table 2. Seasonal differences between the concentrations of DNA copies in PM_1 samples positive for fragments.

PM_1	Season	Spring		Summer		Autumn		Winter	
	p-Value	β	*p*-Value	β	*p*-Value	β	*p*-Value	β	*p*-Value
Fungal species									
Aspergillus fumigatus	**<0.0001**	**1.94**	**<0.0001**	**0.32**	**0.05**	0.103	0.54	Ref.	-
Aspergillus versicolor	0.0757	**2.74**	**0.050**	0.40	0.58	−0.91	0.28	Ref.	-
Cladosporium cladosporioides	0.25	1.44	0.11	0.51	0.30	−0.088	0.87	Ref.	-
Cladosporium herbarum	0.095	2.28	0.07	**1.76**	**0.027**	0.91	0.24	Ref.	-
Cladosporium sphaerospermum	0.17	**1.40**	**0.047**	0.61	0.12	0.38	0.35	Ref.	-
Penicillium expansum	**0.0019**	**2.94**	**0.0002**	0.26	0.43	0.32	0.39	Ref.	-
Ulocladium chartarum	0.76	0.50	0.36	0.08	0.79	−0.06	0.85	Ref.	-
Wallemia sebi	0.23	0.078	0.91	0.12	0.74	0.79	0.075	Ref.	-
Fungal Group									
Asp/Pen/Pae group	0.15	**1.58**	**0.037**	0.56	0.17	0.28	0.52	Ref.	
Universal fungi	0.12	**0.74**	**0.030**	0.22	0.22	0.02	0.91	Ref.	-
Pollen									
Alnus/Corylus	0.26	1.33	0.058	0.255	0.49	0.36	0.39	Ref.	-
Bacterial genus									
Streptomyces spp.	**0.0041**	**2.47**	**0.0035**	−0.21	0.52	**1.10**	**0.015**	Ref.	-

Statistically significant values are in **bold**. β (β-coefficient) is the estimate of each season. A positive β-coefficient means there is an elevated concentration in the season. A mixed-effects linear regression model was used with the home as a random effect and season as a fixed factor. Winter was chosen as the reference (Ref.).

Table 3. Seasonal differences between the concentrations of DNA copies in the GSP samples.

GSP	Season	Spring		Summer		Autumn		Winter	
	p-Value	β	*p*-Value	β	*p*-Value	β	*p*-Value	β	*p*-Value
Fungal species									
Acremonium strictum	0.13	1.74	0.065	0.98	0.071	0.24	0.66	Ref.	-
Aspergillus fumigatus	0.057	0.62	0.22	**0.64**	**0.037**	−0.11	0.69	Ref.	-
Aspergillus niger	**0.0057**	**1.77**	**0.0011**	**0.67**	**0.0087**	**0.47**	**0.05**	Ref.	-
Aspergillus versicolor	0.20	0.77	0.10	0.017	0.95	0.39	0.17	Ref.	-
Chaetomium globosum	0.56	−0.18	0.78	−0.05	0.90	−0.51	0.22	Ref.	-
Cladosporium cladosporioides	**0.0002**	**1.44**	**0.016**	**2.12**	**<0.0001**	**1.37**	**0.0013**	Ref.	-
Cladosporium herbarum	**0.0025**	1.05	0.061	**1.501**	**0.0003**	0.66	0.06	Ref.	-
Cladosporium sphaerospermum	**0.0085**	**1.28**	**0.042**	**1.36**	**0.0014**	0.57	0.13	Ref.	-
Penicillium expansum	**0.014**	0.95	0.15	**−0.93**	**0.02**	0.14	0.73	Ref.	-
Rhizopus stolonifera	0.42	0.82	0.20	−0.17	0.61	−0.12	0.74	Ref.	-
Tricoderma viride	0.29	−0.17	0.53	−0.29	0.07	−0.11	0.48	Ref.	-
Ulocladium chartarum	**0.0048**	**1.39**	**0.008**	−0.19	0.41	**0.74**	**0.016**	Ref.	-
Wallemia sebi	0.13	0.011	0.99	−0.58	0.22	0.59	0.23	Ref.	-
Fungal Groups									
Aspergillus glaucus group	0.44	0.037	0.91	0.0004	0.99	0.27	0.18	Ref.	-
Mucor/Rhizopus group	**0.0003**	**1.78**	**0.0005**	**1.29**	**<0.0001**	**0.64**	**0.018**	Ref.	-
Asp/Pen/Pae group	0.062	**1.27**	**0.01**	0.34	0.17	0.44	0.11	Ref.	-
Universal fungi	**0.018**	**0.86**	**0.029**	**0.77**	**0.004**	**0.55**	**0.03**	Ref.	-
Pollen									
Betula	0.056	1.05	0.059	0.48	0.11	**0.87**	**0.015**	Ref.	-
Alnus/Corylus	0.23	1.58	0.15	−0.061	0.92	0.89	0.18	Ref.	-
Grasses/*Artemisia*	0.13	**2.91**	**0.02**	0.72	0.29	0.59	0.41	Ref.	-
Bacterial genus									
Streptomyces spp.	0.27	1.05	0.096	0.55	0.13	0.31	0.42	Ref.	-

Statistically significant values are in **bold**. β (β-coefficient) is the estimate of the season. A positive β-coefficient means there is an elevated concentration in the season. A mixed-effects linear regression model was used with the home as a random effect and season as a fixed factor. Winter was chosen as the reference (Ref.).

3.4. Pollen

The *Alnus/Corylus* group of pollen was detected in 4 of 15 PM_1 samples, these 4 samples were sampled from 4 different seasons, and 3 of the samples were from the same house, and the fragments might have been re-aerosolized. In contrast, *Betula* and Grasses/*Artemisia* were not found in the PM_1 fraction. The concentration of *Alnus/Corylus* pollen in GSP samples was significantly higher than that in the PM_1 samples ($p < 0.05$, Table 1), but the PM_1/GSP was relatively large (16.7%). Pollen from all three groups,

Alnus/*Corylus*, *Betula*, and Grasses/*Artemisia* were detected in 4, 10, and 6 of the 15 GSP samples, respectively. No significant difference in *Alnus*/*Corylus* concentration was found between seasons in both PM_1 and GSP samples ($p > 0.05$, Tables 2 and 3.). Grasses/*Artemisia* was present in the highest concentration in spring in GSP samples (Table 3).

3.5. Bacteria

The bacterial genus *Streptomyces* was found in 9 of 15 PM_1 samples and in all of the 15 GSP samples. The concentrations of *Streptomyces* spp. in PM_1 and GSP samples were significantly different ($p < 0.05$, Table 1). The concentration of *Streptomyces* spp. in GSP samples correlated significantly with the universal fungi in GSP samples ($r = 0.52$, $p < 0.05$) and in PM_1 samples ($r = 0.61$, $p < 0.05$, Table A2). Significant seasonal differences were found in PM_1 samples ($p = 0.0041$, Table 2), but not in GSP samples ($p = 0.27$, Table 3).

4. Discussion

The result in this study showed that qPCR can be used to identify the genus or species of airborne fungal and pollen fragments in field samples. Fungal fragments from 8 species were detected in PM_1 samples. The knowledge about health effects of exposure to fungal fragments is still not clear, but it can't be ignored, as for example, an in vitro study with human HL-60 cells showed that fungal fragments of PM_1 size induced a pro-inflammatory response [19], and β-glucan has been found in higher concentrations in the fungal fragment size particles in homes with asthmatic children than in homes with non-asthmatic children [21]. To our knowledge, this is the first study to show that *Alnus*/*Corylus* pollen, *Cladosporium herbarum*, *Cladosporium sphaerospermum*, *Penicillium expansum*, and *Wallemia sebi* can be present as airborne particles of PM_1 size.

All the species found in the PM_1 samples were as expected also detected in the GSP samples, although with higher concentrations in the latter. Based on the data for total fungi 2.95% fungal particles in GSP samples were present as fragments with a particle size less than 1 µm. A study using microscopy found that hyphal fragments in indoor air constituted 6.3% of total fungi (spores + hyphal fragments), however, these fragments were large (2–20 µm, i.e., PM_{10} fraction) [46]. The high percentages that PM_1 constituted out of the amount present in the GSPs for *U. chartarum* (39.64%), *Pen. expansum* (16.47%), *W. sebi* (1.16%), and *Cl. Sphaerospermum* (1.08%) indicates that these fungal species were more fragmented than the rest of the targeted species. A review of the literature shows that the share of fungi that are present as fragments are very different from study to study and within a study, and this difference is also seen for both field and laboratory studies (Table 1), and it is not clear how much this fragmentation is related to species. For laboratory-generated aerosols, the share of fungi being present as fragments decrease with exposure of fungal colonies to increasing air velocity [28], but the share seems also to be affected by age of culture and the material the fungus is growing on [30,31]. Fungal fragments of the PM_1 size seem not to be cultivable [10]. In line with this cultivable fungi were in this study detected only in 6 out of 15 PM_1 samples and in concentrations near the detection limit. This corresponds to the aforementioned fraction of larger particles penetrating the cyclone when used for sampling PM_1 particles [39]. Studies using another sampler type, the Andersen six-stage sampler, showed low concentrations of cultivable *Penicillium*, *Aspergillus*, and *Cladosporium* spp. in submicron particle size (0.65–1.1 µm) in samples from e.g., homes [48], public buildings [49], and pig farms [50]. A study using a two-stage bioaerosol cyclone sampler found cultivable fungi in the PM_1 size in 3 out of 4 agricultural farms [12]. For studies using the electrical low-pressure impactor for sampling, few cultivable fungi are also found in the PM_1 size, and the presence of cultivable fungi in that size fraction is suggested to be due to particle bouncing between the different stages of the sampler [6]. Hence, whether some of the small fragments actually can be cultivated or if larger, cultivable fungal spores or hyphae are present by chance or due to bouncing, still need to be further studied.

Thirteen different fungal species were detected in the GSP samples in this study. In a previous study, nineteen samples were taken in the same five living rooms in the same periods, and the dominating fungal species were identified using a combination of culturing and subsequent analysis by MALDI-TOF MS. Compared with analysis by qPCR, MALDI-TOF MS method has the advantage that not only predetermined species are possible to detect. Thus in addition to the species found in the GSP samples in this study, *Asp. flavus*, *Asp. nidulans*, *Asp. glaucus* and *Asp. penicilloides*, *Epicoccum nigrum*, *Fusarium proliferatum*, two *Candida* species, and eight *Penicillium* species were found [51]. The use of DNA-primers for quantification and identification of airborne microorganisms has the advantage that also non-viable species and dormant and dead microorganisms as long as DNA is present can be identified. However, the concentration of DNA from fungal fragments in the tested sample may not be proportional to the concentration of the "true" fungal fragments as some DNA can be lost during purification and some fragments may not contain genomic DNA. Previous studies with aerosolized *Cladosporium* [52] and *Wallemia sebi* [53] from different environments found that PCR quantification detected higher concentrations than CFU counting. Similar results were also found in this study. On the other hand, we found a significant correlation between the concentration of fungi measured as universal fungi by qPCR and measured by cultivation in GSP samples ($r = 0.837$, $p < 0.001$). Similarly, a study found a significant correlation between qPCR and CFU counts for *Thermoactinomyces vulgaris* [54] while a study with surface samples showed the only correlation between the two methods for samples with high fungal and *Streptomyces* spp. concentrations [55]. For future studies, it could be relevant to use e.g., cultivation and MALDI-TOF MS as the first step to obtaining knowledge about dominating viable species in general, and following use primers for the dominating species and/or for the most health-relevant species.

Cladosporium was the most common genus in both sample types, which was also reflected in the correlation between total fungi and each *Cladosporium* species. The dominance of *Cladosporium* spp. is in accordance with a review study about fungi in indoor air [56]. The concentration of total fungi correlated also with *Asp/Pen/Pae* group in both GSP and PM_1 samples. In our previous studies with the cultivation of fungi, we found *Penicillium, Aspergillus*, and *Cladosporium* to be present in the highest concentrations [19].

The fungal species composition in the GSP sampled from indoor air varied between seasons, and similar results have previously been reported in indoor air in homes in the USA [57]. The seasonal variation of fungal species composition and concentration may be due to variation in air change rate and relative humidity through a year [51], and the seasonality in outdoor species [58]. The highest concentration of *Cladosporium* species was found in summer, similar to an outdoor air study in Poland [59]. In this study, seasonality was found for *Asp. niger* and this has also been found in indoor dust in Finland [60]. The allergenic species *U. chartarum* was found in a sample taken in autumn and spring. A study from France has also found this species in indoor air [61]. The species *Pen. expansum* was found in the highest concentrations in spring. This species has previously been found in nursing homes in Denmark [62] and in dwellings in France [63]. In vitro studies with human cell lines show that this species can be cytotoxic [64] and inflammogen [62].

Three groups of pollen, *Alnus*/*Corylus*, *Betula*, and Grasses/*Artemisia*, were detected in GSP samples with the qPCR method. We chose to investigate *Alnus*/*Corylus* as they are among the first pollen to appear in outdoor air in Europe (December to April), and as they act as primers of allergic sensitization to other pollen allergens, like *Betula* allergens [65]. We have chosen to investigated *Betula* pollen as they are considered as major allergens, and they have been long-term monitored in several European countries e.g., Denmark [66], Finland [67], and Croatia [68]. *Artemisia* pollen allergy is also common in Europe [69], and a recent study has identified it as the main vector for airborne endotoxin from gram-negative bacteria [70].

On one hand, pollen fragments of small particle sizes have been described to be only rarely found, probably because the pollen-grain wall resists fragmentation [65]. On

the other hand, some severe weather, like thunderstorms and rain events can cause that pollen to break into small fragments [27]. A study showed that exposure to water can induce the respirable allergen-bearing particles to be released from grass pollens [71]. Furthermore, a review study describes that pollen fragments contain allergens and a range of other compounds [72]. In addition, grass pollen allergens were detected in the fraction with small particles (0.6–1.3 µm) of atmospheric dust [73]. As mentioned, we found pollen fragments of *Alnus*/*Corylus* in four PM_1 samples in normal indoor air. We expected it to be possible to obtain knowledge about the importance of these small particles in environments with complex exposure using the combination of methods used in this study. Thunderstorm-related asthma is a health risk that is suggested to be caused by the small pollen fragments entering the airways [74]. With this study, we show fragments of *Alnus*/*Corylus* pollen can be found in normal indoor air.

The concentration of airborne *Betula* pollen tended to be lower in the winter than during the other seasons, and according to the Danish pollen calendar, the highest concentrations are expected in spring. *Alnus*/*Corylus* pollen and pollen fragments tended to be highest in the spring, and this is in accordance with the Danish pollen calendar. Grasses/*Artemisia* concentrations tended to be highest in spring and summer which is also in accordance with the pollen calendar [75].

In this study, *Streptomyces* were detected in 60% of the PM_1 samples and all the GSP samples. *Streptomyces* has in a previous study been found in the indoor air in up to 8% of the samples [76]. Similar to a previous study [33], we found *Streptomyces* spp. to significantly correlate with total fungi. Of the airborne inhalable *Streptomyces* spp. 3.7% had a particle size of less than 1 µm in our study. At the laboratory level aerosols generated from *Streptomyces albus* had a geometric mean diameter of 0.98 µm [77] and spores an optical diameter of 0.7 µm [78]. Thus, based on data from that species most particles would be expected to belong to the PM_1 fraction. The small share present in the PM_1 fraction in this study shows that spores were present in clusters or associated with other particles, or were present as larger fragments of hyphae.

5. Conclusions

This study showed that it is possible to identify fungi, pollen, and the bacterial genus *Streptomyces* in the PM_1 aerosol fraction using a qPCR assay. Eight fungal species, *Asp. fumigatus*, *Asp. versicolor*, *Cl. cladosporioides*, *Cl. herbarum*, *Cl. sphaerospermum*, *Pen. expansum*, *U. chartarum,* and *W. sebi*, the *Asp/Pen/Pae* group, the universal fungi, pollen from *Alnus*/*Corylus*, and *Streptomyces* spp. were found in both PM_1 and GSP samples from indoor air using a qPCR approach, and this approach can be relevant in future epidemiological studies. *Alnus*/*Corylus* fragments constituted 17% of what was found in the inhalable fraction. Even though spores of *Streptomyces* spp. have an optical diameter smaller than 1 µm only 4% of the inhalable fraction was present in the PM_1 fraction. A significant effect of season was found on fungal species concentrations and composition in the PM_1 and GSP samples. For the universal fungal primers, 3% were present as fragments, and for the individual species, the PM_1 fraction constituted between 0.4 and 40% of what was found in the inhalable fraction.

Using qPCR analysis to detect microorganisms in the PM_1 fraction had some advantages, but there are still factors that can affect the results as e.g., DNA-free fragments in the PM_1 fraction, hence, further studies are needed to focus on the effects of these factors on this method.

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Appendix A

Table A1. The lowest value considered as positive for each organism in PM_1 and GSP samples, and in brackets the highest measured value for organisms below the detection (bd).

	SE */Sample	
	PM_1	**GSP**
Fungal species		
Acremonium strictum	Bd (7)	10
Alternaria alternata	Bd (0)	Bd (1)
Aspergillus fumigatus	14	14
Aspergillus niger	Bd (2)	17
Aspergillus versicolor	9	86
Chaetomium globosum	Bd (2)	14
Cladosporium cladosporioides	17	54
Cladosporium herbarum	12	76
Cladosporium sphaerospermum	9	14
Penicillium chrysogenum	Bd (0)	Bd (0)
Penicillium expansum	12	12
Rhizopus stolonifer	Bd (1)	18
Stachybotrys chartarum	Bd (1)	Bd (2)
Tricoderma viride	11	11
Ulocladium chartarum	8	8
Wallemia sebi	19	55
Fungal Group		
Aspergillus glaucus group	Bd (3)	17
Mucor/Rhizopus group	Bd (13)	30
Asp/Pen/Pae group	50	54
Universal fungi	338	4300
Pollen		
Betula	Bd (14)	25
Alnus/Corylus	15	15
Grasses/Artemisia	Bd (7)	30
Bacterial genus		
Streptomyces spp.	19	50

* SE: spore equivalent.

Appendix B

Table A2. Correlation coefficients (r) between concentrations of fungi and pollen in PM_1 and GSP samples as measured using Pearson's correlation coefficient.

Organisms	Sample Type Number	GSPa	GSPb	GSPc	GSPd	GSPe	GSPf	GSPg	GSPh	GSPi	GSPj	GSPk	GSPl	PMa	PMb	PMc	PMd	PMe	PMf	PMg	PMh	PMi	PMj	PMk	PMl
As. fumigatus	**GSPa**	1.00																							
As. versicolor	**GSPb**	−0.13	1.00																						
***Cl.** cladosporioides*	**GSPc**	0.54 *	0.19	1.00																					
Cl. herbarum	**GSPd**	0.58 *	0.14	0.94 **	1.00																				
Cl. sphaerospermum	**GSPe**	0.67 **	0.08	0.90 **	0.95 **	1.00																			
Alnus/Corylus	**GSPf**	0.39	0.19	0.15	0.05	0.19	1.00																		
Asp/Pen/Pae **group**	**GSPg**	0.39	0.75 **	0.49	0.45	0.42	0.36	1.00																	
Pen. expansum	**GSPh**	−0.23	0.69 **	−0.28	−0.31	−0.29	0.39	0.38	1.00																
***Streptomyces* spp.**	**GSPi**	0.69 **	0.18	0.60 *	0.64 **	0.75 **	0.46	0.54 *	0.05	1.00															
U. chartarum	**GSPj**	0.10	0.33	−0.02	−0.05	−0.07	0.65 **	0.48	0.54 *	0.28	1.00														
Universal fungi	**GSPk**	0.48	0.35	0.87 **	0.85 **	0.78 *	0.37	0.56 *	0.03	0.52 *	0.19	1.00													
W. sebi	**GSPl**	−0.05	0.30	−0.14	−0.22	−0.21	0.46	0.04	0.56*	−0.09	0.32	0.19	1.00												
As. fumigatus	**PMa**	0.43	0.16	0.25	0.26	0.36	0.42	0.41	0.15	0.36	0.47	0.36	−0.04	1.00											
As. versicolor	**PMb**	0.56 *	0.16	0.17	0.30	0.28	0.14	0.48	0.15	0.53 *	0.26	0.26	0.09	0.46	1.00										
***Cl.** cladosporioides*	**PMc**	0.46	0.06	0.37	0.41	0.51	0.36	0.29	0.05	0.58 *	0.14	0.48	0.28	0.48	0.60 *	1.00									
Cl. herbarum	**PMd**	0.32	−0.08	0.58 *	0.61 *	0.62 *	0.12	0.31	−0.39	0.49	0.15	0.46	−0.27	0.46	0.44	0.58 *	1.00								
Cl. sphaerospermum	**PMe**	0.53 *	0.29	0.56 *	0.62 *	0.66 **	0.40	0.62 *	−0.01	0.79 **	0.31	0.54 *	−0.05	0.43	0.69 **	0.58 *	0.77 **	1.00							
Alnus/Corylus	**PMf**	0.54 *	0.15	0.32	0.27	0.37	0.76 **	0.51	0.16	0.71 **	0.58 *	0.37	0.17	0.56 *	0.57 *	0.64 *	0.57 *	0.78 **	1.00						
Asp/Pen/Pae **group**	**PMg**	0.55 *	0.24	0.51	0.56 *	0.64 *	0.38	0.56 *	0.06	0.82 **	0.30	0.45	−0.07	0.52 *	0.73 **	0.62 *	0.75 **	0.95 **	0.79 **	1.00					
Pen. expansum	**PMh**	0.21	0.45	0.09	−0.01	0.08	0.42	0.49	0.38	0.22	0.39	0.23	0.17	0.81 **	0.42	0.32	0.18	0.33	0.48	0.39	1.00				
***Streptomyces* spp.**	**PMi**	0.02	0.54 *	0.16	0.20	0.21	0.25	0.55 *	0.48	0.49	0.64 **	0.17	0.16	0.33	0.37	0.27	0.38	0.57 *	0.46	0.62 *	0.26	1.00			
U. chartarum	**PMj**	0.12	0.33	0.08	0.11	0.07	−0.03	0.41	−0.06	0.24	0.13	−0.05	−0.23	0.33	0.48	0.29	0.42	0.52 *	0.47	0.50	0.34	0.37	1.00		
Universal fungi	**PMk**	0.53 *	0.32	0.51	0.58 *	0.60 *	0.40	0.57 *	0.20	0.61 *	0.31	0.65 **	0.19	0.59 *	0.71 **	0.83 **	0.64 *	0.76 **	0.70 **	0.79 **	0.38	0.46	0.44	1.00	
W. sebi	**PMl**	−0.32	0.28	0.10	0.08	0.06	0.22	0.15	0.08	0.21	0.24	0.13	0.02	−0.22	−0.35	0.04	0.06	0.12	0.10	−0.05	−0.14	0.23	0.04	−0.13	1.00

* Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed). Only the Organisms which were detected both in PM_1 and GSP samples were listed. Sample type number: GSP and PM_1 are two sample types, letters a to l are different organisms that are listed in the left-most column.

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